Federal Agency for Nature Conservation

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Cover picture: Flowering oilseed rape (© B. Groeger)

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Zusammenfassung

In der Europäischen Union ist für die Zulassung gentechnisch veränderter Organismen (GVO) eine Umweltrisikobewertung durchzuführen. Als Startpunkt für diese Risikobewertung dient eine agronomische und phänotypische Charakterisierung des GVO, die Teil einer vergleichenden Bewertung des GVO mit einer nicht gentechnisch veränderten Vergleichspflanze ist. In der Umweltrisikobewertung müssen GVO auch hinsichtlich ihres Potentials persistenter oder invasiver zu werden oder mit kompatiblen Wildpflanzen zu hybridisieren, sowie dadurch bedingte mögliche nachteilige Effekte, bewertet werden. Da das Umweltverhalten von GVO auch von seinem Phänotyp bestimmt wird, ist eine Bewertung seiner agronomischen und phänotypischen Merkmale wesentlich, da diese auch Aussagen hinsichtlich seiner Umweltsicherheit stützt. Gemäß den derzeitigen Leitlinien der EFSA umfasst die Bewertung agronomischer und phänotypischer Pflanzenmerkmale jedoch hauptsächlich landwirtschaftlich wichtige Merkmale, die in Feldversuchen getestet werden. Um auch Aussagen hinsichtlich möglicher beabsichtigter, aber auch unbeabsichtigter Umwelteffekte der genetischen Veränderung treffen zu können, sollte die agronomische und phänotypische Charakterisierung des GVO auch Informationen über umweltrelevante Pflanzencharakteristika beinhalten.

Vor diesem Hintergrund war Ziel des vorliegenden Projektes, Vorschläge für die Verbesserung von Methoden, Parametern und Endpunkten, die in agronomischen und phänotypischen Feldversuchen untersucht werden, zu erarbeiten. Diese Vorschläge können im Falle der Umsetzung dazu dienen, aussagekräftigere Daten für die Umweltrisikobewertung, im speziellen hinsichtlich Risiken der Persistenz und Invasivität von GVP, zu erhalten. Solche Risiken könnten sich in Zukunft erhöhen, wenn GVP mit Toleranzen gegenüber Krankheitserregern oder abiotischem Stress (z. B. Trockentoleranz) entwickelt werden. Im Speziellen lag der Fokus dieses Projekts auf der Bewertung der Überlebensfähigkeit von Samen und Pflanzen, sowie der Reaktion des GVO auf biotische und abiotische Stressoren, die als wesentliche Aspekte für diesen Risikobereich identifiziert wurden. Verbesserungen werden hinsichtlich des allgemeinen Bewertungsansatzes, aber auch für einzelne methodische Ansätze vorgeschlagen. Fünf Arten von Kulturpflanzen (Mais, Raps, Sojabohne, Kartoffel und Kriech-Straußgras), sowie vier gentechnisch veränderte Merkmale (Herbizidtoleranz, Insektenresistenz, Veränderungen von Sameninhaltsstoffen, Trockentoleranz) werden in diesem Bericht diskutiert.

In dieser Studie werden folgende Aspekte analysiert:

- Die derzeitige Praxis der agronomischen und phänotypischen Charakterisierung in GVO Anträgen.
- Die Relevanz phänotypischer Pflanzenmerkmale für das Überleben, die Persistenz und Invasivität.
- Nachteilige Umwelteffekte und Schutzziele im Kontext des Risikobereichs Persistenz und Invasivität des GVO.
- Die Relevanz von Genotyp x Umwelt Interaktionen und ihre derzeitige Bewertungs-praxis in GVO Anträgen.
- Methodische Ansätze für die Phänotypisierung von Pflanzen aus unterschiedlichen Forschungsrichtungen (Grundlagenforschung, angewandte Forschung).
- Empfehlungen hinsichtlich merkmals- und pflanzenspezifischer Charakteristika mit Relevanz für die agronomische und phänotypische Charakterisierung von GVO.

- Empfehlungen für allgemeine Verbesserungen der agronomischen und phänotypischen Charakterisierung, sowie spezifischer methodischer Verbesserungen, um aussagekräftige Daten und Informationen für die Bewertung von Umweltrisiken zu erhalten.

Für diese Studie wurden Informationen aus folgenden Quellen verwendet: Analyse von GVO Anträgen, Literatursuche hinsichtlich relevanter Pflanzenmerkmale und Methoden zur Phänotypisierung aus unterschiedlichen Forschungsrichtungen (Pflanzenökologie, GVO Forschung, Konzept der funktionalen Pflanzenmerkmale, Invasionsbiologie, landwirtschaftliche Sortenprüfung, internationale Protokolle), sowie fachlicher Austausch mit externen Experten und Expertinnen im Rahmen von zwei Workshops und Interviews. Die endgültigen Ergebnisse und Empfehlungen wurden mit nationalen Behörden sowie Vertretern und Vertreterinnen der Europäischen Lebensmittelbehörde und Europäischen Kommission im Rahmen eines Stakeholder-Workshops diskutiert.

Die derzeitige Praxis der agronomischen und phänotypischen Charakterisierung von GVO basiert vorrangig auf Versuchen, die agronomischen Zielen dienen, wie beispielsweise die Sicherstellung der Saatgutqualität oder die Prüfung von Pflanzenmerkmalen, die für die landwirtschaftlichen Ergebnisse der Pflanzen im Feld wesentlich sind. Die vom Antragsteller geprüften Endpunkte folgen im Allgemeinen den Vorgaben der entsprechenden Leitlinien. Die wenigen zusätzlichen und optionalen Prüfungen von Pflanzenmerkmalen, die durchgeführt werden, sind für eine Aussage hinsichtlich Umweltrisiken nur bedingt geeignet. Zusätzlich sind methodische Mängel in den vorgelegten Studien identifizierbar, z. B. bei Laborstudien für die Keimfähigkeit von Samen, zur Überprüfung der Pollenlebensfähigkeit oder für Freilandstudien zur Bewertung biotischer bzw. abiotischer Stressoren. Generell fehlt in den GVO Anträgen eine Überprüfung der Lebensfähigkeit des GVO inner- und außerhalb des agrarischen Kontexts.

Eine Reihe phänotypischer Merkmale sind für Pflanzen wichtig, um in der Umwelt überleben und fortbestehen zu können. Überlebensfähigkeit und Persistenz sowie Invasivität von Pflanzen hängen allerdings auch stark von anderen Faktoren (abgesehen von phänotypischen Merkmalen) ab, insbesondere dem selektiven Wert des neuartigen, gentechnisch veränderten Merkmals und der aufnehmenden Umwelt.

Beobachtete Unterschiede in agronomischen oder phänotypischen Parametern zwischen dem GVO und der nicht-GV Vergleichspflanze müssen hinsichtlich ihrer biologischen Relevanz interpretiert werden. Deshalb muss eine Verbindung zwischen den Ergebnissen der agronomischen und phänotypischen Bewertung und agronomischer Schutzziele sowie Zielen des Umwelt- und Naturschutzes hergestellt werden. Erst diese Verbindung verbessert die Aussagekraft der Ergebnisse der agronomisch und phänotypischen Bewertung und ermöglicht eine Entscheidungsfindung in der Umweltrisikobewertung.

Die derzeitige Praxis der Bewertung von Genotyp x Umwelt Interaktionen in GVO Anträgen fokussiert auf das Verhalten der Pflanze in landwirtschaftlichen Feldern unter optimalen Umweltbedingungen, ohne unbeabsichtigte Effekte der gentechnischen Veränderung zu überprüfen, die vorrangig unter Stressbedingungen sichtbar werden.

Eine Reihe von Methoden zur Phänotypisierung von Pflanzenmerkmalen sind aus unterschiedlichen Forschungsrichtungen verfügbar, inklusive moderner Phänotypisierungsmethoden (z. B. high throughput phenotyping). In diesem Bericht werden Methoden für die Phänotypisierung bzw. die Bewertung von Samen, Überlebensfähigkeit von Pflanzen und Pollen, Konkurrenz und Fitness von Pflanzen, Samenschüttung, vegetatives Wachstum und Antwort auf biotische und abiotische Stressoren diskutiert.

Die in diesem Bericht genannten Verbesserungsvorschläge umfassen allgemeine sowie spezifische Empfehlungen. Grundlegende Pflanzenparameter, die für das Überleben und die Persistenz von Pflanzen relevant sind, sollten in der agronomischen und phänotypischen Charakterisierung generell geprüft werden. Dies umfasst Samenkeimung, Samendormanz sowie die Überlebensfähigkeit von Samen und Pflanzen (inklusive vegetativer Pflanzenteile). Diese Parameter sollten für alle Arten von Kulturpflanzen und Anträgen (Import, Anbau) beurteilt werden. Zusätzlich wird empfohlen, das Studiendesign der agronomischen und phänotypischen Charakterisierung gemäß der Umweltexposition des GVO anzupassen. Das bedeutet, dass für GVO Importanträge auch Studien unter sub-optimalen Umweltbedingungen durchgeführt werden sollten, um unbeabsichtigte Ausbringung von GVO in die Umwelt (außerhalb landwirtschaftlicher Flächen) zu berücksichtigen. Hingegen sind für Anträge, die den Anbau des GVO in der EU inkludieren, sowohl optimale als auch sub-optimale Umweltbedingungen zu berücksichtigen, um sowohl beabsichtigte Ausbringung in landwirtschaftlichen Flächen als auch unbeabsichtigte Ausbringung außerhalb dieser zu berücksichtigen.

Eine weitere Empfehlung betrifft die Durchführung einer fallspezifischen Bewertung der Pflanzenfitness für jene Kulturpflanzen, die derzeit bereits als verwilderte Pflanzen (z. B. Raps) oder Wildpflanzen (z. B. Kriech-Straußgras) in der Umwelt verbreitet sind, insbesondere, sofern diese GV-Merkmale besitzen, die einen Fitnessvorteil bereits unter landwirtschaftlichen Bedingungen aufweisen (z. B. Stresstoleranz). Für diese GVO sind Experimente mit ganzen Pflanzen unter den entsprechenden Umweltbedingungen notwendig, um mögliche Fitnessvorteile auch außerhalb landwirtschaftlicher Flächen zu prüfen.

Zusätzliche Leitlinien sind notwendig, um einsatzfähige Entscheidungskriterien für die Interpretation von beobachteten Unterschieden bzw. Nicht-Äquivalenzen während der agronomischen und phänotypischen Charakterisierung von GVO zu entwickeln. Gemeinsam mit der Auswahl indikativer Merkmale, Parameter und Endpunkte ermöglichen diese, den beobachteten Unterschieden von phänotypischen Merkmalen eine biologische Relevanz zuweisen zu können. Zudem kann damit eine Verbindung zur Problemformulierung der Umweltrisikobewertung hergestellt werden. Für GVO, für die keine konventionellen Vergleichspflanzen verfügbar sind, wird ein neuer Risikobewertungsansatz notwendig sein. Speziell für GVO mit komplexen genetischen Veränderungen oder Merkmalen wie grundlegende Veränderungen der Morphologie oder Physiologie (z. B. *de-novo* domestizierte Tomate, Vitamin B12 Mais) wird eine per-se Bewertung notwendig sein, um die biologische Relevanz vielfältiger und tiefgreifender Veränderungen bewerten zu können. Dies betrifft auch die agronomische und phänotypische Bewertung und deren Ergebnisse.

Vorschläge für Verbesserungen beziehen sich auch auf die derzeit in der agronomischen und phänotypischen Bewertung angewandten Methoden. Vor allem wird eine verbesserte und ausgeweitete Bewertung von Samenmerkmalen empfohlen, ebenso wie die Entwicklung standardisierter Methoden für die Bewertung kurzzeitiger Überlebensfähigkeit des GVO am Feld (Durchwuchs). Genotyp x Umwelt Interaktionen sollten unter Stressbedingungen, gemeinsam mit geeigneten Parametern und Erhebungsmethoden durchgeführt werden, um die Stressantwort des GVO bewerten zu können. Die Bewertung der Antwort des GVO auf biotische Stressoren (Pathogene, Schädlinge) benötigt einen fokussierten und konsistenten Ansatz. Ein verbesserter Bewertungsansatz muss sicherstellen, dass eine Antwort auf einen biotischen Stressor ausgelöst wird, beispielsweise durch die Auswahl relevanter Feldstandorte, durch Experimente, in denen der Schädlingsbefall gezielt herbeigeführt wird, oder durch Abänderung der landwirtschaftlichen Praxis. In diesem Bericht werden Vorschläge für die Auswahl und die Bewertungsmethode relevanter biotischer Stressoren für die in diesem Projekt berücksichtigten Kulturpflanzen unter EU-Umweltbedingungen gemacht. Ebenso muss die Bewertung der Antwort des GVO auf abiotische Stressbedingungen verbessert werden. So sind z. B. Kriterien zur Definition abiotischer Stressbedingungen (v. a. für Trockenheit und Kälte) notwendig, gemeinsam mit Leitlinien wie diese abiotischen Stressbedingungen in der agronomischen und phänotypischen Charakterisierung bewertet, überwacht und berichtet werden sollen. Speziell für stresstolerante GVO sind zusätzliche Leitlinien nötig, wie aussagekräftige Experimente durchzuführen sind. Schließlich sollten auch moderne Phänotypisierungsmethoden ausgelotet werden, inwiefern sie die agronomische und phänotypische Charakterisierung von GVO unterstützen können, insbesondere, wenn neue funktionale Pflanzenphänotypen ins Auge gefasst werden. Dennoch sind auch für neue methodische Ansätze klare Entscheidungskriterien hinsichtlich der biologischen Relevanz beobachteter phänotypischer Veränderungen vonnöten.

Die in diesem Bericht vorgeschlagenen Verbesserungen sind notwendig, um eine agronomische und phänotypische Charakterisierung von GVO sicherzustellen, die als geeigneter Startpunkt für die Umweltrisikobewertung dienen kann und die Risikocharakterisierung des GVO unterstützt. Eine verbesserte Bewertung agronomischer und phänotypischer Pflanzenmerkmale sollte Schlussfolgerungen zum Phänotypen des GVO inner- und außerhalb des agrarischen Kontexts, sowie unter sub-optimalen Umweltbedingungen erlauben. Eine diesbezüglich verbesserte Pflanzencharakterisierung ist dadurch geeignet, über Umweltrisiken zu informieren, indem erste grundlegende Hinweise auf die Überlebens- und Persistenzfähigkeit des GVO in der Umwelt erbracht werden.

Summary

Any authorization for a release of a genetically modified plant (GMP) into the environment in the European Union requires an environmental risk assessment (ERA). As a starting point in the ERA, a phenotypic and agronomic characterisation of the GMP, which is part of the comparative safety assessment, is carried out. In ERA, the risk of the GMP to become more persistent in agricultural habitats or more invasive in natural habitats, including the ability of the GMP to transmit transgene(s) to sexually compatible relatives and the environmental effects thereof have to be assessed. The GMP's behaviour in the environment is linked to its phenotype; therefore the assessment of its agronomic and phenotypic characteristics can be used to inform the ERA. According to the current guidance provided by EFSA, this assessment of agronomic and phenotypic plant traits comprises agriculturally important traits evaluated in field trials. In order to inform the risk assessment, not only regarding intended but also unintended effects of the genetic modification, the agronomic and phenotypic characterisation of the GMP should also provide information on environmentally relevant characteristics of the plant.

Against this background, the aim of this study was to provide suggestions for improvement of methods, parameters and endpoints assessed in agronomic and phenotypic field trials in order to gain conclusive data for use in ERA, specifically with respect to risks related to persistence and invasiveness of the GMP. These risks may become more important in the future if GMPs with tolerance to pathogens or abiotic stress are developed. Specifically, the focus of this study was on the assessment of the survivability of seeds and plants and the response of the GMP to biotic and abiotic stressors, which were identified as the main aspects with relevance for this risk area. We focused on five crop types (maize, oilseed rape, soybean, potato and creeping bentgrass) and four GM traits (herbicide tolerance, insect resistance, changes in seed composition and drought tolerance).

In this project, we

- Evaluated and scrutinized the current practice of the agronomic and phenotypic characterisation of GMPs as carried out in GMP applications.
- Discussed which phenotypic traits are considered relevant for survival, persistence and invasiveness of plants.
- Outlined environmental harm and protection goals relevant in the context of risks of a GMP to persist, outcross or become invasive.
- Discussed the relevance of Genotype x Environment (GxE) interactions and their assessment in current ERA practice.
- Evaluated methodological approaches for plant phenotyping from different research areas.
- Made recommendations for important GM trait- and plant-specific characteristics with relevance for the agronomic and phenotypic characterisation of GMPs.
- Made recommendations how to improve the agronomic and phenotypic characterisation in general, but also specifically with respect to methodological approaches, in order to achieve relevant data and information that can be used for problem formulation in ERA.

Information used in this study was gained from the analysis of agronomic and phenotypic assessments as presented in GMO applications. In addition a literature search regarding relevant plant traits and methods for phenotyping from different basic and applied research areas (e.g. plant ecology, GMO research, plant functional trait concept, invasion biology, plant variety testing, international protocols, e.g. of the European and Mediterranean Plant Protection Organization or the International Seed Testing Association) was carried out. We interviewed and discussed results with experts in the context of two workshops. We finally discussed the outcome and, specifically, the recommendations of this project with national authorities and representatives of EFSA and the European Commission in a stakeholder workshop.

The current practice of the agronomic and phenotypic characterisation of a GMP is mainly restricted to assessments that serve agronomic purposes, such as the assessment of seed quality and of plant traits that are relevant for the crop performance in the field. The chosen endpoints in these assessments follow the respective guidance document. The few additional and optional assessments of plant traits are of limited relevance for environmental risks. In addition, methodological shortcomings in the presented assessments are evident, e.g. laboratory assessments of seed germination and pollen viability or field assessments of biotic and abiotic stressors. In general, an assessment of the survivability of the GMP in and outside the agricultural context is lacking in GMP applications.

Plant phenotypic traits are important for the ability of a plant to survive and persist in the environment. Survivability and persistence as well as invasiveness also depend on other factors than phenotypic traits alone, such as the selective value of novel GM trait(s) and, importantly, the receiving environment.

Observed differences in agronomic and phenotypic parameters between the GMP and its non-GM counterpart should be interpreted in view of their biological relevance. Therefore, a link between the results of agronomic and phenotypic assessments and agronomic and environmental protection goals in ERA is necessary. This improves the informative value of the agronomic and phenotypic assessment and facilitates decision making in ERA.

The currently applied approach to evaluate Genotype x Environment interactions in GMP applications focusses on the assessment of the performance of the GMP under optimal environmental conditions, rather than an assessment of unintended effects of the genetic modification, which become apparent only under stress conditions.

A range of methods for phenotyping plant traits can be identified from different basic and applied research areas, including modern phenotyping methods (e.g. high throughput phenotyping). This report discusses methods for phenotyping and assessment of seeds, plant survival, plant competition and fitness, pollen viability, seed shattering, vegetative growth, and response of the GMP to biotic and abiotic stressors.

Recommendations for improvements comprise general recommendations, recommendations with respect to the assessment approach as well as methodological recommendations. In general, we recommend that basic plant parameters should be assessed in the agronomic and phenotypic characterisation relevant for survival and persistence of plants. These comprise seed germination, seed dormancy, as well as seed and plant survival (including vegetative plant parts) and should be assessed for all crops and types of applications (import and cultivation). In addition, we recommend adapting the study design of agronomic and phenotypic assessments according to relevant exposure scenarios. This implies that for GMP applications including import also non-optimal environmental conditions should be considered in the study design in order to reflect accidental spillage outside agricultural fields. For applications that include cultivation purposes, optimal (i.e. managed field) and non-optimal (i.e. unmanaged field) environmental conditions should be considered to reflect both, cultivation of the GMP in agricultural fields as well as accidental spillage outside fields. We also recommend to define important terms used in the context of the respective risk area such as ferality, persistence or invasiveness in order to delineate spatial and temporal scales during the agronomic and phenotypic assessments.

Another recommendation refers to the need for a case-specific assessment of plant fitness for GMPs with a history of occurrence as feral or wild plant (e.g. in the case of oilseed rape or creeping bentgrass), particularly if these are GMPs with input traits that confer a fitness benefit already under managed conditions (e.g. stress tolerance). For these GMPs, manipulative whole plant experiments under the respective environmental conditions are necessary in order to evaluate potential fitness benefits of these plants also outside agricultural fields.

Further guidance should be developed to derive operable decision-making criteria for the interpretation of observed differences and/or non-equivalences during the agronomic and phenotypic characterisation of GMPs. Together with the selection of indicative traits, parameters and endpoints. This would enable risk assessors to assign biological relevance to observed changes in phenotypic plant traits and provide a link to the problem formulation in ERA. For GMPs for which no comparator exists, a novel ERA approach may be needed. Specifically, for GMPs with complex genetic modifications or traits, e.g. with profound modifications of the morphology or physiology (e.g. *de-novo* domesticated tomato, vitamin B12 maize), *per-se* assessments will become necessary for evaluation of the biological relevance of observed changes. This will also affect the agronomic and phenotypic characterisation and results observed therein.

Suggestions for improvements also refer to the methodology applied in agronomic and phenotypic assessments. In particular, an improved and extended assessment of seed traits, as well as the development of standardized methods for the assessment of short-term persistence of the GMP in field (volunteer assessments) is required. Assessments of Genotype x Environment interactions need to consider environmental stress conditions, together with the choice of appropriate parameters and methods to evaluate the stress response of the GMP. The assessment of the response of the GMP to biotic stressors requires a more focused and consistent approach. An improved assessment approach must ensure a response of the GMP to biotic stressors, e.g. by selection of relevant field trial locations, targeted infestation experiments or the amendment of standard agricultural practices. Suggestions for the selection and assessment methodology of relevant biotic stressors for the crop types considered in this project under EU conditions are proposed. Similarly, the assessment of the response of the GMP to abiotic stressors needs to be improved e.g. by defining relevant abiotic stress conditions (particularly for drought and cold conditions), together with guidance how to assess, monitor and report abiotic stress conditions during the agronomic and phenotypic characterisation. Specifically for stress-tolerant GMPs, additional guidance is needed on how to carry out manipulative stress experiments. Last but not least, modern phenotypic approaches should be explored whether they could support agronomic and phenotypic characterisation of GMPs, specifically if plants with new functional phenotypes are envisaged. Nevertheless, also for new methodological approaches operable decision-making criteria are needed in case changes in phenotypic plant traits are observed.

The suggested improvements are necessary in order to obtain an agronomic and phenotypic characterisation that serves as an appropriate starting point for ERA and supports the risk characterisation of GMPs. An improved assessment of agronomic and phenotypic plant traits should enable conclusions on the GMP's phenotype in and outside the agricultural context and under non-optimal environmental conditions. Such an improved phenotypic plant characterisation is necessary to inform risk assessors about environmental risks by providing first, but substantiated, indications on the GMP's ability to survive and persist in the environment.

1 Introduction

1.1 Background of the study

In the European Union, according to Commission Directive (EU) 2018/350, amending Directive 2001/18/EC as regards the environmental risk assessment of genetically modified organisms, potential risks to human health and the environment have to be assessed before a genetically modified organism (GMO) is deliberately released into the environment. The legislative provisions for the environmental risk assessment are defined in Commission Directive (EU) 2018/350, Regulation (EC) No. 1829/2003 and the Commission Implementing Regulation No. 503/2013.

The environmental risk assessment (ERA) of GMOs comprises the assessment of specific areas of risk of a genetically modified plant (GMP). According to Directive 2001/18/EC and Commission Directive (EC) 2018/350, respectively, this includes “Any change to the persistence or invasiveness of the GMHP (genetically modified higher plant), and its ability to transfer genetic material to sexually compatible relatives and the adverse environmental effects thereof”. Hence, conclusions on the environmental impact from the release or the placing on the market of a GMP shall include information the i) the spread of the GMO(s) in the environment and ii) persistence and invasiveness of the GMO including plant-to-plant gene transfer.

According to Regulation (EC) No. 1829/2003, the European Food Safety Authority EFSA plays a central role in this context and carries out GMO risk assessments in collaboration with the EU Member States. Based on its competence, EFSA publishes a range of different guidance documents for the risk assessment of GMOs, which also include environmental risk assessment aspects (EFSA 2010, 2011a, 2015).

Conclusion on environmental risks of the GMO are tightly linked to the agronomic and phenotypic characterisation the crop plant that is carried out in the context of the comparative assessment of the GMP and its non-GM comparator. This evaluation of agronomic and phenotypic traits of the GM and non-GM crop plants is a starting point in the ERA and usually carried out in field trials. It aims to assess any changes due to the intended genetic modification of the GMO but also unintended effects, which might occur and lead to changes in genotypic or phenotypic traits. For this assessment, EFSA has published a specific guidance (EFSA 2015). The guidance document covers a minimum set of agronomic and phenotypic parameters, which should be assessed to conclude on potential intended and unintended effects (EFSA 2015). In addition, the results of the comparative assessment of agronomic and phenotypic traits of the GMP compared with its non-GM counterpart are used for the conclusion on and characterisation of environmental risks, specifically risks related to persistence and invasiveness of the respective GMP.

1.2 Aim of the study

The aim of this study is to scrutinize if the agronomic and phenotypic characterisation of the GMP, as currently carried out, can inform the environmental risk assessment. The focus is on risks regarding persistence and invasiveness of the GMP, specifically the plant traits and parameters assessed and the methodological approaches used. For this purpose and as a starting point, we scrutinized the current practice of carrying out the agronomic and phenotypic assessment in selected GMO applications.

Another aim of this study is to provide suggestions for improvements of the agronomic and phenotypic assessments with respect to individual crop types and GM traits. Five different plants (maize, oilseed rape, soybean, potato and creeping bentgrass) and four different GM traits (herbicide tolerance, insect resistance, drought tolerance and compositional changes) are addressed. The suggestions refer to the assessment of specific plant traits and characteristics but also to improvements of methodological approaches, based on currently available plant phenotyping approaches as applied in basic and applied research and plant breeding practice.

In addition, the study will discuss different aspects related to the environmental harm due to persistence and invasiveness of a GMP and the definition of thresholds of acceptable effects (i.e. Limits of Concern) in ERA. Such Limits of Concern are necessary as decision criteria in ERA in order to evaluate differences observed between a GMP and its non-GM counterpart and potential adverse effects on relevant protection goals in European agro-environments.

The focus of this study is on the improvement of the agronomic and phenotypic characterisation to support the assessment of risks due to persistence and invasiveness of the GMP. A comprehensive discussion of the assessment of risks relating to persistence and invasiveness of the GMP (see EFSA 2010) is, however, beyond the scope of this study.

1.3 Structure and methodology

This report is structured into different chapters with different purposes.

Chapter 2 provides background information on the agronomic and phenotypic characterisation of GMPs, by outlining the regulatory requirements and the practice of implementation of these requirements as carried out in selected GMP applications.

In Chapter 3, the relevance of phenotypic plant traits for the ability of a plant to become persistent or invasive is discussed based on evidence from the scientific literature and invasion biology.

Chapter 4 discusses environmental harm due to persistence and invasiveness of GMPs and the related protection goals as discussed either in the scientific literature or in EFSA guidance documents.

Chapter 5 discusses the relevance of Genotype x Environment (GxE) interactions for plant phenotypes. Existing concepts from the scientific literature and GMP risk assessment are discussed. Specific links are made to the current practice in GMP applications of selected crops. The appropriateness of the concepts for GMP risk assessment is evaluated and apparent conceptual and implementation gaps are also considered.

Chapter 6 focusses on methods for phenotyping plant traits. It comprises current knowledge and state-of-the-art approaches from general plant ecology literature (specifically from GMP research) but also from research related to functional plant traits and plant trait databases. In addition, methods from plant breeding are discussed. As there is a broad range of phenotypic characteristics in plants, which are of relevance with regard to persistence, invasiveness and plant-to-plant gene flow, the focus was put on selected plant traits, which were considered to be most relevant. For this purpose, the discussion of methodological approaches, but also suggestions for improvements and recommendations refer to:

- The survival of seeds and whole plants (in and outside of agricultural fields) including plant competition and fitness assessments

- The response of the GMP to biotic stressors (e.g. pests, pathogens)
- The response of the GMP to abiotic stressors (e.g. cold or drought stress)

Chapter 7 analyses plant- and GM trait-specific characteristics, which are important for the agronomic and phenotypic characterisation.

Chapter 8 contains recommendations for the ERA with regard to necessary improvements of the agronomic and phenotypic characterisation.

The literature sources used for the analyses differed according to the specific research question in each chapter. Information, data and literature sources used were:

- EFSA guidance documents and GMP applications (Chapter 2)
- Scientific literature databases (Chapters 3, 4, 5 and 6)
- Plant variety protocols from different countries (Chapter 6)
- EPPO protocols (Chapter 6)
- OECD Consensus Documents (Chapter 7)
- Plant trait databases (Chapter 6)
- Expert interviews (Chapters 3 and 6)

In addition, during the research phase two workshops were held in which the preliminary results were discussed with external experts. At the end of the project, a stakeholder workshop was organised to present and discuss the results and recommendations with national authorities of Member States involved in the authorization of GMOs as well as other relevant stakeholders, such as EFSA and the European Commission.

2 Current practice of the agronomic and phenotypic characterisation of GMPs

2.1 Why the plant's phenotype?

The phenotype (from Greek pheno = showing) generally refers to all observable characteristics or traits of an organism. A plant's phenotype covers all levels – starting from the cell level - the transcriptome, the proteome, the metabolome - to diverse morphological and functional aspects of the plant (e.g. plant biomass, root morphology, leaf characteristics, and fruit traits). Moreover, this term may also refer to (bio)chemical characteristics (e.g. secondary metabolites or volatile organic compounds) and other functional traits (e.g. Martin and Isaac 2015; Costa et al. 2018).

In the context of risk assessment of GMPs, the phenotype is important when assessing risks for human health and the environment as the underlying genetic modification of the GMP may not only have intended effects (e.g. the expression of a novel protein), but also unintended phenotypic effects (EFSA 2010). Hence, for GMPs, not only the assessment of the novel (introduced) trait but also of the whole plant is crucial to detect such unintended effects, which may also vary with changing environmental conditions (Hilbeck et al. 2011). This is also important as GMPs are produced by using novel genome editing techniques (e.g. SDN1, SDN2) introducing modifications in the genome without the insertion of genes conferring a novel trait (e.g. a toxin) or with simultaneous modification or knock out of multiple genes with possible unintended effects (Kawall et al. 2020).

A key question in this context is how the phenotype affects the environmental performance of a GMP, including its ability to spread in the environment, to become persistent or invasive or to hybridise with wild relatives. This question requires considering the whole plant and the whole phenotype including all plant characteristics, which can contribute to these processes. In this report, the focus is on vegetative and generative traits of the plant, which may affect the ability of the GMP to spread, outcross, and persist in the environment. This includes all characteristics that can be assessed under agronomic conditions, e.g. by field-testing or under contained conditions (greenhouse). In addition, specific aspects will be addressed that require testing in the laboratory (e.g. seed testing).

2.2 Assessment of agronomic and phenotypic traits from the regulatory perspective

The GMP`s phenotype has to be characterised in the context of the agronomic and phenotypic characterisation of the GMP according to the regulatory provisions in the EU (see 1.1). This characterisation of the GMP serves – together with the compositional and the molecular characterisation of the GMP – as the starting point in the ERA as part of the "comparative safety assessment" (EFSA 2010). The ERA guidance recognises that, specifically, the assessment of agronomic or phenotypic traits is one source of data that can provide information on unintended effects, e.g. linked to morphological alterations. Such changes in specific agronomic or phenotypic characteristics can be indicative for an altered weediness or invasive-ness and thus for the potential of the GMP to cause environmental harm (EFSA 2010).

To support the agronomic and phenotypic characterisation of the GMP, EFSA has published a specific guidance document (EFSA 2015). It supplements the general ERA recommendations outlined in EFSA (2010) and Regulation (EU) No 503/2013 and focuses on data collected in field trials. Also in this guidance, EFSA emphasizes the importance of the agronomic and phenotypic assessment providing information relevant to the assessment of persistence and invasiveness of GMP, which can indicate a change in persistence and/or invasiveness of the plant (EFSA 2015). In this context, EFSA (2010 and 2015) refers to predictive plant traits with relevance for persistence and invasiveness considered relevant for specific species which can persist in agricultural fields (e.g. potato, oilseed rape) and/or species which are able to establish temporary or persistent feral populations, e.g. oilseed rape (Table 1).

Other specific recommendations for the agronomic and phenotypic characterisation include recommendations for field-testing, such as the selection of test sites and test materials, the quality and design of field trials, the selection of relevant agronomic and phenotypic endpoints and the data analysis (EFSA 2015).

For the appropriate characterisation and description of the biology of the plant as well as information on its performance under representative environmental conditions, a range of plant characteristics (endpoints) are to be measured. These should take the objectives of the agronomic and phenotypic characterisation, the biology of the crop species, the novel trait intentionally introduced as well as the scope of the GMP applications into account (EFSA 2015). These agronomic and phenotypic characteristics referred to in the guidance are:

- plant vigour
- growth and development
- morphology
- yield
- crop characteristics
- pest and disease susceptibility
- fertility
- seed and pollen characteristics

The guidance distinguishes between:

- Generic endpoints: these endpoints shall always be measured in accordance with the scope of the GMP application.
- Case-specific endpoints: those are crop or trait related. Applicants can decide on a case-by-case basis whether they are considered further. In that case, a scientific rationale has to be provided by the applicant justifying inclusion or exclusion.

2.2.1 Assessment of generic endpoints

For import/processing for food and feed uses, the following generic endpoints are recommended by EFSA (2015):

- Seed characteristics
- Early stand count
- Days to flowering
- Lodging
- Final stand count

- Plant height
- Days to maturity
- Fruit count
- Seed moisture
- Seed weight
- Yield
- Biotic interactions
- Abiotic interactions

For applications for cultivation, the list of generic endpoints is complemented with:

- Crop development (as a measure of vigour and vegetative growth)
- Duration of flowering (to assess differences in the duration of the flowering phase)
- Seed loss (as an indicative endpoint for the potential of a plant to build up a seed bank, emphasizing the possible correlation between seed loss and relative survival and the occurrence of volunteers or feral GM plants).

The guidance includes details for the assessment of the agronomic and phenotypic endpoints measured in field trials, e.g. the phenological or growth stage of the plant, the type of measurement (e.g. visual estimation or measurement), the unit to be used and methodological recommendations.

Seed characteristics

For the assessment of seed characteristics, EFSA refers to laboratory studies that should be performed to demonstrate the quality of the seed used in field trials. In this context, the GMP and the conventional plants should follow international rules for seed testing set by the International Seed Testing Association (ISTA 2015) when testing for seed health, germination and seed viability/vigour.

Biotic/abiotic interactions of the GMP

The biotic and abiotic interactions of the GMP are two of the generic assessment endpoints listed above. For cultivation applications, a cross-reference is made to EFSA (2010).

Abiotic stress

The evaluation of the plant's response to abiotic stress, applicants should routinely record damage due to abiotic stress. Specific attention should be given to GMPs with traits intended to reduce the susceptibility of the plant to a defined stressor, e.g. by including case-specific endpoints by the applicant. The specific assessment method should be justified by the applicant. Damage should be assessed at plot, plant and organ levels and likely causes defined. In addition, damage to leaves, stems and reproductive structures that are related to abiotic stressors should be recorded visually. EFSA (2015) provides some general examples as well as specific aspects related to herbicide and pesticide injury.

Biotic stressors

Guidance for the assessment of plant responses to biotic stressors is only provided for applications for import/processing for food and feed uses. For cultivation purposes, EFSA (2015) refers to the general ERA guidelines of GMOs (EFSA 2010).

For applications for import/processing, EFSA (2015) states the following:

- Common pest species should be assessed, considering the expected or actual presence of plant pests locally (considering the feeding mode and the biological/economic relevance); assessment methods of arthropods will vary depending on the species and should be determined on a case-by-case basis.
- All "relevant" plant diseases should be measured or estimated visually (disease incidence or severity per plot), considering diverse categories of etiological agents (virus, fungus, bacterial) and/or mechanisms of pathogenesis as well as the biological and economic impact of the respective disease. The applicant must provide a justification of the selection of the assessed diseases.

For applications for cultivation of the GMP, the general ERA guidelines (EFSA 2010) foresee an assessment of interactions of the GMP with target organisms (TOs). In this assessment, the focus is on the specific pest or pathogen species being the target of the genetic modification. All other organisms are considered non-target organisms. The assessment of interactions of the GMP with non-target organisms (NTOs) addresses potential impacts of the GMP on population levels of non-target biotic stressors such as herbivores, parasites and pathogens. A representative subset of NTO species, referred to as "focal species" shall be selected on a case-by-case basis. In this context, a stepwise selection procedure is recommended, considering relevant functional groups. At least one focal species from each relevant functional group should be further considered in the ERA. No further provisions are specifically made for biotic stressors.

In addition, the stage 1 information ERA requirements for assessing persistence and invasiveness of the GMP in the general ERA guidelines (EFSA 2010) include information on the response of the GMP to naturally occurring insects, diseases and/or abiotic stressors (e.g. heat, drought, excess of water). These must be provided for all GM plant applications, independent of the scope of application (EFSA 2010).

2.2.2 Assessment of case-specific endpoints

In addition to the generic endpoints, case-specific endpoints can be included by applicants. EFSA (2015) considers the following categories of case-specific endpoints to be taken into account:

- Trait-specific endpoints (depending on the intentionally introduced GM trait)
    - The assessment of the efficacy of male sterility or altered pollen characteristics (for male sterile GMP).
    - Additional endpoints associated with the intended compositional change, e.g. pest susceptibility for GMP with altered levels of anti-nutrients (for GMP with altered composition).
    - Assessment of further pollen characteristics (for GMP with specific pollen characteristics).

  - Field trials over a natural gradient of the stressor or through local manipulation (for GMPs resistant to abiotic stress).
- Endpoints related to potential unintended effects (e.g. potential unintended effects identified in the molecular characterisation)
- Endpoints related to persistence and invasiveness

Assessment of endpoints related to the persistence and invasiveness of the plant

Additional measurements relevant to the assessment of persistence and invasiveness of the GMP may be needed, depending on the plant species, the intended traits and the scope of the application. In this context, EFSA (2015) refers to important plant characteristics that may be predictive for the persistence and invasiveness (see also Table 1). Indicative plant traits are:

- the ability to make a long-lived soil seed bank
- small seeds
- a short vegetative period before seeds are produced
- a long flowering period
- a very high seed output
- seed shattering

Measurements are recommended for species that can persist in agricultural fields (e.g. potato, oilseed rape under cultivation conditions) and/or such species able to build feral populations (oilseed rape under cultivation or import conditions). In this context, the importance of the ability of the GMP to build up a persistent seed bank is emphasized by EFSA (2015) and a link is made to the staged approach of the persistence/invasiveness assessment as outlined in EFSA (2010). For species without the ability to persist in agricultural fields and/or establish feral populations (e.g. maize, soybean under EU conditions) such additional measurements are not considered necessary. Suggestions for additional experiments are also made, e.g. for seeds or for the whole GMP (Table 2).

Tab. 1: Typical plant characteristics that affect the vegetative or reproductive phenotype of the plant and are indicative for the persistence and invasiveness of the GMP (according to EFSA 2010, 2015).

Life cycle stage	Plant characteristic
seedling	plant establishment growth rate/duration
	early ground cover
mature plant	plant vigour growth rate/duration (period)/development
	plant size/height/biomass/yield/dry matter
	flower biology/time to flowering or maturity/flowering period
	fertility/vernalisation requirement attractiveness to pollinators

Life cycle stage	Plant characteristic
	pollen shed/viability/compatibility/morphology
	seed dispersal ability/seed shatter ability
seed	seed size/morphology/moisture
	seed number/weight
	seed longevity/survival
	seed germination characteristics
	primary/secondary dormancy
	volunteers in subsequent crops
all stages	response to naturally occurring insects and pathogens (biotic) response to abiotic stress (heat, drought, and excess of water)

Tab. 2: Experiments suggested in the context of the assessment the persistence and invasiveness of the GMP (according to EFSA 2010)

Life cycle stage	Suggested experiments
seed	Seed burial experiment
	seed germination in growth chamber experiments or field trials (under various conditions)
	seed dormancy potential under controlled conditions (viability testing of dormant seeds)
	seed survival under field conditions (viability testing of buried seeds and survey of volunteers in subsequent years)
whole plant	plant vigour testing under (extreme) environmental conditions
	biotic and abiotic stress responses (stress response tests under greenhouse conditions with diff environmental conditions)
	surveys of feral (GM) plants
	surveys of volunteer (GM) plants in subsequent years in field trials (left unmanaged)
	vegetation competition studies
	manipulative field experiments
	fitness experiments (glasshouse, growth chamber and microcosm)
	population modelling

2.3 Assessment of agronomic and phenotypic traits - the applicants' practice

In this chapter, the practice of assessing agronomic and phenotypic traits as carried out by GM applicants is outlined, based on the selection of 17 GMP applications in the EU. For detailed results of the analysis, see Annex A).

The studies provided by the applicants comprise ecological and environmental interactions of the GMP, germination and dormancy, pollen characteristics and volunteers. The main results are:

- The assessments including endpoints used exclusively aim at the evaluation of the agronomic performance of the GMP.
- The conclusions regarding risks with respect to persistence and invasiveness of GMPs are based on assessments of a limited number of agronomic and phenotypic traits.
- No rationale is provided for the selection of assessments or endpoints.
- A range of methodological shortcomings in the assessments are evident:
    - The use of standard germination tests do not give an indication of the ability of the GMP to germinate under suboptimal (field) conditions.
    - The survivability of the GMP is not assessed.
    - Pollen characteristics are assessed under laboratory conditions only.
    - The assessment of biotic stressors is based on observation of their random occurrence in combination with qualitative ratings of the plants' response. Artificial infestation studies are generally not carried out.
    - As for biotic stressors, the assessment of the GMPs' response to abiotic stress is also based on observation of rather than experimental approaches. Prevalent stress conditions were not systematically measured and reported. Generally, manipulative experiments are not carried out.

2.4 Conclusions

The assessment of the plant phenotype is important for the ERA of GMPs as it affects the environmental performance of a plant, including its ability to spread, survive, become persistent or invasive, or hybridise with wild relatives.

According to regulatory provisions and ERA guidance, the GMP's phenotype has to be characterised during the agronomic and phenotypic assessment, serving as a starting point for the ERA. The rationale behind is that thereby unintended effects due to the genetic modification can be detected which can also indicate alterations in survival, spread, weediness or invasiveness of the GMP. The corresponding guidance document recommends a range of assessments and experiments that could be indicative for such changes and therefore environ-mental harm. However, these are optional and the decision to carry out these assessments is left to the applicant.

In ERA practice, the agronomic and phenotypic assessment focusses on a few plant traits and assessments, which are important to demonstrate the agronomic performance of the GMP. The assessments made are only of limited usefulness to indicate potential changes in the GMP's ability to survive, spread or persist in the environment. In addition, considerable methodological shortcomings are evident, in particular to with regard to the assessment of environmental interactions of the GMP (biotic and abiotic stressors).

3 Phenotypic traits indicative for persistence and invasiveness

3.1 Terminology

Terms related to persistence and invasiveness of plants are often used in the context of GMO risk assessment. These terms are, however, not used in a consistent way and may lead to diverging interpretations of the reader. For this reason, and to achieve a common understanding, the following definitions of certain terms are provided.

Volunteer (weeds): Plant that grows within the agricultural field (in season after a crop had been cultivated), derives from seed of a crop plant (before or during harvest), and thrives through max. one to two seasons (Gressel 2005).

Weed: Plant that grows predominantly in situations disturbed by man, e.g. on agricultural land (agrestals), on disturbed land such as waste places, along roadsides (ruderals) (see references cited in Gressel 2005)

Feral plant: Plant that grows in semi-natural or natural habitats, derives from crop plants (volunteers or seed loss), is in part or fully de-domesticated. It is not dependent on management measures, shows cultivation-independent reproduction and can persist outside arable fields (Gressel 2005). The ability to build up self-sustaining populations should last for at least three years (according to Huiting et al. (2018).

Persistence: The ability of a plant to build up sustained and permanent populations, which are no longer dependent of the supply of diaspores from crop cultivation (Kowarik et al. 2008a).

Invasiveness: This terminology is derived from invasion biology (see Chapter 5.3.1), referring to non-native species but can have a different meaning. According to the Convention on Biological Diversity, an invasive species refers to a non-native species, which threatens biodiversity. Also in EU legislation, invasiveness refers to an organism outside its natural range, which threatens biodiversity, or ecosystem services (Regulation (EU) No 1143/2014). In scientific literature, however, invasive species often refer to the spread of non-native species without the potential for adverse effects on biodiversity (Pyšek et al. 2004). Others have defined “invasive species” as those species that increase in numbers and spread, in addition to outcompeting other species for resources (Hancock 2003).

Introgression: refers to “the permanent incorporation of genes from one set of differentiated populations (species, subspecies, races) into another” (Stewart et al. 2003)

Fitness: refers to the potential evolutionary success of a genotype, which is defined as the reproductive success or the proportion of genes that an individual leaves in the gene pool of a population. The individuals with the greatest fitness leave the largest number of offspring (Stewart et al. 2003). In the context of environmental risk assessment of GMPs, fitness is defined as the number of seeds (or propagules) produced per seed sown, and includes the whole life cycle of the plant (EFSA 2010, referring to Crawley et al. 1993).

3.2 Persistence and invasiveness – evidence from scientific literature

Environmental risks due to persistence and invasiveness of a GMP in the environment are strongly linked to the plant’s ability to become a volunteer or a feral plant. The build-up of a volunteer population (per definition occurring in arable fields, not deliberately planted) or of a feral population outside agricultural fields (e.g. semi-natural habitats, ruderal sites), may need management methods (other than with non-GM) that cause harm to the environment, e.g. due to exacerbated weed problems (EFSA 2016). In addition, the adaptive introgression of transgenes into weed populations could alter (the relative) persistence or invasiveness of the feral plant or crop-wild hybrids and cause adverse environmental impacts, such as extinction of wild taxa (for discussion see Ellstrand et al. 1999, Guadagnuolo et al. 2006).

Gressel (2005) describes the process of de-domestication of crop plants to volunteer and feral plants (Figure 1). Feral plants are more likely to evolve from volunteer weeds. This may be facilitated by species de-domestication on its own, e.g. back mutations (endoferality) or by gene flow from sexually compatible wild relatives (exoferality). While the volunteer thrives in agricultural fields or nearby semi-natural habitats (e.g. field margins) with human intervention, the feral plant itself may thrive in disturbed or ruderal habitats or in undisturbed, natural habitats.

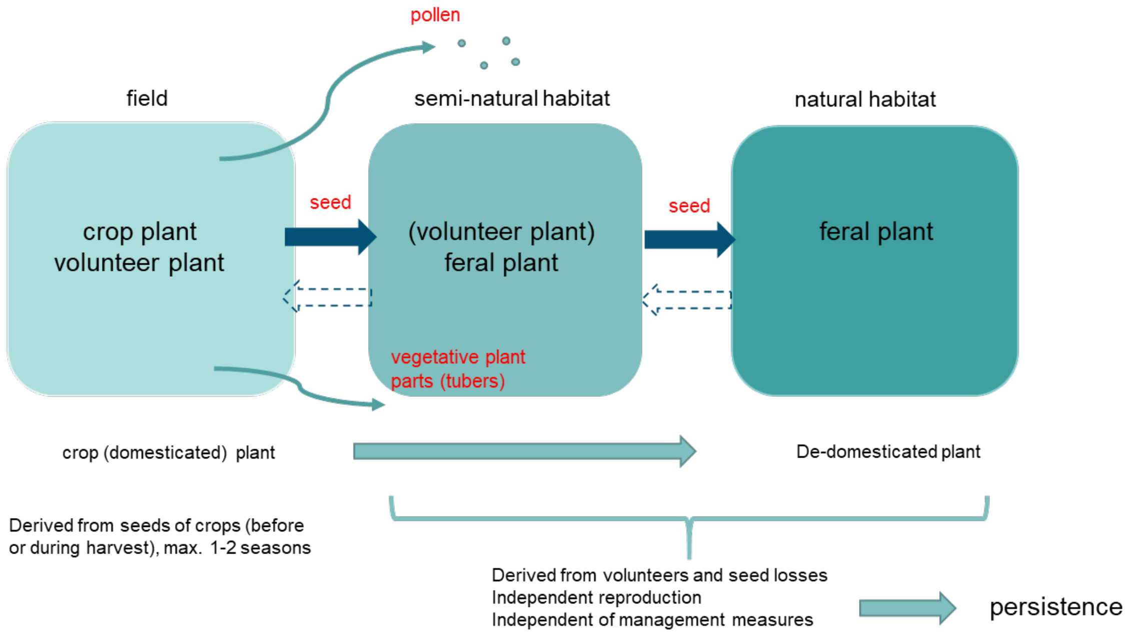

Fig. 1: The process of de-domestication of crop plants to feral forms (modified after Gressel 2005).

In his review, Gressel (2005) summarizes the process of crops becoming weeds and ferals in general by use of several examples. The author describes traits that are so called “weedy or invasive” traits as opposed to domestication traits and traits of wild non-weedy plants. In addition, other authors have discussed plant traits that are considered relevant for a plant to establish or become persistent in the environment, specifically with reference to GMPs (Table 3).

Ellstrand (2018) describes 14 cases of free-living GM populations of crop plants, among other *Brassica napus*, *Brassica rapa*, *Agrostis stolonifera*, and *Zea mays*. For some of these cases, environmental or agricultural problems have been described, such as multiple herbicide tolerant oilseed rape in Canada, herbicide tolerant creeping bentgrass in the US or glyphosate-resistant *B. rapa* in Argentina (Ellstrand 2018, Pandolfo et al. 2016). In most cases, seed dispersal was the dominant mechanism that lead to dispersal and establishment of populations, however, in two cases pollen flow was also involved (e.g. in creeping bentgrass in the US and multiple herbicide-tolerant canola in Canada). A pre-existing tendency for ferality in a particular crop type facilitates the establishment of GMPs and their transgenes in free-living populations, which is a consequence of certain biological traits. In addition, the selective value of the GM trait plays an important role, which itself depends on the respective environment in which the plant thrives (Ellstrand 2018). Already one single trait alteration can affect plant competitiveness, as was shown with the American chestnut tree, which lacked resistance to a pathogen (Andow 1994). Ellstrand et al. (1999), Ellstrand (2018) and Le Corre et al. (2020) discuss adaptive introgression of (transgenic) crop traits into wild relatives.

Tab. 3: Plant traits depending on developmental stage that are considered “weedy traits” or “feral traits” (after Gressel 2005, Claessen et al. 2005b, Bagavathiannan and van Acker 2008, Ellstrand 2018).

Plant stage	**trait**
Seedling/vegetative	Seedling survival
growth stage	Rapid growth to flowering (annual plants)
	Vigorous vegetative reproduction (perennial)
	Short vegetative phase
	Competitive ability
	Growth plasticity
	Adaptation to disturbed habitats
Mature plant/generative	Seed production under a wide range of environmental conditions
growth stage	Seed shattering/easy distribution of seeds
	Continuous flowering/seed production
	High seed output/fertility
	Pollen distribution over large distances
	No obligate selfer (but self-compatible) or apomictic
	Unspecialized pollinators
	Deep root system
	Sexual and vegetative reproduction
	Allelopathy
Seed characteristics	Small seed size
	Seed survival/longevity of seeds/seed bank
	Seed dormancy/secondary dormancy
	Broad germination requirements
	Long-distance dispersal of seeds
	Bitter substances in seed (or fruit)

While seed dispersal is one of the major mechanisms to enable persistence and establishment of GMP in the environment, gene flow between GM crops and wild relatives is also important and has extensively been studied since the introduction of GMPs. Ellstrand et al. (1999) provide one of the first syntheses on gene flow between domesticated plants into wild relatives and its evolutionary consequences. The authors summarize the spontaneous hybridization between the most important food crops and their wild relatives. Such gene flow can be neutral, detrimental or beneficial for a crop-wild hybrid population. Potentially harmful consequences of such gene flow, e.g. the evolution of more aggressive weeds and increased weediness in weedy relatives is well documented in wild relatives of 7 of the world`s 13 most important crops (Ellstrand et al. 1999). Hybridisation between species or between different populations of a species can serve as a stimulus for the evolution of invasiveness of non-native plants (Ellstrand and Schierenbeck 2000). This is also the case for invasive *Helianthus annuus* which evolved after intertaxon hybridization as well as for other agriculturally relevant taxa such as Beta vulgaris, *Oryza sativa* or *Raphanus raphanistrum*, all having weed status (Schierenbeck and Ellstrand 2009). Ellstrand et al. (1999) describe another potential problematic consequence of gene flow, the risk of extinction of rare wild relatives, in two out of 13 crop species.

In general, the ability and extent of plant-to-plant gene flow depends on the biology of a certain taxon (e.g. crop species) and the presence of wild relatives. Several crop plants have been assessed for spontaneous hybridization (see e.g. Ellstrand et al. 1999, Stewart et al. 2003). Maize can hybridize with the interspecific wild relative teosinte. For oilseed rape (*B. napus*) there is good molecular evidence for introgression into field mustard (*B. rapa*), while hybridization with other relatives is either rare, e.g. *Raphanus raphanistrum*, or absent for *Sinapis arvensis* or *Erucastrum gallicum* (Warwick et al. 2003). Hybridization between *B. napus* and *B. rapa* has been estimated to be extensive by some authors (Wilkinson et al. 2003) but others could not confirm the presence of advanced generation backcrosses (Luijten et al. 2015). For soybean, there is evidence for hybridization with wild soybean *Glycine soja* (Guan et al. 2015; Kim et al. 2019). Ellstrand (2018) considered that crops with a pollen dispersal of 1 km or more can contribute to transgene escape to wild populations. Field-based experiments are generally used to measure spontaneous hybridization rates between crops and wild relatives (Ellstrand et al. 1999). The authors propose that only experimental stands of crop and weeds under realistic conditions can realistically assess the extent of crop-wild hybridization. Sample sizes must be large enough to detect gene flow also at low levels. When addressing effects on rare species, measurements of hybridization rates as well as estimation of population sizes of these species are critical (Ellstrand et al. 1999).

Gene flow is generally more effective from crop to wild (feral) populations, although this cannot be generalized (Gressel 2005). For example in maize, gene flow was thought to occur mostly from teosinte to maize (Ellstrand et al. 1999), however, recently genetic introgression of two adaptive traits of European maize varieties into weedy European teosinte populations has been shown (Le Corre et al. 2020).

Assessing plant-to-plant gene flow and hybridization alone does not allow an evaluation of the ecological impact of the GMP as the formation of further backcross generations is often uncertain (Luijten et al. 2014). Therefore also the hybrid fitness and the reproductive ability of further generations is critical to determine the impact of crop-wild introgression, e.g. by comparing the fitness of weed-crop hybrids with that of the weedy parents under field conditions (Ellstrand et al. 1999). The fitness of different hybrid generations (F2, BC1 or higher back-cross generations) is critical for a successful introgression, as F1 hybrids often have reduced fertility and fitness which is restored in later generations (Warwick et al. 2008). On the contrary, some F1 hybrids are as fit as their parents but later generations have reduced fitness (Hauser et al.

1998a; Hauser et al. 1998b). In the few studies Ellstrand et al. (1999) evaluated, the fitness of the hybrids tended to be higher than that of the wild parent. Introgression of crop genes into populations of wild relatives is a dynamic process with several hybrid generations, which can take many years before a transgene is fixed in the target population and stable introgression occurs (see overview in Stewart et al. 2003). The recently described introgression of crop genes from maize into teosinte resulted in higher fitness of first generation hybrids (Guadagnuolo et al. 2006) and in beneficial traits that enabled the establishment of a new weed (Le Corre et al. 2020). The persistence of a transgene over a period of 6 years in a weedy *B. rapa* population has been shown by Warwick et al. (2008).

Annex E (Table 25 and Table 26) provides an overview of selected studies on plant fitness, persistence, survival or weediness of GMPs.

3.3 Persistence and invasiveness – experience from invasion biology

In this chapter, the relevance of phenotypic traits for the invasiveness of a plant is discussed, based on assessment frameworks for alien species (see Annex B) as well as the scientific literature.

3.3.1 Terminology in invasion biology

As already mentioned, the terms “persistence” and “invasiveness” are not defined in the context of the environmental risk assessment of GMOs, neither by the regulative framework, nor by EFSA in its respective guidance documents. When using these terms for GMO risk assessment, it needs to be kept in mind, that these are used differently in other contexts. Invasion biology uses the terms “establishment” and “spread” (see below). In the context of invasion biology, establishment of an alien species occurs before it spreads, while in the GMO context a GM plant spreads from its original habitat (e.g. agroecosystem) to natural habitats where it can potentially build up a population. Respective definitions were developed in the context of the Convention on Biological Diversity, the IUCN and to some extent in Regulation (EU) No 1143/2014 on the prevention and management of the introduction and spread of invasive alien species (see also Blackburn et al. 2014). In these policies, invasiveness relates to harm to biological diversity and ecosystem services. In the following, some important definitions are provided:

Alien species: refers to “a species, subspecies or lower taxon, introduced outside its natural past or present distribution; includes any part, gametes, seeds, eggs, or propagules of such species that might survive and subsequently reproduce” (CBD).

Invasive alien species: means an “alien species whose introduction and/or spread threaten biological diversity” (CBD).

Establishment: refers to “the process of an alien species in a new habitat successfully producing viable offspring with the likelihood of continued survival” (CBD).

Widely spread: means an “invasive alien species whose population has gone beyond the naturalisation stage, in which a population is self-sustaining, and has spread to colonise a large part of the potential range where it can survive and reproduce” (Regulation (EU) No 1143/2014, Article 3 (16)).

3.3.2 The relevance of individual plant characteristics in invasion biology

In invasion biology, no set of plant characteristics has been defined or agreed upon so far that specifically promotes invasion. However, many authors discuss aspects that are associated with establishment and spread of a species or subspecies. This includes characteristics that are likely to make a plant becoming a weed. This was already discussed in the 1960s by Baker, who published a list of plant characteristics that characterised the ideal weed (Baker 1965 cited in Kos et al. 2012). However, this list was based on the characteristics of only a few weedy species (Sutherland 2004). However, although research is still ongoing, this list is often cited. In the context of GMOs, Kos et al. (2012) discussed plant characteristics that could be used for pre-screening weediness. Based on the list of Baker and other literature, they also defined 17 plant characters as relevant for weediness.

Sutherland (2004) assessed whether there are specific life history traits for weeds. According to the author, the weedy plants need to have a competitive advantage over non-weeds. He compared weeds and non-weeds based on information provided by several weed-databases in the US and identified ten life history traits, i.e. vegetative reproduction, breeding system, compatibility, pollination system, shade tolerance, habitat, life span, life form, morphology and toxicity. In general, weeds were more likely to be annuals or biennials and adapted to wetlands, armed (e.g. plants with thorns) and toxic. Not all of Baker's predictions were supported by their results, as some traits, e.g. vegetative reproduction, breeding system, self-compatibility, or wind pollination, were found not to be significant for weeds.

In general, drawing conclusions on individual plant characteristics and their relevance for invasiveness is challenging, because different studies examine different plant characteristics and parameters. Although a vast number of publications are available on this topic, their results vary and can be contradicting. In addition, the spatial scale or study design and the statistics applied differ.

In addition, comparisons are not only performed between invasive and non-invasive alien species, but also between invasive and native species (native in the invaded area) or invasive species and native species that are themselves being invasive elsewhere. Other common garden experiments compare individuals of the invasive population of a certain species with individuals from the original population. In addition, the number of species included in meta-analysis comparing various plant characteristics vary. It has been suggested that characteristics promoting invasiveness could depend on a certain taxonomically or ecologically defined group (Pyšek et al. 2014).

It also seems to make a difference which geographic area is examined. Van Kleunen et al. (2010) suggest that differences in plant characteristics between invasive and non-invasive alien species depend on the climatic region. Available studies differ also in the area studied: the invaded area or the native area of an invasive species. In addition, it remains often un-clear whether identified characteristics directly confer invasiveness or only correlate with it.

According to Divíšek et al. (2018), results regarding whether or not a plant characteristic promotes invasiveness of a plant species are contradicting because different plant characteristics are important in the different phases of becoming invasive. For the successful introduction into a habitat, other characteristics might be crucial than for later processes of invasion. Certain plant characteristics may enable a non-native species to establish and persist in one particular habitat. However, those characteristics may not provide an advantage in another habitat.

As summarised by Milanović et al. (2020), certain characteristics promote the success of alien species. For example, the specific leaf area (SLA) is related to stress tolerance, height and seed size is related to environmental disturbance, and height is also related to competitiveness. The flowering period is also important with early or longer flowering providing an advantage. In addition, self-pollination can support the spread of neophytes.

In a meta-analysis of garden experiments, van Kleunen et al. (2010) assessed six categories of plant characteristics and related them to the performance of a plant. Those were physiology, leaf-area allocation, shoot allocation, growth rate, size and fitness (e.g. number of flowers or seeds per plant, per flower head, per inflorescence, per fruit; seed germination traits; survival). Overall, invasive alien species showed higher values and clear differences for the six categories of performance-related plant characteristic than non-invasive species (van Kleunen 2010).

Jelbert et al. (2015) showed that invasive plants are larger than their non-invasive relatives. In addition, they produce more seeds and so show higher fecundity. The basis of their comparison were data from five pairs of plant species generated in their native range and not in the invaded area. Baker (1965 cited in Jelbert et al. 2015) also considered growth and fecundity when describing weed characteristics.

Hejda et al. (2009) reported that many invasive alien species originating from Europe populate disturbed and eutrophic habitats in their native range being rapidly growing and spreading by very effectively using resources. Native and invasive species, which have a negative impact on biodiversity and species richness show high competitiveness, and can successfully spread and persist in a new area (e.g. forming rhizomes, showing fast juvenile growth, high number of seeds or low seed weight). Another important plant characteristic is the root-shoot ratio. Such plants benefit from land use changes, which can result in eutrophic or homogenous habitats. Changes affecting resource availability also support the invasion success of alien species.

Invasiveness is not only discussed on the species level, but also on the level of intra-specific and inter-specific entities (e.g. cultivars, hybrids; Datta et al. 2020). The underlying reason is that invasion is considered to take place on the level of a population and that the risk for becoming invasive can differ between such entities (e.g. a cultivar). In order to separate invasive genetic entities from less invasive but closely related genetic entities, a set of six questions determining the risk of invasion posed by a cultivar or hybrid is proposed by the authors, focussing on ornamental plants. This includes the discussion of differences in plant characteristics between a cultivar or a hybrid that is considered safe and the corresponding invasive species. These can be vegetative (e.g. leaf size, height, growth form) or reproductive characteristics (e.g. number of fruits or seeds). Relevant characteristics are those related to fecundity, such as pollination length of flowering time, number of flowers, fertilisation, seed production, germination success, survival rate, and vegetative reproduction as well as allelopathic potential, mycorrhizal mutualisms and defence mechanisms against herbivores. In order to assess whether the differences are spatially and temporally stable, Datta et al. (2020) recommend long-term common garden experiments under different conditions.

3.3.3 The role of habitat and environment for invasiveness

The success of an alien species and the possibility to become invasive depend not only on specific plant characteristics, but also on environmental factors like characteristics of the new habitat and its plant community. Thus, not only the invasiveness of species are discussed but also the invasibility of communities. Richardson and Pyšek (2006) give an overview on respective invasiveness concepts.

Divíšek et al. (2018) compared invasive and non-invasive alien plants, focusing not only on plant characteristics, but considered also the habitat with its native species and the available niches therein. They considered that functional traits of an alien species may be relevant for a certain habitat but may not be important in another. They showed that the invasion process is not only determined by the characteristics of the alien species but also by the characteristics of the invaded community. For six habitat types in temperate Central Europe, they demonstrated that non-invasive alien plants are functionally similar to native species occurring in the same habitat type. In contrast, invasive alien species (those spreading from the introduction site) are functionally different.

In order to be able to establish and persist in a habitat, alien species need so share some characteristics with native species occurring in the same habitat (depending on its characteristics). However, in order to become invasive (expand, spread or become dominant), alien and native species need to be different enough. This difference allows the alien species to occupy novel niche space in the respective habitat or the alien species must be able to out-compete the native species. In Divíšek et al. (2018) the invasive species studied mostly occupy the periphery of the functional trait space represented in each of the studied habitats. Of the three traits examined (specific leaf area, maximum plant height, seed weight), maximum plant height was the most important factor suggesting that stronger competitive ability is an important factor for an alien species becoming invasive.

Milanovic et al. (2020) assessed interactions between invasive species and the environment, covering native plant species, archaeophytes (introduced before 1500) and neophytes together with the environmental factors climate, land cover and bedrock. They concluded that the success of alien species depends on the environment. Compared to native species neophytes showed a strong relation to environmental factors for several plant characteristics (e.g. specific leaf area, storage organs and beginning of flowering). This relationship was strongest in invasive plants. Neophytes where mostly affected by climate and geology.

Interactions between native and alien species may also be influenced by climate change as presented by Dukes et al. (2011). Altered environmental factors may have a positive effect on the invasiveness of a plant species. In their field and mesocosm studies, they studied the response of *Centaurea solstitialis* (a weed species in North America) to five environmental factors and showed a positive response (increased biomass and height) to elevated CO_2 levels and nitrate deposition. Native plant species in the same area responded less or not at all (Dukes et al. 2011).

3.3.4 Phenotypic plasticity and invasiveness

The phenotype of an organism is not only the result of its genotype; the phenotype can vary in response to environmental factors. Phenotypic plasticity is the variation of a phenotype under different environments.

In a meta-analysis, Davidson et al. (2011) compared invasive and non-invasive plant species regarding their phenotypic plasticity. Their result shows that invasive species show higher phenotypic plasticity for all characteristics evaluated (biomass, nitrogen content, and nitrogen use efficiency, phosphor content, photosynthesis, root biomass, relative growth rate, root-shoot ratio, shoot length, specific leaf area and water use efficiency). However, in response to increasing resource availability this resulted only sometimes in fitness benefits. Under resource-limiting conditions, non-invasive species performed similar or better. The authors, however, therefore called for caution regarding the interpretation of the results regarding fitness effects due to data limitations.

In a common garden experiment, Caño et al. (2008) compared the performance of an invasive population of *Senecio pterophorus* with a population from the native area. Based on experimental changes of disturbance (no vegetation) and water availably, fitness traits (survival, flowering, number of seed heads), leaf parameters related to fitness and chlorophyll florescence parameters were measured. The result showed that the invasive population performed better compared to the native population from the original area (e.g. higher biomass, greater reproductive fitness) and showed higher plasticity of fitness traits. The authors concluded that the genetic differentiation between the original population and the invasive one played a role in whether or not a plant becomes invasive. Also experiments with the invasive grass *Imperata cylindrica* conducted by Hiatt and Flory (2020) showed greater phenotypic plasticity in invasive populations than the six native species tested.

In addition to the native or new environment, also management practices (e.g. water supply) can influence the phenotypic plasticity and the adaptive potential as shown in greenhouse experiments with *Brassica tournefortii* (Alfaro and Marshall 2019). Differences in phenotypic plasticity were found in phenology, leaf morphology, branch architecture, size, and reproduction between native, invasive, and landraces of *Brassica tournefortii*.

3.3.5 Hybridisation as a pathway to invasiveness

Ellstrand and Schierenbeck (2000) and Schierenbeck and Ellstrand (2009) discuss the role of hybridisation after the establishment of a species (between species of between different populations) in the evolution of invasiveness. They provide several examples where hybridisation was followed by invasiveness of the population. Hybridisation can lead to adaptive evolution and increased fitness, e.g. by the generation of novel phenotypes or genetic variation.

3.4 Conclusions

Phenotypic traits are important for invasiveness of plants. There are a range of reproductive and vegetative plant traits that are relevant for promoting persistence and invasiveness. Although experience from invasion biology can give some indications regarding the importance of certain phenotypic plant traits for invasiveness; there is no agreed set of life history traits of plants that determine the ability of a plant to become persistent or invasive. Most GM crops that build up free-ranging populations so far have spread and established by seed dispersal although pollen dispersal also plays a role. For the agronomic and phenotypic characterisation, in particular seed and pollen characteristics as well as vegetative plant traits relevant for spread are considered useful. Nevertheless, the receiving environment as well as the selective value of the specific GM trait and other factors such as phenotypic plasticity as well as hybridisation ability with wild relatives also play an important role for spread, survival, persistence and invasiveness of a plant.

In addition, for ERA purposes, it is important to define terms like invasiveness, particularly considering that the plants' spread and occurrence in the environment may entail an adverse effect on biodiversity or ecosystem services (see Chapter 4).

4 Environmental harm due to persistence and invasiveness of GMP

4.1 Environmental harm of GMPs in the scientific literature

The spread of GM plants in the environment or transgene spread from crops to wild relatives are biological processes, which may have deleterious consequences for biodiversity, ecosystem services or functions or specific objects of protection (e.g. endangered and protected species).

Definition of environmental harm requires three important definitions: 1) the selection and identification of protection goals and entities, 2) the definition of changes that are adverse and 3) the definition of negative changes that exceed a certain level or threshold (Bartz et al. 2010).

Since the beginning of planting of GM crops in the early 1990ies, scientists have addressed questions of environmental harm of GMPs due to their ability to outcross, become persistent or invasive in agricultural or natural habitats. Generally, environmental risks due to GM crops that can spread and establish in the environment have been discussed extensively in the scientific literature (e.g. summarized in e.g. Bauer-Panskus et al. 2013) and general harm scenarios have been outlined (e.g. Raybould 2010). For example, a range of authors addressed the environmental consequences of gene flow of transgenes from crops to wild relatives (Ellstrand et al. 1999; Hancock 2003; Stewart et al. 2003; Marvier and van Acker 2005; Andow and Zwahlen 2006; Bauer-Panskus et al. 2020). These include:

- Genetic assimilation (replacement of wild genes by crop genes) and reduction of the genetic diversity of wild populations
- Demographic swamping, if hybrids have lower fitness than their wild parents) resulting in a shrinking of wild populations or extinction of vulnerable species
- Replacing the wild population and other plants (if hybrids have higher fit-ness than wild parents) in agricultural land or natural areas
- Adverse environmental effects through changed fitness of siblings (i.e. “next genera-tion effects”)
- Evolution of new plant pests, more persistent/aggressive weeds, loss of existing weed control options/additional herbicide loads
- Contamination of seed pools/seed production/other varieties/land races affecting seed quality

Specifically for oilseed rape, a crop that is known to build up persistent feral populations in Europe, Dolezel et al. (2018) have outlined relevant protection goals, related to protected taxa and habitats, thereby specifying protection goals which are only broadly defined in GMO legislation (Kowarik et al. 2008b, Bartz et al. 2010).

4.2 Protection goals and environmental harm in EFSA Documents

In EU legislation, protection goals are broadly formulated, such as “human health” or “the environment”. Further refinements of the term protection goal or specifications of environmental have been made in a range of guidance documents provided by EFSA. In its guidance to develop specific protection goals for biodiversity and ecosystem services, EFSA Scientific Committee (EFSA 2016) provided a harmonized procedure to derive specific protection goals

(SPGs) for problem formulation for different stressors under EFSA's remit. These SPGs constitute explicitly the environmental aspects, which need to be protected together with an indication of the impact that is to be tolerated, also referred to as the magnitude of tolerable effects (EFSA 2016). In this context, EFSA (2016) calls for the definition of the tolerable impact and the avoidance of qualitative terms such as "negligible" or "large". For GMOs, the Ecosystem Service approach is exemplified by the use of Lepidoptera but may also be applied for other relevant protection goals. In addition, EFSA Scientific Committee recommends developing SPGs for endangered species (EFSA 2016).

Based on the ecosystem service concept, thresholds for acceptable adverse effects have already been set in other risk assessment areas at the EU level (plant protection products, EFSA 2013). The approach has been appraised (Devos et al. 2015), but specific thresholds defined for a single protection entity were also criticized (Simon-Delso et al. 2021).

In this context, the EFSA Scientific Committee recommends to consider the relevant biological effect and its size already during study design (EFSA 2011b). Testing should have sufficient statistical power in order to detect the biologically relevant effects. The biological relevance of a biological effect and its size (the effect size) will be determined by expert judgement and is considered an effect which is important and meaningful for human, animal, plant and environmental health (EFSA 2011b, 2017a). If no consensus on the relevant effect size can be reached, the use of default values is recommended. For the assessment of biological relevance, the EFSA Scientific Committee has provided a separate guidance document (EFSA 2017a). Environmental harm in the ERA context is considered as "the measurable ad-verse change in a natural resource or the measurable impairment of a natural resource service. It may occur as a measurable or observable loss or damage that has adverse and significant impact upon conservation and sustainable use of biodiversity" (EFSA 2017a).

In specific guidance documents for GMP risk assessment, the need to identify protection goals during the problem formulation step is outlined and a translation into measurable assessment endpoints in order to facilitate decision-making required (EFSA 2010, 2016). In this context, these protection goals comprise natural resources (e.g. arthropod natural enemies, bees) or natural resource services (e.g. regulation of arthropod pest populations, pollination), species richness, ecological functions, ecosystem services, species of conservation concern such as red list species or sustainable land use (EFSA 2010, EFSA 2016).

The guidance document on Environmental Risk Assessment (ERA) of GMPs provides detailed guidance regarding the assessment of persistence and invasiveness, which include environmental concerns that should be addressed with the assessment (EFSA 2010, Table 4). The concerns refer to the ability of a GMP to exacerbate weed problems within production regions thereby making novel weed control strategies necessary that may cause more harm to the environment. In addition, outside production areas, in natural or semi-natural habitats, GMPs (GM ferals) or their hybrids with wild relatives (GM hybrids) may reduce the diversity or abundance of valued flora and fauna (EFSA 2010). In addition, a decrease in the fitness of GM-wild hybrid offspring resulting in a decline or loss of populations of wild relatives is a further concern.

Tab. 4: Environmental concerns related to the persistence and invasiveness of GMPs, including plant-to-plant gene transfer (according to EFSA 2010)

Environmental concerns
Exacerbation of weed problems in production areas requiring novel weed control strategies
Reduction of diversity or abundance of valued flora and fauna
Decline or loss of populations of wild relatives

The guidance document describes a staged approach with different stages of information requirements to test hypotheses concerning persistence and invasiveness of a GMP or any of its wild relatives, if vertical gene flow occurs (see Table 5). In addition, phenotypic data are required for the assessment.

Stage 1 information requirements refer to data relevant for the reproductive biology of the plant, characteristics associated with weediness and invasiveness (e.g. seed dormancy, germination and persistence), hybridisation and introgression potential (e.g. flowering synchrony), or information on the phenotype under agronomic conditions. For plants with the ability to overwinter in the EU or outcross and hybridise with wild relatives, which are able to overwinter, stage 2 information requirements apply. These foresee data on persistence and fitness of the GMP and GM volunteers under agronomic conditions, in order to evaluate the environmental behaviour of the GMP within the production site, but also data on whether GM ferals can occur or if the GMP can hybridise with wild relatives outside production systems. Stage 3 information requirements refer to the assessment of fitness of the GM feral plant or GM hybrids in semi-natural habitats, while stage 4 information requirements refer to GM feral plants or GM wild relatives with altered fitness or increases in the habitat range. In addition, regarding the assessment methods, EFSA (2010) refers to greenhouse, microcosm and growth chamber experiments in order to assess fitness of ferals or wild relatives, to population models and the exploration of worst-case scenarios.

The staged information requirements provide some links to the assessment of environmental harm. For example, the environmental impact has to be determined in case the GMP has an increased fitness or is more persistent under agricultural conditions (stage 2). In addition, if population changes occur in feral plants or wild relatives with a GM trait then the potential environmental damage has to be assessed (stage 4). However, no further guidance is provided on how to assess such harm scenarios.

Tab. 5: Information stages to assess risks concerning persistence and invasiveness including plant-to-plant gene flow for GMPs (accord. to EFSA 2010).

Information stage	Information points (on GMP)
Stage 1	Growing of plant
	Growth characteristics different
	Overwintering ability
	Reproduction and hybridising ability with wild relatives

Information stage	Information points (on GMP)
Stage 2	Persistence under agricultural conditions
	Fitness under agricultural conditions (of GM trait)
	Ability to form feral populations
	Hybridisation ability outside production systems
Stage 3	Changed fitness of ferals or wild relatives in semi-natural habitats
	Altered range of population of ferals or wild relatives
Stage 4	Changes in population size of ferals or wild relatives

In its guidance document for the ERA of GMOs, EFSA (2010) introduced "limits of concern" as a concept to evaluate the potential environmental harm of a GMO during ERA. Based on an assessment of differences between the GMO and the non-GM comparator (the comparative approach), EFSA requires that the biological relevance of statistically significant differences should be assessed. EFSA defines Limits of Concern as "the minimum ecological effects that are deemed biologically relevant and that are deemed of sufficient magnitude to cause harm". During ERA testing the biological relevance of an observed effect has to be determined. For example, for food-feed purposes, significant compositional differences be-tween a GMO and a non-GM counterpart are evaluated whether they fall within so called "equivalence limits", taking the variability between commercial crop varieties into consideration (van der Voet et al. 2011).

So far, no such LoC for the ERA of GMPs have been defined or applied, although for non-target organisms the setting of such thresholds and related statistical aspects have been dis-cussed in the literature (Perry et al. 2009, Semenov et al. 2013, Goedhart et al. 2014, van der Voet and Goedhart 2015; Andow et al. 2016). Thresholds for the acceptability of adverse effects can either be formulated for affected species and habitats, based on specific and de-fined effects on biodiversity, or for the biological processes that may lead to those effects, e.g. if species or habitats of conservation concern are affected (Kowarik et al. 2008b).

As outlined by Dolezel et al. (2017, 2018), a range of aspects need to be clarified before the LoC concept can be made operational. In particular, determining what constitutes environmental harm where and when in the context of GMPs, which are able outcross, persist and become invasive is required in order to enable decision-making. This has to be explicitly stated at the beginning of the ERA in the problem formulation and provide a normative framework within which risk assessors can formulate testable risk hypotheses (Devos et al. 2013, Raybould 2010). Dolezel et al. (2018) have made suggestions for the operationalization of protection goals for ERA purposes using GM oilseed rape as a case study.

4.3 Conclusions

Considering environmental harm to protection goals is crucial in ERA of GMPs. Both, EU legislation as well as guidance documents for ERA have addressed this necessity to introduce thresholds for acceptability of adverse effects in order to define environmental harm. Specifically, the concept of Limits of Concern (LoC) in ERA guidance of GMOs has provided the basis for the consideration of environmental harm when assessing risks of GMOs. Nevertheless, so far, this concept has not been implemented in ERA practice.

For the agronomic and phenotypic characterisation of GMPs, such thresholds for the acceptability of changes in plant trait measurements have so far not been proposed or discussed. It is recommended to develop and set values for individual plant parameters when changes in agronomic or phenotypic plant traits are observed. This will facilitate problem formulation in ERA but also support decision making with respect to environmental risks.

5 The phenotype and GxE interactions

The phenotype of a plant is influenced, both, by its genotype and by the physical and biological environment in which the plant thrives. Genotype-by-Environment (GxE) interactions are a central concept in ecology and evolutionary biology and refer to the fact that (plant) traits vary in different environments based on genetic variation (Saltz et al. 2018). Such interactions have been defined as "… *a non-linear response of genotypes to environmental conditions*..." (Hufford et al. 2019). The responses of different genotypes can be parallel or non-parallel to different environmental conditions (Saltz et al. 2018). In the plant-breeding context, such interactions are considered as crossover and non-crossover interactions (Kang 2002). Such crossover interactions are usually assessed to evaluate whether identical plant genotypes perform constantly across environments. However, due to (sometimes substantial) GxE interactions also in genotypically-uniform breeding lines (e.g. for yield, see Kang 2002), multi-environment testing of cultivars over a range of environments and locations is carried out in modern breeding in order to identify cultivars with good performance across a range of environments or under specific environmental conditions (Kang 2002).

Genomic regions for fitness-related plant traits can be selected differently under contained conditions compared to field conditions and when comparing normal versus stressful, non-optimal conditions. In non-familiar, non-uniform or under non-optimal conditions, GxE interactions can influence which alleles are selected in a specific crop (Mercer et al. 2007, Hartman et al. 2012). Such novel environments can considerably determine the type and size of GxE interactions. While in familiar environments the reaction norms of a set of genotypes often is relatively uniform, in novel environments the responses may be considerably different - also referred to as cryptic genetic variants (Saltz et al. 2018).

5.1 Evidence for GxE interactions

Evidence for GxE interactions is available from research on GM crop plants, but also GM animals.

For example, Hartman et al. (2012) analysed 49 quantitative trait loci (QTL) for fitness-related traits of crop and wild lettuce and its crosses (*Lactuca sativa* x *serriola*) to evaluate environmental effects on the QTL. Under greenhouse conditions, other QTL patterns were observed than under field conditions, due to different selection pressures under different containment levels. They concluded that GxE interactions cause changes in selection pressures and consequently affect the selection of (crop) alleles. For GM ERA and the interpretation of the selective value of crop genes, assessing plants under realistic field conditions rather than extrapolating results from the greenhouse is needed (Hartman et al. 2012).

GxE interactions have also been shown to be relevant for the expression of *Bacillus thuringiensis* (*Bt*) toxins in insect tolerant GM maize. It is known that *Bt* toxin content and expression levels vary with environmental (stress) conditions (Dong and Li 2007, Dutton et al. 2004, LIU et al. 2019; Trtikova et al. 2015). For example, Trtikova et al. (2015) grew *Bt* plants in climate chamber experiments under optimal conditions. Then, plants were either kept under optimal conditions or exposed to hot, dry or cold, wet conditions. Under the hot and dry stress conditions, *Bt* expression was reduced. The authors concluded that the *Bt* content is influenced by environmental conditions and that these effects are generally difficult to predict. Biotic and abiotic stressors can also differently affect the fitness of GM plants under glasshouse and field conditions (Zeller et al. 2010). Hence, such GxE interactions are relevant if non-target pests vary with location or region where the GMP is grown (e.g. Catarino et al. 2019) or if wild relatives are present (e.g. Le Corre et al. 2020).

GxE interactions have been shown also for GM animals. Sundström et al. (2007) showed phenotypic differences in GM fish due to different environmental conditions (rearing in artificial vs natural conditions) with different consequences for predation rates and therefore eco-logical risks.

5.2 Assessment of GxE interactions according to ERA guidance

The fitness of a plant varies, depending on the environmental context, specifically with regard to the presence of competitors, herbivores, pathogens and abiotic conditions. A potentially enhanced fitness of the GMP, the GM feral plant, or GM crop-wild hybrids can lead to an increase in persistence or invasiveness of the respective plant with adverse effects on biodiversity and related flora and fauna in production areas, semi-natural or natural habitats (EFSA 2010). Such differences in fitness due to differences in biotic and abiotic conditions are referred to as 'Genotype by Environment (GxE) interactions' (EFSA 2010).

During the agronomic and phenotypic characterisation of the GMP, such GxE interactions have to be assessed, particularly if significant differences and/or lack of equivalence of plant characteristics have been observed (EFSA 2015). When carrying out field tests, the applicant has to consider whether potential differences between the GMP and its non-GM counterpart vary across sites. Therefore, a range of environmental conditions should be considered during the assessment. In general, these show up as statistical interactions between test material and environmental factors (EFSA 2010). Only in case of significant differences and/or a lack of equivalence for a specific endpoint between the GMP and the non-GM plant, an analysis for each test site (per-site analysis) is requested from the applicant. Applicants have to assess whether the identified differences are related to specific characteristics of the receiving environment and have to determine the respective implications for the risk assessment.

EFSA (2015) requires the applicant to choose representative locations and managements systems as different receiving environments, which are able to capture the variability of meteorological and agronomic conditions (EFSA 2015). Applicants have to justify the selection of sites and provide respective information to demonstrate suitability and representativeness of sites. Factors like growing area, climatic conditions, soil moisture, weed profile, presence/absence of pests and natural enemies are of relevance. The description of the receiving environments is requested for all applications, irrespective of the scope. The following information is required:

- Geographical location of field trials
- Agrometeorological data
- Soil type and soil characteristics
- Cropping history
- Crop management
- Post-harvest storage conditions for harvested materials to be used for further testing

For each of the aspects, the information to be provided are described in detail by EFSA (2015a). Additional data might be needed on a case-by-case basis, e.g. for specific crops or traits or in case of GxE interactions. For example, a description of soil characteristics should include soil texture, soil organic carbon, pH and soil bulk density. On a case-by-case basis, information on incidence and severity of plant disease epidemics and pest outbreaks is considered useful.

Regarding crop management EFSA (2015) states, that excessive use of plant protection products might affect the evaluation of pathogen/pest-plant interactions. Therefore, the principles of `Good Agricultural Practice´ should be followed, in order to keep the use of pesticides to a minimum. However, a lack of pest management is not considered to reflect normal agricultural practice. Recommendations on the herbicide regimes applied in field trails with herbicide tolerant GMP include an assessment of the possible influence of expected practice on the expression of the studied endpoints.

5.3 The consideration of GxE interactions in GMP applications

For agronomic and phenotypic field trials, applicants of GMPs generally provide information on field trial sites. This includes information on the location of field sites, planting dates/depths, soil descriptions, plot sizes and cropping history (e.g. type of crop planted in previous one to two years), cultivation practices (including pesticides, fertilization), information on weather during cultivation, sometimes including precipitation and information on harvest.

The selection of field trial sites is generally not justified by applicants. Generally, applicants choose locations with a range of environmental and agronomic conditions that are representative of major maize growing regions in the US. The selection of field trial sites is argued e.g. by "… widely distributed locations in the US Corn Belt to ensure (…) harvestable locations that are in agricultural regions where climatic conditions and soil types are typical for commercial maize production, and that are suitable for the cultivation of the hybrid maize lines" (e.g. for GM maize).

Applicants do not specifically choose locations with specific biotic or abiotic stress conditions, such as e.g. drought conditions or the presence or absence of certain pests and/or pathogens. Regarding agronomic practices such as pesticide use, applicants refer to "agronomic practices used to prepare and maintain each field site were characteristic of each respective region". For GMPs with insect resistance traits, sites with known insect pressure are not specifically selected (e.g. lepidopteran insect pests for *Bt* maize).

Only in the case of drought tolerant maize, some of the field trial locations were selected based on a high likelihood of drought stress, as the applicant expected phenotypic changes in the GMP under limited moisture conditions.

Applicants generally assess whether there are any statistical differences between the agronomic and phenotypic endpoints across all test sites. Due to the lack of a quantitative assessment of the relevant agronomic and/or phenotypic traits, specifically the response to biotic – pests and diseases – and abiotic stressors (yes/no classification, ordinal scales etc.), no differences are usually observed. If differences are observed, these are interpreted as follows (selected examples):

- The detected differences between GM and non-GM plant are randomly distributed among the measured characteristics with no trend among sites.
- Different incidences of stressors occur only in some of the replications at some sites.
- Difference incidences of stressors occur only at some sites, not all sites.
- The observed qualitative difference refers only to consecutive rating categories (e.g. none-slight vs. none-moderate).
- The incidence of each stressor lies within the range of incidence observed for the reference hybrids.
- No trends in susceptibility to the observed stressors across sites is evident, therefore a more quantitative assessment was not carried out.
- The observed differences are likely an artefact of the assessment method (i.e. qualitative assessment of spatially variable pests).
- The observed differences do not indicate a biologically meaningful result.

Hence the conclusion of applicants generally is, that the introduction of the GM trait does not unexpectedly alter the phenotype or ecological interactions of the GMP compared to the conventional counterpart.

5.4 Concepts for the classification of receiving environments in the EU

According to Directive 2001/18/EC, GMPs have to be assessed on a case-by-case basis. Thus, the ERA has to consider the plant, the novel trait and the potential receiving environment (RE) where the GMP could be grown. EFSA (2010) defines three elements that characterise the receiving environment(s):

- the GMP (including the crop species, its GM modification and the intended use),
- the geographical zone (defined by climate, soil, flora, and fauna) and
- the management system (e.g. production system, pest management)

Based on these three elements, when defining the RE in ERA the following aspects have to be considered:

- biotic and abiotic interactions of the GMP
- occurrence of compatible relatives and feral populations of the GMP
- protection goals
- likelihood of cultivation of the GMP

- risk implications due to the presence of other GM plants already cultivated in a specific RE
- predicted trends and changes to receiving environments

The necessary three steps to select appropriate RE comprise: i) the distribution of the crop plant (step 1), ii) the cultivation areas and their production systems relevant for the plant x trait combination (step 2) and iii) the selection of the RE for each environmental issue of concern identified in the problem formulation (see Table 2 in EFSA 2010). Appendix A of the EFSA guidance document provides further background information for the geographical zones in the RE in Europe, referring to plant protection product registration-based zoning, phytogeographic zoning, Natura 2000 zones, SEAMLESS zoning approach and LANMAP (EFSA 2010).

Also the CBD adopted the concept of receiving environments in its risk assessment guidance for living modified organisms (CBD 2016). One of the principles of the risk assessment is that "…risks associated with living modified organisms (…) should be considered in the context of the risks posed by the non-modified recipients or parental organisms in the likely potential receiving environment". Elements for consideration are given specifically for GM trees, among other the presence and proximity of species in the receiving environment with which the LM tree may hybridize (CBD 2016).

According to Jänsch et al. (2011), the concept of receiving environments is insufficiently implemented in GM ERA, as the ERA does not differentiate between the different receiving environments in which a GMP could be used. They therefore propose an ecologically relevant classification for non-target invertebrates on the European scale and focussing on non-target invertebrates used in ERA (Jänsch et al. 2011). Various already existing biogeographical classification concepts in Europe could also be used to classify receiving environments in the EU for ERA purposes. Important aspects to be used for classification are vegetation as well as climate and soil parameters. The classification should not include more than 20 units. Jänsch et al. (2011) recommended using the indicative map of European biogeographical regions (IMEBR) due to the ecological approach of the classification and its feasibility for use in ERA. The proposed nine biogeographical regions in the EU are seen as manageable for ERA purposes from a regulatory point of view. However, this approach does not include soil parameters, which would be important for the selection of non-target invertebrates living below ground. In the practical implementation, the area where the GMP is likely to grow should overlap with the nine biogeographical regions, resulting in the number of receiving environments that should be included in the ERA. In this respect, each combination of plant, trait and receiving environment forms a case that should undergo a specific ERA process (Jänsch et al. 2011). For example, for potato, this overlap would result in eight or nine different cases to be considered in the ERA, for grain maize five to nine cased were identified. Since 2015, EU Member States have the possibility to ask the applicant to remove their territory from the cultivation application of a certain GMO in the EU (Directive 2015/412/EU). Thus, the number of cases as described by Jänsch et al. (2011) might also depend on the area covered by the authorisation of the GM crop.

In the context of the INSPIRE Directive and the need to create a spatial data infrastructure for EU environmental policies and activities which may have an impact on the environment, Metzger et al. (2012) provided a classification scheme for the Environmental Stratification of Europe (EnS). The aim was to classify the biogeophysical environment of Europe into homogenous zones for analysis of ecological and environmental aspects but also for the selection of study sites across the continent and environmental reporting. Descriptions of the 13 environmental zones are made for climate, geomorphology, vegetation, and land cover, but not for biotic factors.

Arpaia (2021) discussed challenges when selecting receiving environments in the ERA. He considered the expected scale of environmental release of the GMP and the flexibility in se-lection of non-target focal species for e.g. toxicity tests as important aspects. In addition, he pointed out the relevance of the differences in agricultural systems throughout the EU with diverse cultivation, cropping and pest control practices that may also affect the potential benefits and use of GM crops. Not only is the cultivation area of the GMP of importance but also the presence of pest species in the selected environments. Due to climate change and the production of new varieties, the cultivation area of a specific crop species may also change. Climate change could also have an effect on the distribution of pest species or species that potentially form hybrids with the GMP under assessment. Agro-climatic changes can therefore affect crop production patterns in the EU and consequently potential overlaps with the occurrence of non-target species (Dolezel et al. 2018). In addition, depending on climate suit-ability, invasive agricultural pests may also affect agricultural areas in the EU differently (EF-SA PLH 2018). Different GM phenotypic traits or different product uses may also have an effect on the RE. Hence, applying pre-defined REs for ERA purposes may therefore be challenging, requiring a periodic re-assessment (Arpaia 2021).

Classification of receiving environments in ERA or monitoring of GMPs based on biogeographic regions in the EU have been used by Dolezel et al. (2018) and Lang et al. (2019) to evaluate risks to non-target butterflies.

5.5 Conclusions

Evaluating Genotype x Environment interactions of GMPs in ERA is important, as these can affect the selective value of the GM trait or GM crop alleles. The ERA guidance requires the applicant to consider GxE interactions during the agronomic and phenotypic characterisation of the GMP, e.g. by choosing representative locations and management systems in different receiving environments. However, specific environmental stress conditions (e.g. biotic, abiotic stress) are usually not specifically addressed when applicants choose testing sites. In addition, monitoring of and reporting on specific stress conditions is not carried out for the selected trial sites. Observed differences in the plants' responses are generally dismissed as not relevant. For the European Union, a classification scheme of representative receiving environments, e.g. with respect to the occurrence of specific biotic (e.g. pests, wild relatives) or abiotic (e.g. drought) conditions is still to be developed.

6 Methods for plant phenotyping

6.1 Phenotyping in plant breeding

In this chapter, methodologies used in plant breeding are scrutinized for their appropriateness for the phenotypic characterisation of GMPs with focus on the crop species maize, oilseed rape, soybean, potato and creeping bentgrass. During field-testing, the value for cultivation and use (VCU) of agricultural species, phenotypic assessments on cultivation, disease, yield and quality traits are carried out prior to variety registration. The analysis considers such methods as laid down in a range of different VCU protocols from different EU countries in terms of their applicability to assess invasiveness, persistence, and environmental interactions. Furthermore, plant traits and aspects that are insufficiently covered by the VCU test are identified. The detailed results of the analysis can be found in Annex 0 to this report.

6.1.1 Conclusions

The plant traits and characteristics assessed when evaluating a novel agricultural variety focus on aspects that are agronomically relevant, particularly establishment of seedlings, crop development and growth in the field, or yield parameters. For these plant traits, established methods are available. Some of these plant traits, such as (premature) grain loss, pod bursting, tuber size distribution, flowering phenology etc. are also useful to conclude on potential changes in the phenotype, which might affect the persistence or invasiveness of the GMP. However, specific approaches for the assessment of the potential persistence or invasiveness of the novel variety, e.g. changed dormancy, seed longevity or volunteer occurrence in subsequent crops, is not foreseen in VCU testing. The evaluations are carried out in a single season (e.g. no assessment of volunteers in the following season) and within the agricultural field only. The assessment of differences between varieties regarding infestation by pests and diseases or the resistance to abiotic stress is, however, an important assessment in VCU testing. Appropriate methodological approaches are therefore available for specific aspects and useful for application in the agronomic and phenotypic characterisation.

With respect to individual crop species, the analysis shows that certain critical traits and aspects are not routinely assessed in VCU testing, specifically:

- The survivability of tubers and the occurrence of volunteers from (residual) tubers left in fields (and berries) in the subsequent growing season as well as dormancy of seeds (potato).
- The survivability of grains/seeds or the occurrence of volunteer plants (soybean, oilseed rape, maize, creeping bentgrass, potato).
- Vernalisation requirements (oilseed rape, maize, potato).
- Dormancy of seeds (oilseed rape, maize, soybean, potato, creeping bentgrass))
- General seed characteristics (creeping bentgrass).
- Pollen shed, dispersal and characteristics such as pollen viability/compatibility/morphology (oilseed rape, maize, soybean, potato, creeping bentgrass).
- Response to biotic stressors, particularly naturally occurring insects, and abiotic stress (creeping bentgrass).

In general, few modern methodological approaches are used in plant variety testing. Most assessments are based on visual inspection under field conditions using a qualitative assessment method based on a 1-9 scale or the quantitative measurement of yield (Annex 0).

6.2 Phenotyping in plant ecology

6.2.1 The functional trait concept

Plant functional traits are defined as "morphological, physiological or phenological features, measurable for individual plants, at the cell to the whole-organism level, which potentially affects its fitness…or its environment" (Pérez-Harguindeguy et al. 2013). Plant functional traits considerably affect and are affected by environmental conditions and ecosystem processes. Data on plant traits (morphological, anatomical, physiological, biochemical and phenological) have been used in a range of basic and applied research areas – including ecology, invasion biology and agro-ecology (see e.g. Martin and Isaac 2015, Garnier & Navas 2012, Drenovsky et al. 2012, Alfaro and Marshall 2019). In addition, efforts have been made to compile information on plant traits in a regional or worldwide context (e.g. Kattge et al. 2020, Kleyer et al. 2008) and to standardize measurements and protocols under different geographical and environmental contexts (Perez-Harguindeguy et al. 2013). In the agro-ecological con-text, functional trait-based approaches are being used for research to better understand responses of plants or plant communities, e.g. to different management practices or environmental changes but also how crop plants influence agro-ecosystem functioning (for overview see Martin & Isaac 2015, Garnier & Navas 2012). For this purpose, plant trait databases have been compiled which contain large datasets on life-history plant traits for a variety of species, freely available for the research community.

6.2.2 TRY plant trait database

The TRY database[1] is a worldwide plant trait database, which covers approx. 2000 plant traits of 280.000 species (Kattge et al. 2020). It covers both, quantitative and qualitative plant traits, although the focus is on the latter, in combination with the necessary environmental covariates. The focus of this database is on wild plant taxa, with few well-covered species, but many species still being underrepresented. The database contains information of common crop species, however, being still limited and patchy (Martin & Isaac 2015).

Specific plant traits relevant for the purpose of this report, such as seed and pollen traits are generally covered by the database, e.g. seed dry mass, seed germination rate or seed storage behaviour.

For example, for *Zea mays*, 1011 observations of 199 traits are contained in the database. The traits comprise e.g. a range of seed traits, such as seed (seedbank) longevity, seed dry mass, germination rate, seed length and width, seed morphology, seed oil content, seedling vigour etc. Seedbank longevity contains nine measurements.

[1] www.try-db.org/TryWeb/Home.php

6.2.3 LEDA Traitbase

The LEDA traitbase[2] is managed by the University Oldenburg, Germany. It covers life-history traits of approx. 3000 species of the Northwest European flora (Kleyer et al. 2008). Information on traits contained in this database focus specifically on 26 plant traits relevant for persistence, regeneration and dispersal, such as clonal traits, leaf, tis-sue traits (relevant for persistence) or seed traits (relevant for regeneration) and dispersal unit and vector traits (relevant for dispersability).

Information on seed bank longevity is contained in this database, e.g. a seed bank longevity index (e.g. approx. 44.000 records on 1.500 species), as well as (seed) dispersal traits that are otherwise hardly available. The “seed bank longevity index” is a categorical trait that differentiates short-lived and long-lived species.

The Leda traitbase project also issues trait standards, defining the specific trait and giving advice with respect to standardized measurement protocols. For seed traits, such protocols are available for seed number per ramet, seed crop frequency and seed shedding, seed weight and shape, seed longevity, morphology dispersal unit. For seed longevity traits, the soil seed banks are divided into three categories: transient banks with seeds of species that persists less than one year, short-term persistent banks with seeds of species that persist between one and five years and long-term persistent banks with seeds that persist at least five years. Different types of seed bank methods are included in the database and recommendations for soil seed bank sampling protocols are given.

6.2.4 CROP-Trait Database (Crop Ontology Project)

The Crop Ontology project (www.cropontology.org) is run by the Consultative Group on International Agricultural Research (CGIAR) and focuses on traits of crop plants. It comprises approx. 4.000 traits for 31 plant species. The database contains information on morphological and phenological (including stress) plant traits relevant for plant breeding. The aim of the project is to set up a digital breeding tool with lists of defined and standardised crop traits. The aggregated trait data of crops should also allow the evaluation of varieties across multi-locations.

6.2.5 Conclusions

The usefulness of plant trait databases for GM ERA and the assessment of phenotypic traits are in general limited due to their focus on wild plant taxa (e.g. TRY and LEDA trait database). Although information on some of the relevant traits (e.g. dispersal, regeneration traits) of crop plants are contained in these databases, for some crop types (potato, soybean) only few or no entries are available (e.g. seed longevity for potato has 1 entry, soybean has no entries at all). Seed longevity traits, as contained in the LEDA database is of high relevance also for crop plants, but crop data are outdated. For example, for maize seed, longevity data in the database are reported from the DUVEL experiments carried out in the 1940s. Although data on seed longevity for crop plants is generally limited in the scientific literature, information that is more recent is available from published GMO research. Data on wild plants may be relevant in the future, in case these plants are also subject to genetic modification. In addition, it has been argued that the classification of seed bank persistence, as provided by the LEDA Trait-

[2] www.leda-traitbase.org

base, is inappropriate, as, according to current scientific standards, seed longevity is categorized into five classes. Instead of "longer than 5 years" another three categories (5-20 years, 20-50 years und > 50 years) should be used to account for seeds with very long-term seed survival capacity. In addition, methodological improvements for seed burial experiments are needed, specifically regarding the area from which seeds are retrieved, as many seeds are not recovered if sampled soil areas are too small (Prof. Poschlod, pers. Comm).

Other crop-specific databases such as the Crop-Ontology database focus on breeding-relevant plant traits rather than ecologically relevant traits. However, the database information may be useful as reference values for the phenotypic variability of a specific trait under certain conditions. In addition, it contains useful information on phenotyping methodologies of individual plant traits.

6.3 Phenotyping plant seeds

6.3.1 Laboratory seed testing

6.3.1.1 Seed viability and germination ability

Usually the viability of seeds and their ability to germinate is assessed using standardized germination tests in the laboratory (see Annex 0). Laboratory germination test systems test single parameters that influence germination in a controlled environment. These tests are available from the International Seed Testing Association (ISTA), the Association of Official Seed Analyst (AOSA) and the Society of Commercial Seed Technologists (SCST) in the US, organizations that develop rules and procedures for seed testing and the standardization of their interpretation. Applicants of GMOs refer to and use the standard seed germination tests and protocols as outlined by ISTA or AOSA. In general, the methods for seed germination tests are comparable between AOSA and ISTA rules. In addition, the seed laboratory of the Iowa State University is a public seed-testing laboratory in the USA and offers seed testing services for industry for more than 300 crop and other plant species. It publishes a range of seed testing methods (https://seedlab.iastate.edu/portfolio-items/). Such laboratory seed testing methods to assess the germination ability of plants are also being used in many ecological research questions.

Specifically for GMPs, Kjellson and Simonsen (1994) described a general laboratory germination test system to study a range of aspects concerning seed germination and dormancy regulation for use in ERA of GMPs. They propose a basic method that can be adjusted when addressing specific questions (e.g. effect of cold stratification, temperature fluctuations, stress effects on seeds etc.).

In general, in these standardized test systems, the plant seeds are usually put on wet filter paper and incubated in growth chambers at the prescribed temperature regimes. After a short incubation time, the percentage of germinated and/or viable seeds is determined. In some protocols, germination curves and a t50 value (time to 50% germination) is proposed as assessment endpoint (Kjellson & Simonsen 1994). For some species break of dormancy is required before viability can be tested.

6.3.1.2 Possibilities to improve seed germination testing

The main purpose of seed germination tests is to assess the seed quality (i.e. seed viability and health) for international seed trade. The aim of these tests is to ensure that seeds germinate uniformly and at a high percentage.

Field seedling emergence can differ significantly from seed germination rates, as measured in the laboratory, with seedling emergence often being much lower (Finch-Savage & Bassel 2016). Hence, seedling emergence under non-optimal conditions can hardly be predicted from seed germination rates assessed in laboratory testing. Specifically for maize seeds, such correlation studies were carried out between seed vigour tests, the standard seed germination test and field emergence (Aliloo & Shokati 2011). The standard germination test was no good predictor for field emergence while the vigour tests were better indicators. In a comparative assessment with Lucerne seeds, the standard germination test did not show a correlation with seedling emergence in the glasshouse or in the field (Wang et al. 1996). In contrast, seed vigour tests (electrical conductivity, controlled deterioration test) better predicted seedling emergence (Wang et al. 1996).

An overview of standardized methods based on international protocols (ISTA, AOSA) can be found in Annex 0. Standard germination tests could be complemented by seed vigour tests to better predict the germination ability of the seed under different environmental conditions. In addition, assessing seedling normality and further categorizing normal seeds into “strong and weak” based on morphological characteristics may also be a better predictor of performance in the field (https://seedlab.oregonstate.edu/importance-seed-vigor-testing).

Kjellson & Simonsen (1994) propose a seedling emergence test in the greenhouse. It is a method to assess seed survival in different ecosystems. The test can be carried out either with a single species or samples with seeds of several species. Soil samples, from soil sampling, seed burial experiments or seed traps, are used and cold stratified before the test (e.g. stored in a cold chamber for 1-3 months). Soil samples are spread out over sterilized soil in trays in a greenhouse at 16/22°C (night/day) in 16 h daylight. Test period is 3 months.

In addition to seed vigour tests, also seedling vigour can be assessed in the greenhouse under controlled conditions at 20°-24°C day/12-16°C night (12 light period) and constant water capacity at 75% for 7 days. The assessment endpoint is fresh weight of the seedlings. This method is able to discriminate between seedlings derived from seeds from non-stressed versus stressed mother plants (Hatzig et al. 2018).

6.3.1.3 Assessing seed germination under drought stress

The effects of drought stress on seed germination can be assessed by using osmotic regulation substances (e.g. Mannitol, Polyethylenglycol PEG) creating osmotic stress potentials, thereby simulating drought conditions (e.g. Hatzig et al. 2018, Liu et al. 2015a, Schuab et al. 2007, Basal et al. 2020, Saffariha et al. 2020). Saffariha et al. (2020) assessed the effect of seven different drought stress conditions (with different osmotic potentials) on seed germination under controlled laboratory conditions to derive a model that can be used as a decision support system for predicting the seed germination success of a certain plant in agricultural or natural ecosystems under certain abiotic stress conditions.

In wild plants the seed survival of desiccation is an important functional trait (e.g. for pioneer species). For crop plants, environmental stress conditions of the plant can affect seed quality, seed germination or seed vigour. For example, in oilseed rape drought stress can affect seed oil content, protein content or fatty acid composition as well as seed germination parameters (Hatzig et al. 2018). Also Awan et al. (2018) showed the importance of environmental stress (drought) on mother plants of *Brassica oleracea* on seed performance parameters (e.g. germination speed, resistance to controlled deterioration, induction of secondary dormancy).

6.3.1.4 Assessing seed germination under water stress

Grass seeds like creeping bentgrass have a long seed viability with a high percentage of seeds known to germinate after five up to 11 years of storage under dry conditions (Zapiola & Mallory-Smith 2010). As creeping bentgrass seeds can also be dispersed in waterways and channels, Zapiola & Mallory-Smith (2010) suggested to conduct a germination test with soaked panicles at two different temperature regimes (20°C and 4°C) to reflect water stress during water dispersal. Standard germination tests were carried out with dried panicles, which were previously soaked in water for one, two, four, six, eight, 12 and 17 weeks and germination rates assessed. The authors showed a negative effect of the water soaking at low temperature on seed germination of creeping bentgrass. Germination was reduced due to induction of secondary dormancy in experiments with the cold-water temperature regime.

6.3.1.5 Modern phenotyping methods for seed testing

Digital image analysis has been a popular approach to support automated seed germination and vigour testing. A range of crop species including maize and oilseed rape have been frequently phenotyped for germination traits (see references in Matthews and Powell 2011). Jahnke et al. (2016) used an automated phenotyping system to measure biometric traits of seeds. Hatzig et al. (2018) evaluated mean germination time, germination rate within 96 hrs and the uniformity of germination (time difference to reach 10% and 90% of germination) by use of an automated phenotyping platform of the variety control office of the French national seed-testing agency. In recent years a range of novel and high throughput phenotyping methods have been developed which allow the phenotyping and quantification of morphological seed traits (e.g. size, shape) as well as seed quality traits including germination, viability and vigour. Colmer et al. (2020) describe a phenotyping platform for the analysis of crop seed germination and seed germination phenotypic traits. Also Merieux et al. (2021) propose a high throughput phenotyping tool for seed germination (ScreenSeed technology) in order to assess germination behaviour.

Some of these systems can score germination parameters, measure morphological changes for a range of crop species and are able to discriminate between genotypes and even identify associations between genomic regions and differences in germination traits (e.g. Colmer et al. 2020). Such methods have been proposed for use in breeding and research activities (Colmer et al. 2020).

Also for potato tuber characterisation, modern phenotyping approaches are available. For example, Neilson et al. (2021) used RGB imaging to phenotype potato tuber shape, specifically length to width ratio. In a similar study, Liu et al. (2021a) used 3D image analysis for counting potato eyes and estimating eye depth. However, the aim of tuber phenotyping studies is generally related to assess crop traits, which are important for breeding and marketing and to replace manual assessment and scoring methods, rather than aspects related to the ability of the crop to survive in the field.

6.3.2 Assessing seed survival *in-situ*

Assessments of seed survival under field conditions (e.g. in soil seed bank) has been carried out in the context of GMO research, research on weeds or on invasive plant species.

For weeds, seed bank persistence, seed longevity or seed decay has been assessed (e.g. Saatkamp et al. 2009; Ullrich et al. 2011; Buhk and Hensen 2008). Experimental approaches to assess weed seed mortality and viability in fields are also available (Gardarin et al. 2010). In order to assess the long-term survivability of seeds of an invasive plant, Karrer et al. (2016) carried out a field experiment over several years. Each year seeds buried in bags at different depths were excavated and tested by standard germination tests.

Kjellson and Simonsen (1994) propose a general method for a field germination test for GM ERA purposes. The test may be used to study long-term seed viability (three to five years), germination and plant establishment in the field, especially in cultivated ecosystems. Cylinders (25 cm diameter, 30 cm long) are buried in the ground, filled with sterilized soil and the upper surface mixed with seed of the target species. Cylinders are covered with nets to exclude seed predators. The upper 25 cm of soil is mixed three times per year and emerged seedlings counted and removed each month. In addition, the authors recommend a seed burial test that gives information on seed survival and germination in the field depending on soils, depth and burial duration (Kjellson & Simonsen 1994). These experiments are carried out with seed in soil-filled containers or enclosed in nylon or fiberglass-mesh bags buried in the soil. They can be used for short-term (less than 3-5 years) and long-term assessment of seed survival (more than 3-5 years). As a standard, usually 50-100 seeds are covered in a 5x5 cm bag and buried in the soil. After the necessary burial time, bags are removed from the soil and seed viability is tested by a germination test in the lab (see above). This test system excludes seed predators but allows natural processes in the soil to occur, such as colonization of seeds with fungi and microorganisms.

Specifically for GMOs, one of the earliest assessments in the context of seed survivability and invasiveness of GMPs was carried out by Crawley et al. (1993) using GM oilseed rape for experimental studies in natural habitats in the UK. The authors used seed survival and seed production to calculate the finite rate of increase of oilseed rape plants. The seed sowing experiment estimated germination and dormancy, plant survival and fecundity. In another experimental approach, Crawley et al. (2001) assessed survivability of seeds (and tubers) of different crop plants in natural habitats. The natural habitats were located in the UK with rather mild winter conditions. Depending on the location, also long-term survival rates (e.g. 10 years for potatoes) were found. Specifically for oilseed rape such long term assessments of seeds to survive and persist in the soil seed bank have been carried out and extensively discussed in the scientific literature (e.g. Linder and Schmitt 1995; LUTMAN et al. 2003; Walker et al. 2004). Seed burial experiments for oilseed rape range from six months (Walker et al. 2004) to 11 years (Lutman et al. 2003).

Also for perennial plants such as creeping bentgrass seed survival in a natural habitat has been assessed. Garrison and Stier (2010) put 100 seeds into a nylon mesh bag together with soil and buried them in the natural habitat. The seeds were then evaluated at 6, 12 and 22 months after planting and seed viability determined in the lab by using a standard seed germination test and staining on ungerminated seeds.

For assessment of seed survival in agricultural fields (i.e. volunteers), specific protocols are still to be developed. In this context, high-throughput phenotyping methods have been proposed. For example, unmanned aerial vehicle (UAV) imaging is being used to estimate potato crop emergence in the field (Sankaran et al. 2017). Li et al. (2019) used RGB image methods in order to assess potato emergence rate and uniformity as well as crop canopy cover in the field. Although their purpose was to optimize field management and yield, this method allowed discriminating emergence rates between cultivars or fertilizer inputs. Nieuwenhuizen et al. (2010) proposed a high-throughput phenotyping method for the detection of volunteer potatoes. The authors developed an automated detection method based on ground imagery in order to classify volunteer potato plants in a sugar beet crop. However, the methods applied aim at agronomic aspects of the potato crop (e.g. nutrient requirements, plant stress or specific phenotypic traits) rather than the assessment of environmental behaviour.

6.3.3 Conclusions

In contrast to seeds of wild plants, which are adapted to natural conditions and avoid uniform germination after seed set (i.e. a bet-hedging strategy), crop plants tend to germinate uniformly. Seed germination assessed under laboratory conditions is therefore only one aspect to predict seed survival in the field. Laboratory test systems to evaluate seed germination focus on agronomic and seed quality aspects of crop plants, but do not consider the environmental variability. Hence, the testing conditions are generally adapted to standard (optimal) field conditions considering uniform germination of the seed. These seed germination tests with the applied test designs are therefore of limited relevance for the assessment of the seeds' ability to germinate outside of cultivated areas. This is particularly relevant for species, which can persist or occur outside agricultural fields. For example, pioneer plant species often have fast germinating seeds in high-stress environments or dimorphic seeds, both fast germinating and seeds with dormancy to build up a seed bank (Finch-Savage & Bassel 2016). Seed germination tests alone cannot predict the establishment of GM seeds, particularly under suboptimal conditions (e.g. in non-managed habitats, under other than optimal temperature regimes, e.g. if spilled during harvest). In addition to standard germination tests, other test systems are available that assess seed vigour under different environmental conditions.

In addition to seed germination seedling performance or seed survival should be assessed also *in-situ* as there is a weak correlation between these two traits (Hatzig et al. 2018, Song et al. 2004). The assessment of seed survival *in-situ* is crucial to be able to conclude on the potential survivability of the GMP under natural (managed and unmanaged) conditions. Experimental assessment approaches (e.g. seed burial experiments) are available from the scientific literature.

The following recommendations are made when assessing seed survival either in the laboratory or under field conditions.

Laboratory assessments:

- Assess not only the percentage of viable seeds but also the ability of seeds to produce normal seedlings, also under sub-optimal or stressful conditions.
- Consider relevant environmental (stress) conditions when testing seeds depending on the plant taxon (e.g. drought for field crops, water stress for seeds that are dispersed in waterways).
- To mimic natural conditions, use alternating temperatures and light conditions reflecting natural conditions (Baskin et al. 2006).
- Consider residual dormancy in crops (e.g. oilseed rape; see Baskin et al. 2006 for discussion of testing non-dormancy).
- For species without official seed germination testing rules, e.g. *Agrostis* sp., methods are also published e.g. by AOSA.
- Test seeds without seed treatments to simulate harvest losses. Soil fungi are important mortality factors for soil-buried seeds (Wagner & Mitschunas 2008). A range of effects of seed treatment and seed priming on germination and seedling emergence have been

shown (Lamichhane et al. 2018). Fungicide treatments can also induce secondary dormancy but do not necessarily affect seed mortality (Mitschunas et al. 2009).

- Assess several germination endpoints. Germination rate (i.e. germinated seed number/test seed number) does not necessarily allow discriminating between two plant varieties, while other indices (germination index or vigour index) or the assessment of root-shoot ratio showed marked differences between two maize varieties (Liu et al. 2015a). Assess also mean germination time (in days) instead of proportion of seeds that germinate only.
- Consider stress conditions in maternal plants and their effects on germination (Hatzig et al. 2018).

Seed survival *in-situ*:

- Survival and persistence of seeds is context-specific and should be assessed in different habitat types (Linder & Schmitt 1995). Specifically, environmental and test conditions (moisture, soil type, burial depth of seeds) affect long-term seed persistence (Mašková et al. 2022; Mašková & Poschlod 2021). Survival and persistence of seeds should therefore be assessed not only in agricultural plots under optimal conditions (Hails et al. 1997; Crawley et al. 1993).
- Choose different habitats used for experiments, e.g. a competitive environment (interspecific plant competition) as opposed to a competition-free environment or mechanical disturbance (Crawley et al. 1993; Lutman et al. 2003). The use of a disturbed habitat type (e.g. ruderal site without weed competition) may give a maximum estimate of seed survival of volunteers (Walker et al. 2004).
- Assess seed survival over a long experimental period (one to two years).
- Assess the proportion of dormant seeds also under field conditions (Linder & Schmitt 1995). Non-germination may indicate dormancy; therefore, seeds may persist for a longer time-period. Higher dormancy can indicate increased seed bank persistence (Linder 1998). Inclusion of a weedy relative (with dormancy) may serve as an indicator of local seed-preservation conditions (Crawley et al. 1993).
- Assess hybrid seed survival for crop species that are able to hybridize with wild relatives (e.g. Linder & Schmitt 1995; Mercer et al. 2006; Pace et al. 2015; Yang et al. 2017). Hybridization of crop and wild relatives can increase germination and decrease dormancy, which can facilitate transgene introgression into wild populations. Besides higher germination and lower dormancy in crop-wild hybrids than in wild seeds, variability in germination is also possible.
- GMPs with different transgenes but with similar function (e.g. herbicide tolerance) must by tested individually as different GM lines may have different rates of seed survival (Linder & Schmitt 1995, Hails et al. 1997).

6.4 Assessing seed shattering

Seed shattering is an important phenotypic characteristic of plants that disperse seeds. The shattering of seeds is the first process that mediates seed dispersal. It is a weedy trait of wild plants and many crop plants are domesticated for seed or pod shatter resistance as seed and pod shattering is one of the causes of yield losses, particular in soybean or oilseed rape (Maity et al. 2021). In soybeans, pod burst occurs predominantly during warm and persistently dry weather during maturing. Also transgenic and gene editing approaches have been used to improve shatter resistance in crops, e.g. in Brassicaceae (Maity et al. 2021).

Any changes (particularly increases) in the seed shattering ability of a GMP could increase the ability of the GMP to disperse its seeds into the environment. Pod and seed shattering is a complex polygenic trait, which varies between cultivars and is influenced by Genotype x Environment interactions. Specifically, temperature and humidity play an important role for seed shattering and temperature stress can lead to changes in the seed shattering phenotype (see examples in Maity et al. 2021). Seed shattering in crop x wild hybrids can be different from crops, e.g. being intermediate between maize and teosinte (Chavez et al. 2012).

6.4.1 Methods to assess seed shattering

Laboratory and field methods have been described for the evaluation of shatter resistance in plant breeding but also when assessing weed seed shattering (see below). However, to the authors' knowledge, no standardized methodology is currently available. Field methods may be inaccurate due to varying weather conditions and laboratory testing may give results that are more reliable. A standardized protocol for the evaluation of the effectiveness of two different types of seed traps commonly used (sticky traps and funnel traps) is available (Arruda et al. 2020).

One method frequently mentioned by the technical experts and frequently cited in the literature to detect the propensity of pod bursting is the oven drying method (Tiwari 1997, Tukamuhabwa et al. 2002, Bhor et al. 2014, Barate et al. 2019, Krisnawati & Adie 2020). For example, Krisnawati & Adie (2020) used an oven-dry method to evaluate pod shattering incidence and shattering severity in soybean. At full maturity sample plants were dried at room temperature, pods placed in petri dishes, dried at different temperature (30°, 40°, 50° and 60°C) and the shattering incidence calculated (number of shattered pods to total pod number). Pod shattering severity refers to the length of the opening pod ventrally in relation to the total pod length (ventrally). Barate et al. (2019) mention a 1-5 scale for classifying the percentage of burst pods. With the oven-drying method, genotypes at high risk for pod shattering are well detected.

Chavez et al. (2012) used a force gauge to measure the force needed to free the maize fruits from the corncob by pulling fruits from the different areas of the maize infructescence. Similarly, Jeon et al. (2021) used a strain gauge to measure the pulling and bending strength differences in GM and non-GM rice.

In the field, seed shattering can also be measured, e.g. seed trap experiments. Already Kjellson & Simonsen (1994) proposed a seed trap method that can be used to assess seed production of GMPs in the field. Different types of traps (petri dishes, trays, plastic containers) are placed at suitable heights in the field (e.g. at soil surface level). Nets can be used to exclude seed predators. Such catch trays have been used to assess seed shattering and pre-harvest losses in Brassicaceae (Gan et al. 2008). Catch trays were placed between plant rows between the end of flowering time and harvest of the crop. Also Schwartz-Lazaro et al. (2021) used seed collection trays placed around weed plants in crop fields to assess weed seed shattering.

Another possibility is to select field trial locations with medium to light soils and dry ripening conditions, under which pod shattering is more likely to occur. In this case, the survey date should be set about two weeks after the possible harvest date. However, this requires the establishment of additional observation plots during field testing.

6.4.2 Conclusions

Seed shattering is the first step for dispersal of GM seeds into the environment. Seed shattering is only relevant if cultivation of a GMP is envisaged. Changes in seed shattering of a GMP could affect the ability of the plant to disperse, survive and persist in the environment. Methods to assess seed shattering are available, both under contained and field conditions.

6.5 Assessing pollen viability

Pollen viability is usually assessed in agronomic evaluations due to its relevance for guaranteeing a uniform fertilization within the crop stand and hence adequate seed set and crop yield. For the assessment of persistence, hybridization and invasiveness, changes in pollen viability is one parameter that can affect the outcrossing ability of a plant. The viability of the pollen is one important aspect to enviable cross-fertilization and possibly hybridization with wild relative plants, which is an important driver of invasiveness (see Chapter 3.3.5). Changes in this plant characteristic may give an indication of potential unintended effects of the genetic modification on male fitness. Reduced pollen viability may be a barrier to successful fertilization and hence gene flow between species, similar to zygotic incompatibility. In addition, the fertilization success may be different for heterospecific and conspecific pollen in hybrids (see Pertl et al. 2002 and references therein). In crop x wild hybrids, changes in pollen fertility compared to the parental plants have been observed indicating partial sterility in F1 hybrids and therefore reduced pollen viability can be seen as a barrier to introgression (Mercer et al. 2006, Song et al. 2004, Pertl et al. 2002).

An assessment of pollen viability can therefore serve as a proxy for male fitness (Guadagnuolo et al. 2006, Pertl et al. 2002). In a range of studies assessing crop-wild hybrid fitness, male fertility was evaluated by assessing pollen viability, (e.g. Guadagnuolo et al. 2006, Allainguillaume et al. 2006, Snow et al. 1999). In addition, knowledge on pollen viability can also play a role to limit gene flow in GM crop species (Wang et al. 2004). Kjellson & Simonsen (1994) suggested pollen germination and pollen viability test systems, specifically for the ERA of GMOs.

In general, pollen viability is highly influenced by environmental parameters, specifically heat stress. In GMO applications, pollen is usually collected from plants grown in field trials, however, with unknown environmental conditions (see 4.3). Pollen is considered viable if it can fertilize the ovaries in the female flowers under natural conditions. Pollen viability is determined by many different factors; mainly physical factors like water balance and humidity, temperature stress and UV-radiation but there are also species-specific aspects of viability of pollen (see review in Bots & Mariani 2005; Wang et al. 2004; Ge et al. 2011). In addition, for viability the (de-)hydration state of the pollen after dehiscence is an important factor. Partially hydrated and metabolically active pollen can accelerate pollen tube formation (see references in Bots & Mariani 2005). Not only the conditions after dehiscence but also before dehiscence can affect pollen viability, in particular drought and heat stress during pollen development (Bots & Mariani 2005). For example in maize, pollen formation starts at growth stage V8 until the mature pollen is produced at V17 (Begcy & Dresselhaus 2017). Longevity of pollen can be drastically reduced under ambient atmospheric conditions with a loss of pollen viability within 20-30 minutes up to one to two hours, depending on the species and the prevailing weather conditions (Fonseca & Westgate 2005, Fei & Nelson 2003, Ge et al. 2011, Rodriguez-Riano & Dafni 2000).

6.5.1 Methods to assess pollen viability

Firmage & Dafni (2001) and Bots & Mariani (2005) summarize the most commonly used methods to determine pollen viability, including their advantages and disadvantages. Often, pollen-staining techniques (e.g. vital stains to detect enzymatic activity, or presence of cytoplasma, callose or starch) are used to determine pollen viability. These staining techniques are generally conducted in laboratory, but can have a high false positive rate, thereby overestimating pollen viability, which may not be correlated with seed set (Bots & Mariani 2005). Certain staining methods are not suitable for some species, as they do not differentiate between viable and non-viable pollen (Wang et al. 2004), indicate stainability rather than viability (Firmage & Dafni 2001) or even stain dead pollen (Rodriguez-Riano & Dafni 2000). Firmage & Dafni (2001) compared four staining methods for a range of species which could be used in the field (X-Gal-test, MTT, Baker`s solution, and isatin staining). The Baker`s and the MTT stains were suitable for 10 out of 17 species. Rodriguez-Riano & Dafni (2000) suggested a new peroxidase test and MTT (testing for dehydrogenase) as the best staining methods for pollen viability as they showed highest correlation with the germination of the pollen. For *Festuca arundinacea* no staining method was found to distinguish between viable and non-viable pollen (Wang et al. 2004).

In-vitro and in-vivo germination techniques (e.g. germination of pollen grains on media immediately after collection in the field) can be used and are relatively accurate to estimate viability of pollen (Fonseca & Westgate 2005; Fei & Nelson 2003; Bots & Mariani 2005). However, these methods require optimally composed growth media (e.g. Fei & Nelson 2003). The germination rates are considered more reliable in predicting pollen viability than staining methodologies (Bots & Mariani 2005). In addition, chemical conductivity or respiration of pollen leachates or proline content are also used to estimate pollen viability (Dafni & Firmage 2000).

Experimental approaches have been used to estimate male fitness and paternity of crop, wild and crop-wild hybrids (Pertl et al. 2002). Analysing the final seed set of a species in the field (e.g. via manual pollinations) has been suggested as a more accurate method to determine pollen viability (Bots & Mariani 2005; Dreccer et al. 2019; Wang et al. 2004). Such experimental pollination test systems have already been proposed by Kjellson & Simonsen (1994) to study gene flow and hybridization. This can be done in the greenhouse or under field conditions.

6.5.2 Conclusions

In order to reliable determine pollen viability, pollen-staining methods have to be scrutinized whether they are able to effectively distinguish viable and dead pollen for each individual crop species. In-vitro germination tests may give a more reliable assessment of pollen viability if an optimized growth medium is available for the respective species (e.g. Wang et al. 2004).

Environmental conditions affect pollen viability, which changes over time under atmospheric conditions. These effects can be assessed under controlled conditions in growth chambers but also under atmospheric conditions. Different types of environmental stress conditions (e.g. temperature, humidity, UV-B radiation) can be included (for protocols see Wang et al. 2004). Final seed set assessments can be used to correlate pollen viability under realistic field conditions.

Further suggestions for the assessment of pollen viability comprise (see Wang et al. 2004; Rodriguez-Riano & Dafni 2000; Firmage & Dafni 2001):

- Optimization of media and conditions for pollen germination for individual crop species.

- Consideration of environmental factors that may affect pollen viability and longevity (e.g. temperature, UV-B radiation).
- Correlation of pollen viability assessed in the laboratory with seed set assessed under realistic conditions.
- Recording of collection and storage conditions prior to testing (e.g. temperature, relative humidity).
- Use of fresh pollen.
- Dead pollen as well as hydrated/dehydrated pollen should be included in testing as control.
- Combination of several tests to find out the best for the respective crop species.
- Use of high-throughput phenotyping methods, e.g. automated image analysis or flow cytometry techniques for assessment of pollen viability instead of manual microscopic assessments (Dreccer et al. 2019; Ascari et al. 2020).

6.6 Assessing vegetative growth

Perennial grasses such as creeping bentgrass can spread vegetatively. For agronomic and phenotypic characterisation of perennial plants, also clonal reproduction endpoints are important as these contribute to survival by vegetative reproduction (Song et al. 2004). These endpoints can be e.g. the number of tillers, panicles or spikelets (Yang et al. 2017; Song et al. 2004).

6.6.1 Methods to assess vegetative growth

In plant variety testing, VCU protocols of some countries outline visual recording methods for assessing vegetative growth of *Agrostis*, focussing on characteristics, which describe the density of grass cover or tillering density after each cut or toward the end of the vegetation. These assessments use a 1-to-9 scale and give an indication of the vegetative growth potential of the grass species (Bundesamt für Ernährungssicherheit 2015; Bundessortenamt 2008; Groupe d´étude et de contrôle des variétiés et des semences 2020).

As an example, the Austrian VCU protocol uses experimental plots with row sowing. A representative part of the plot without animal or wintering damage is selected for the survey of the sward density. Criteria for this assessment are tillering density in the rows, and, especially in case of stoloniferous species, also between the rows. The assessment is made after each cut in the vegetation period. In the protocol, the grade 1 means a very dense sward, 3 approx. 10% open sward, 5 approx. 25% open sward, 7 approx. 50% open sward with grade 9 standing for a very loose sward.

Further approaches for measuring the lateral spread in creeping bentgrass with a more targeted trial setting are described by Gardner et al. (2004). In this study, glyphosate resistant transgenic lines were compared with a conventional control and reference varieties by planting vegetative plugs of bentgrass stands into plots covered with stands of 1-year-old perennial ryegrass (*Lolium perenne*), 10-year-old Bermudagrass (*Cynodon dactylon*) and 10-year old St. Augustine gras (*Stenotaphrum secundatum*). Monthly data recording aimed at the mean diameter of the bentgrass plugs by measuring the longest and shortest spread.

Jones & Christians (2010) tested bentgrass varieties for their lateral spread and divot recovery potential. Similar to the trials of Gardner et al. (2004), they transplanted bentgrass plugs from established stands in the centre of one m² weedless plots. Additionally, sward injuries were simulated by digging out divots of turf and replacing the soil under the open patches. Lateral growth of the bentgrass plugs and the ability to recuperation were detected by digital imaging.

Lootens et al. (2016) developed a high-throughput phenotyping tool based on digital image analysis to measure (re)growth phenotypci characteristics of a range of *Lolium perenne* genotypes, focussing on lateral expansion. The method was sensitive enough to discriminate between different groups of genotypes.

6.6.2 Conclusions

For vegetatively reproducing, perennial plant species, additional guidance is needed in ERA. Assessment endpoints have been proposed and methods are available from plant breeding protocols or the published literature.

6.7 Assessing plant competition and fitness

This chapter summarizes scientific literature regarding two major types of experimental approaches in plant ecology to assess survival of plants under natural conditions: a) competition experiments (with other plants as competitors) and b) fitness experiments (often considering specific environmental stressors, such as pests or herbicide treatments).

6.7.1 Plant competition

In plant ecology, competition can be defined as "an interaction between individuals or populations under a shared resource limitation and leading to a performance reduction of individuals" (Weiner 1993; Weigelt & Jolliffe 2003). Intraspecific competition, i.e. competition between individuals of the same plant taxon has to be distinguished from interspecific competition be-tween individuals of different taxa. Interspecific competition is one of the key processes that enables plant invasions. Particularly if competition is decreased, e.g. in disturbed habitats, then the likelihood of invasion increases (Crawley 1990 cited in Vilà & Weiner 2004).

The aim of plant competition studies in plant ecology is to predict the composition of species in a plant community, if e.g. two taxa compete with each other. Usually, the probabilities of different possible outcomes are assessed, such as i) coexistence of species ii) species 1 succeeds iii) species 2 succeeds iv) species 1 or 2 succeeds. Often researchers use a Bayesian statistical approach (Damgaard 1998).

Experimental studies to assess plant competition usually use mixtures of two different plant taxa in variable densities or in different environments and assess fitness-relevant traits such as seed yield or biomass. For the assessment of the competitive superiorship (e.g. of invasive alien species), generally, three types of experiments are conducted: replacement series, additive experiments or removal experiments (Vilà & Weiner 2004). These experimental approaches are also used for crop-weed competition assessments (Swanton et al. 2015). Competition experiments can be conducted in greenhouses or in the field (Goldberg and Werner 1983). In the most frequently used replacement series experiments, the density of two plant species is kept constant with varying relative frequencies. The effects of competition on yield parameters are then reported by use of models using indices (Damgaard 1998; Damgaard & Kjaer 2009; Weis & Hochberg 2000). The plant performance in species mixtures is then compared to monocultures or other controls. Weigelt & Jolliffe (2003) discuss the usefulness of a range of plant competition indices.

Plant competition experiments have also been conducted in the context of the fitness assessment of GMPs compared with competitors (e.g. other *Brassica* weeds), often in combination with other stressors such as herbivores. Damgaard & Kjaer (2009) used a response surface competition experiment in the greenhouse, where both, plant densities and proportions, were manipulated to assess effects of the insect resistance in *Bt Brassica napus* on the competitors *Brassica rapa* and *Lolium perenne* under insect pressure. Liu et al. (2015b) assessed fitness effects of a *Bt* oilseed rape and a wild relative (*B. juncea*) in a replacement series experiment in the greenhouse and field at 5 plant densities in combination with herbivory. Also Vacher et al. (2004) used a (microcosm) greenhouse experiment with five plant densities and three herbivory levels to assess competition effects of *Bt* oilseed rape on a wild relative (*B. rapa*). Competitive effects of vegetation are also important for the survival of scattered GM seeds, e.g. by assessing seed survival of GM and non-GM seeds in cultivated and uncultivated habitats (see e.g. Walker et al. 2004). Weed competition has also been taken into account when assessing volunteers of potato under field conditions (Mustonen et al. 2009).

Vila & Weiner (2004) give some recommendations for the competitive assessment of invasive (alien) species in pairwise experiments that may also be of relevance for assessing the competitive ability of GMPs, such as:

- Compare similar taxa or closely related plants with GMPs (regarding their competitive ability).
- Compare the effect of a GMP on a native (target) species with the effect of another coexisting native on the native (target) species in order to assess the relative impact.
- Assess competition under realistic conditions (in field context, outside field context).

6.7.2 Plant fitness

Fitness is a composite character that is determined by many genotypic and phenotypic characteristics of the plant in conjunction with the environmental context. In order to assess the fitness of a plant comprehensively, it is not sufficient to assess a single character, although some characters may be indicative for fitness. In the scientific literature, fitness assessments of plants are generally done by measuring female fecundity (e.g. seed output) of a plant, but also vegetative fecundity (e.g. growth rate, biomass, seedling height etc., see also below). If a single component of fitness is measured, this is mostly fecundity (Snow et al. 2003). If other parameters are unchanged, an increase in fecundity can lead to an increase in fitness. Enhanced fitness can be defined as a characteristic of an individual or subpopulation of individuals that consistently contribute more offspring to the subsequent generation (Wilkinson & Tepfer 2009). Fitness will vary depending upon the environmental context, particularly upon the presence of inter and intra-specific competitors, herbivores and pathogens, and the abiotic conditions. The variation in fitness according to biotic and abiotic conditions is often referred to as the “genotype by environment interaction” (see Chapter 7). It is therefore relevant that an appropriate range of environmental conditions is considered when assessing the fitness of a plant.

Fitness is usually defined as “relative fitness”, i.e. the contribution of a specific taxon, genotype or individual to the gene pool of the next generation in comparison to other taxa, genotypes or individuals (e.g. non-GM plants, parental plants). Fitness assessments are also often referred to as “performance” assessments in the scientific literature, e.g. when assessing the fitness of hybrids in relation to their crop or wild parents. In general, fitness can be defined for a range of scales and metrics used, e.g. individual fitness, population fitness, lifetime fitness, composite fitness, reproductive fitness, vegetative fitness etc.

Experience with experimental approaches of fitness assessments is available from different research areas, such as:

- Fitness of weeds, e.g. when evaluating fitness costs due to herbicide tolerance (Vila-Aiub et al. 2015; Vila-Aiub 2019)
- Comparison of crop plants (either GM or non-GM) with crop-wild hybrids for plant taxa that are able to hybridize and form crop-wild hybrids which are volunteers or can build up feral populations in a specific region (e.g. sunflower in the USA, oilseed rape in Europe and soybean and rice in Asia, see Annex E)
  - Soybean (e.g. Guan et al. 2015; Liu et al. 2021b)
  - Sunflower (e.g. Snow et al. 2003; Mercer et al. 2006; Mercer et al. 2007).
  - Oilseed rape (e.g. Hauser et al. 1998a; Hauser et al. 1998b; Stewart et al. 1997; Londo et al. 2011; Vacher et al. 2004; Snow et al. 1999; Allainguillaume et al. 2006; Moon et al. 2007)
  - Rice (e.g. Yang et al. 2017; Jeon et al. 2021, Song et al. 2004)
  - Maize (Guadagnuolo et al. 2006)
- Fitness comparison of native and introduced (invasive) plant populations (Caño et al. 2008)

There are several different experimental approaches to assess plant fitness. For example, common garden field experiments or field experiments with or without plant competition under agricultural conditions are carried out (see above). Experiments in greenhouses with plants in pots (e.g. Guan et al. 2015; Liu et al. 2021b, Vacher et al. 2004) or in growth rooms (e.g. Snow et al. 1999) are also performed. Mesocosm experiments similar to small field plots are also carried out. For example, Waschmann et al. (2010) have modified outdoor sunlit open top chambers usually used for studies of effects of atmospheric pollutants. The sunlit mesocosm can be used for a range of studies for risk assessment of GMPs, as confined testing system as these are specially designed with pollen filters to avoid escape of pollen or seeds, thereby supplementing field tests. For example, Londo et al. (2011) used these mesocosm testing systems to assess fitness effects of stacked GM traits in oilseed rape. In addition, there are combinations of contained and field assessments (see e.g. Guan et al. 2015, Moon et al. 2007).

In plant fitness experiments, many surrogate parameters for plant fitness are used. Younginger et al. (2017) give an overview of the fitness parameters used in plant research. They report that most studies use seed-related fitness parameter, but also biomass is positively correlated with fecundity and therefore may serve as proxy for plant fitness. The most direct fitness estimate, however, would be to measure the parental contribution to offspring in successive generations.

In the above-mentioned studies assessing the fitness of GM plants, non-GM plants, weeds or invasive species, most frequently parameters relating to seed production are used (e.g. seed mass, seed number, seed biomass, relative seed production per g vegetative biomass or per plant, but also seed viability/germination, flower number, flowering time, fruit number). In addition, vegetative fitness is assessed, e.g. growth rate, biomass, relative growth. In contrast, male fitness parameters are rarely assessed, if, then pollen production and/or pollen viability are used as surrogates.

In some cases, a “composite” fitness measure is used that combine several fitness-related traits, e.g. across life-history stages (e.g. by calculating the mean values of variables of germination, growth, reproduction etc.) or across vegetative and reproductive parameters (Song et al. 2004; Guan et al. 2015; Liu et al. 2021b). However, these composite measures (e.g. indices across life history traits) may not necessarily result in differences in plant comparisons, which is often in contrast to vegetative and reproductive fitness parameters (Song et al. 2004).

Fitness assessments under specific selection pressures can affect plant fitness. These selection pressures relate to:

- Herbicide application compared to no application in a herbicide tolerant plant, in order to evaluate the fitness cost of the transgene (Mercer et al. 2007 Li et al. 2021; Londo et al. 2011).
- Competitors, including weedy or crop competitors (Mercer et al. 2007; Mercer et al. 2006; Liu et al. 2021b; Londo et al. 2011).
- Herbivore pressure, e.g. at different strengths (Xia et al. 2016; Letourneau et al. 2003; Stewart et al. 1997; Moon et al. 2007; Londo et al. 2011) or disease pressure (Burke & Rieseberg 2003).
- A combination of stress factors, e.g. competition and herbivory Mercer et al. 2007; Stewart et al. 1997; Londo et al. 2011, Vacher et al. 2004).

When conducting fitness assessments in the GM ERA context, the following aspects should be considered:

- Assess fitness of the GM crop (or the GM hybrid) relative to the non-GM crop (or hybrid) and fitness of GM crop-wild hybrid(s) relative to the GM parental taxa.
- Assess reproductive fitness (i.e. male and female fitness).
- Assess also vegetative fitness for perennial species (e.g. biomass, tiller number, lateral growth, colony diameter).
- Assess several fitness-related endpoints to cover the entire life history and the main components of fitness (dormancy, germination, survival and fecundity.
- Develop demographic models to evaluate population level impacts of plant fitness changes.
- When assessing hybrid fitness, test more than one generation and consider different backcrosses or filial generations (BC1/BC2, F1/F2) as lower performance of e.g. F1 hybrids can be offset in the F2 progeny.
- Consider strong genotype x environment effects on fitness. Certain traits enhance fit-ness depending on the environment and the prevailing stress conditions, e.g. drought.
- Consider traits already prevailing in wild populations, such as a certain degree of tolerance to herbicide damage already present in wild soybean or pest resistance present in teosinte population.
- Consider the influence of the genetic backgrounds of parental lines of crop and wild plants used.

- Consider intraspecific or interspecific competitive interactions on fitness. Measured fitness components under controlled conditions may give other results under field conditions due to plant competitive interactions.
- Select relevant environments in which the plant is expected to thrive, e.g. agricultural environments but also habitat types, which typically occur near agricultural fields, e.g. disturbed sites.
- Assess fitness separately for different herbicide tolerance traits as beneficial effects on fitness cannot be generalized for a specific GM trait.
- Consider artificial inoculation under presence and absence of pest/pathogen pressure for pest or pathogen resistant GMPs, such as manipulation experiments with different herbivore pressure levels and different herbivore combinations, in combination with the assessment of plant damage.
- Assessments without selection pressure should be made for herbicide tolerance traits to assess effect of transgene on the relative plant fitness.
- For the evaluation of hybridisation and introgression into wild relatives, the fecundity of the plant (pollen viability as well as flowering characteristics) in addition to germination, dormancy, survival parameters are important.

6.7.3 Conclusions

Plant competition and fitness comparisons between the GMP and the non-GM counterpart can only be assessed in manipulative experiments, under either contained (greenhouse) or field conditions. Such experimental assessments are common in plant ecology and have also been applied for GMPs. EFSA (2010) refers to such experiments for the assessment of the persistence and invasiveness of GMPs (see Table 2). However, due to the need of a specific experimental setup, such assessments cannot be carried out in the context of standard agronomic field trials, but need to be addressed in separate risk assessment studies.

6.8 Phenotyping abiotic stress responses

Abiotic stress, i.e. frost, drought, heat, salinity or water stress, can seriously affect crop yield and productivity and therefore negatively affect a plant’s fitness (Liang et al. 2014). Changes in the plant’s response to abiotic stress can be either intended (e.g. stress-tolerant GMP) or due to unintended effects of the genetic modification.

Abiotic stress tolerances are quantitative traits with stress-responsive genes involved in several stress response metabolic pathways (Zhao et al. 2019). For example, regulatory proteins such as transcription factors affect expression of genes involved in abiotic stress tolerance. Therefore, a specific abiotic stress tolerance may also (predictably or unpredictably) confer tolerances to other abiotic stress conditions (Khan 2011). For example, the salt-tolerance-inducing coda gene in GMPs also conferred tolerances to other abiotic stress conditions (Chen & Murata 2008 cited in Khan 2011). The drought tolerance gene ABF3 inserted in drought tolerant potato is known to increase also cold tolerance (Kim et al. 2010). A transgenic creeping bentgrass overexpressing a microRNA conferred multiple abiotic stress tolerances (Zhao et al. 2019).

If plant hormones (e.g. abscisic acid, jasmonic acid, ethylene or salicylic acid) are affected in abiotic stress tolerant GMPs, this can also impact abiotic and biotic stress signalling or even phenotypic traits related to plant growth or seed-related parameters such as dormancy (Khan et al. 2011, Zhao et al. 2019). For example, an herbicide tolerance trait in *Arabidopsis* sp. affected the germination characteristics of the plant under drought conditions, even without application of the complementary herbicide (Fang et al. 2018). If the genetic modification unintendedly affects certain plant signalling pathways that are important for mediating the response to abiotic stressors, then such effects may become evident. Such changes can be relevant for the ability of the plant to become weedy (persistent) or invasive, if e.g. reproductive characters, vegetative growth or the competitive ability of the GMP are affected in a specific environmental context (Liang 2016; Khan 2011).

Due to interactions between abiotic and biotic stress signalling pathways, also changes in responses to biotic stressors, such as pathogens or pests, are possible. However, although one of the aims of the agronomic and phenotypic evaluation of the GMP is to identify such unintended effects in the response of the GMP to abiotic stressors, such interactive effects have not yet been addressed in risk assessment studies of abiotic stress tolerant GMPs (Khan 2011).

So far, a range of GMPs with abiotic stress tolerance have been developed and field tested, most of them by addressing regulatory or metabolic genes, transcription factors or specific enzymes such as heat shock proteins (see Liang et al. 2014, Khan 2011, Zhao et al. 2019). Despite many efforts to develop GMPs with abiotic stress tolerance, few have made it to the market so far. The only abiotic stress tolerant GM crop which has been approved for import and processing in the European Union is drought tolerant maize MON87460 (see also below – Example maize).

6.8.1 General methods

Approaches to evaluate the response of a plant to abiotic stress in the scientific literature refer to methods used for breeding purposes, applied plant stress research of crops in general (Rapacz et al. 2015), stress-tolerant GM crops (Zhao et al. 2019) or invasive alien species (Bykova & Sage 2012).

A few selected methods to evaluate abiotic stress responses for agricultural and applied plant stress research are available at https://plantstress.com/. General methods to evaluate plant stress include e.g. chlorophyll fluorescence, leaf canopy temperature, thermal and spectral imaging of plant stress or methods to evaluate changes in phytohormones. Some methods refer to specific stress types, such as drought stress, heat stress, salinity stress, stress due to mineral deficiency or toxicity, oxidative stress, cold stress, and water logging. In the examples outlined below, drought stress and cold stress are addressed.

Marchin et al. (2019) developed a simple glasshouse (pot) experiment in order to assess drought responses of different plant species or genotypes using different drought stress intensities and in combination with other abiotic or biotic factors.

A reliable parameter to quantify any stress impact on the plant is the abundance and in-plant localization of reactive oxygen species and the associated anti-oxidant enzymes. The most important enzymes are superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), which all have available protocols for *in-situ* staining and can be indirectly quantified on a plant by plant or organ by organ basis through image analysis software (Abdel Latef & Tran 2016 and references therein). Chlorophyll contents, which also sometimes are used as a parameter to measure stress impact, can be measured spectrophotometrically after pigment extraction

(Abdel Latef & Tran, 2016), via Chlorophyll meter (e.g. SPAD) or remote sensing (Miao et al. 2009).

6.8.2 Methods to assess cold stress response

Cold stress is an important abiotic stress factor, which affects the performance of crop plants, specifically if it occurs during late frosts in spring causing damages in crop plant tissues. However, plants are able to adapt to low temperature stress, also known as cold acclimation.

Cold and frost damage in plants occurs due to the ice formation in tissues, which damages cell membranes, or other cell components (see Chen et al. 2014 and references therein). Tolerance to cold stress is mediated by an array of functional genes that protect cell membranes and regulation genes that regulate cold signalling pathways. In contrast to freezing tolerance, measured in the laboratory, winter survival under field conditions necessitates that plants not only resist to the low or freezing temperatures, but also to other (abiotic and biotic) stress factors. Therefore, in plant breeding, field-testing to evaluate winter survival is often preferred over assessing freezing tolerance (Reynolds et al. 2001). For this purpose, recommendations for field-testing have been formulated to be used in combination with laboratory techniques to assess freezing resistance in crops (e.g. for cereals, see Saulescu & Braun in Reynolds et al. 2001). The focus is on multilocational testing due to the variability between locations and fields regarding soil preparation, soil and plant moisture and environmental aspects such as snow cover. Also combined assessments of freezing tolerance in the climate chamber and field survival studies are recommended due to the weak correlation of winter hardiness of plants in the lab and actual winter survival in the field (Rapacz et al. 2015).

As a general approach for freezing tests, tissues or plants undergo a freeze-thaw-cycle after which the plant injury is evaluated. Cold hardening can be done beforehand. The effective temperature regime used will depend on the plant species. The following tests to assess the injury after cold stress have been recommended: visual observation, measuring plant regrowth and measurement of ion leakage from tissue (https://plantstress.com/cold-methods/). In addition, indirect measurements of freezing tolerance (e.g. chlorophyll fluorescence or electrolyte leakage method) are frequently used (Rapacz et al. 2015; Bykova & Sage 2012).

Example: cold stress in potato

Li et al. (1981) extensively discuss frost resistance and cold acclimation in tuber-bearing *Solanum* species. Frost resistance (also referred to as frost hardiness) is given if species can survive freezing temperatures of -4°C or colder, while frost sensitivity (non-hardiness) refers to species that can survive temperatures of -2,5°C or warmer. The authors report a 3-4°C difference between frost resistant and frost sensitive potato species. Cultivated *S. tuberosum* cannot survive below -2.5°C and cannot acclimate well to cold conditions. However, *S. tuberosum* has a relatively high heat resistance level.

Factors contributing to winter survival in potato plants are the rate of acclimation in fall (if any), variation in snow cover, lowest temperature, midwinter thaw periods followed by very cold temperatures, and rate of de-acclimation in spring (see Vega et al. 2000 and references therein). The ability to acclimate rapidly during early fall and to de-acclimate slowly in response to midwinter thaw is of great importance for winter survival.

Common laboratory tests to evaluate cold stress in plants are also used for potato, such as the TTC (triphenyl tetrazolium chloride) reduction and the conductivity test (Chen et al. 1979). The TTC test is usually used to assess viability of seeds that failed to germinate or the metabolic activity of biologically active plant parts (Del Lopez Egido et al. 2017 and references therein). The conductivity test measures electrical conductivity of plant tissue extracts. Also Waterer et al. (2010) assessed cold stress effects at the tissue level. Young potato leaves (from either greenhouse or field grown plants) were exposed for 1 h to each test temperature (1°C steps from 0 to -8°C) and the conductivity of the leaf leachate was assessed.

Mustonen et al. (2009) assessed winter survival of potato tubers and seedlings under controlled lab conditions and under field conditions in Finland. Field assessments monitored seedling numbers from true potato seeds as well as size and numbers of tubers derived from these seedlings. Depending on the soil temperature regimes (and snow cover) up to 3.5% of the tubers survived winter conditions. Potato seeds remained viable over the winter and produced seedlings (300-700 seedlings/ha) which themselves also produced tubers (three to nine tubers per seedling).

Also Boydston et al. (2006) assessed freezing tolerance of potato tubers. They quantified the freezing injury of tubers at sub-zero temperatures in field trials over 6 years, by assessing tuber mortality and viability. Significant tuber mortality occurred at -2.8°C across all depths, sites and years but tuber survival largely depended on soil depth with almost all tubers survived which were buried at 20 cm. Potato tubers are reported to have the ability to supercool to several degrees below their freezing point (up to -8°C), at least under laboratory conditions. Under field conditions, however, supercooling is not relevant due to the presence of soil. In addition, tubers in the field do not fully freeze but only in part.

6.8.3 Methods to assess drought stress response

Drought stress is one of the most important abiotic stressors for crop plants as it can reduce crop yield significantly. For example, the annual yield loss of maize due to drought has been estimated at 15% (Liang 2016). Soybean is even more drought-sensitive with estimated 40 % crop losses under drought-conditions (Liang 2016). For agricultural purposes, drought is defined as “a period of below-average precipitation when the amounts of available water in the plant rhizosphere drop below the limits required for efficient growth and biomass production” (Osmolovskaya et al. 2018 and references therein).

GM drought-tolerant maize, soybean and potato have already been developed and commercialized in some countries (see below). GM crop plants developed for drought tolerance include the modification of the stress response to absicic acid (ABA) or modification of ABA-independent gene regulatory pathways, mostly by modulating specific transcription factors that induce the expression of stress response genes (https://www.isaaa.org/resources/publications/pocketk/32/default.asp).

General methods

Laboratory experimental approaches in drought stress research are explored in detail by Osmolovskaya et al. (2018), referring specifically to soil-based models, hydroponic aqueous culture, and Agar-based experimental setups. Monneveux et al. (2014) give an overview of plant phenotyping methodology in general and for specific crop types.

General methods to evaluate the response of plants to drought stress include (https://plantstress.com):

- The estimation of soil water content in the field, based on a field manual of the IAEA (2008).

- The estimation of the Relative Water Content of the plant as a measure of plant water status.
- The calculation of the drought resistance index in terms of yield.

Field capacity (FC) and the permanent wilting point (PWP) are also used to define the intensity of drought stress. Field capacity is influenced by many factors, e.g. the status of the soil before irrigation (Kirkham 2005). Assessing an earlier onset of maturity of the plant is covered indirectly by the VCU methods and determination of BBCH stages.

The least work intensive options is to quantify the evapotranspiration (e.g. Yuan et al. 2019), e.g. by measuring the potential evapotranspiration (without drought stress) and actual evapotranspiration in combination with soil moisture availability and summing up the deficit over time similar to degree-days (Shaw and Newman 1991). This can be done by use of lysimeters or modelled via the Penman equation (Penman and Keen 1948). This leads to a comparable quantification of the drought stress, which can then be used to better compare other parameters, like yield, reactive oxygen species or chlorophyll content.

Decisive for the plant reaction is also in which growth phase drought prevails and how long it lasts. An early summer drought can significantly affect winter oilseed rape yields, while soil water may still be sufficient for young maize and soybean plants. For an informative experiment, it is crucial to ensure drought stress exposure to an appropriate extent. Trials under controlled environmental conditions (rainout shelters, phenotyping platforms) meet these requirements. Additionally they offer the possibility of positioning cameras for imaging of the plants' development and reaction (Kant et al. 2017). However, these devices are also costly and their results (e.g. variety ranking) must ultimately be confirmed under field conditions.

A good compromise in the experimental set-up is realized by trial sites in the cultivation area that reliably allow drought stress situations to be expected during the whole season and permit the control of water scarcity or supply in time and intensity by irrigation (Tuberosa 2012). Such trials in water scarce regions must be combined with an exhaustive monitoring of the environmental conditions given during the vegetation period, such as the hydrogeological characterisation of the experimental sites (Struckmeier & Margat 1995) and the continuous monitoring of the actual growing conditions during the season. These weather and soil data form the basis for the interpretation of the results and differences between the test substances and reference varieties.

Plant architecture changes would also still be a good approach, since they can partially be conducted using machine learning and Deep Convolutional Neural Network (DCNN) approaches (e.g. An et al. 2019). Changes in plant architecture, size, shape, and colour of plants or plant parts in reaction to drought stress as can be detected and analysed well and in repeated sequences with digital imaging using RGB cameras. Leaf temperature measured by thermal imaging is a well-suited approach for estimation the water supply status of plants (Prashar & Jones 2014). Based on the development of leaf temperature during the vegetation period, the ability of the plants to use water following periods of drought stress can be surveyed. Hyperspectral imaging provides the data for calculating fitness indices for the plants such as the Normalized Difference Vegetation Index (NDVI), providing information on the health status of the observed plant population.

Investigations in changes of root system architecture need a lot of manual labour to extract wash and then further analyse the roots in a field experiment (e.g. Kumar et al. 2015) or a rhizo-box approach under comparable lab/greenhouse conditions (e.g. Dermenjiev et al. 2021).

Data recording aided by modern phenotyping techniques such as RGB digital imaging, thermal imaging (leaf temperature) and hyperspectral imaging (Normalized Difference Vegetation Index NDVI) help to indicate the drought stress level plants had to suffer but at least yield figures as an allover index of the situation are essential. One example for oilseed rape utilizes hyperspectral imaging to quantify healthy and damaged tissue in seedlings (Żelazny & Lukáš 2020).

Example: drought stress in maize

For breeding purposes of drought resistant maize varieties, the use of targeted (drought) stress conditions is common practice. Campos et al. (2006) evaluated 18 maize hybrids under drought stress conditions with overlapping periods of water deficit. Except from pre-flowering, also the late grain filling development stage is a sensitive period under drought conditions. From the breeder's perspective, the yield components and anthesis-silking intervals are important as target assessment endpoints. Zaman-Allah et al. (2016)) provide a field manual for the identification and selection of superior maize lines for use in breeding drought tolerant maize varieties, which is a priority issue of the International Maize and Wheat Improvement Center (CIMMYT). The manual identifies basic requirements for generating high-quality phenotyping data for drought stress under field conditions, referring to the selection of the field trial site, the crop management, the recording of weather data, the management of drought stress and data collection, specifically with respect to the phenotypic traits (Zaman-Allah et al. 2016).

Other authors assessed different maize hybrids for their drought tolerance under greenhouse (Pires et al. 2020) or field conditions (Adebayo & Menkir 2014; Su et al. 2019; Sammons et al. 2014; Traore et al. 2000). The authors evaluated different maize hybrids under a well-watered and a water-limited treatment, usually by suspending irrigation treatments at certain growth stages (usually around the time of flowering of the maize or at the late vegetative to early grain-fill period).

Drought stress can be assessed by visual inspection of plants. For example in maize, the anthesis-silking synchronicity may be affected (Harrison et al. 2014). Generally, extended drought and heat stress is displayed as stunted growth and temporary leaf rolling. The presence or absence of leaf rolling alone is, however, no reliable indicator of drought stress. Additionally, in case of maize and other Poaceae, plant-available silicon plays an important role in dealing with abiotic and biotic stress (Abdel Latef & Tran 2016). Similarly, plant-synthesized antioxidants are important in reducing the damage caused by stress-induced reactive oxygen species (ROS) on tissue and the photosynthetic pathways (Gill & Tuteja 2010). Other visible symptoms of drought stress are reduced number and size of leaves. This symptom can be used additionally to reactive oxygen species- (ROS) and chlorophyll-quantification to assess drought stress impact in long-term experiments (Qaderi et al. 2006).

6.8.4 Modern phenotyping methods for assessing plant stress

High-throughput phenotyping methods are increasingly being used to assess plant responses to abiotic stress and to identify robust phenotypes for further breeding, including approaches that use machine learning for stress phenotyping in plants (Humplík et al. 2015; Campbell et al. 2018; Smith et al. 2021; Veley et al. 2017). For example, in order to identify plants with drought tolerant traits, Su et al. (2019) were able to discriminate drought tolerant maize phenotypes assessing vegetative traits like plant height, plant area, leaf area under drought conditions by use of terrestrial LIDAR technology. Wasaya et al. (2018) give an overview of a range of laboratory, greenhouse or field based phenotyping methods for a range of root traits that are sensitive to drought conditions, specifically fine root diameter, root length and area or root length density or root angle. Campbell et al. (2018) summarize common high-throughput phenotyping platforms to assess tolerance to different abiotic stressors of a range of crops.

High-throughput phenotyping of crop plants are also being used to screen and select genotypes for drought tolerance and to reduce the manual labour involved in the screening step (see summary in Arya et al. 2021). This involves, remote sensing techniques including thermal, spectral and hyperspectral imaging techniques for e.g. estimation of the chlorophyll con-tent or to distinguish genotypes under different watering conditions and at different phenological stages or to estimate yield under drought stress.

Musse et al. (2021) applied a holistic approach for phenotyping drought stress responses of potato using three different watering regimes. They used modern methods in a controlled and a semi-controlled environment (greenhouse). Physiological measurements referred to soil and leaf water potential, osmotic potential of leaves, leaf water deficit and tuber biomass. The stress phytohormone ABA (abscisic acid) and genes responsible for adaptive stress responses (i.e. gene expression analysis, e.g. heat shock proteins) were also assessed. By use of a high-throughput phenotyping platform monitored shoot morphology and plant colour. By use of NMR (nuclear magnetic resonance relaxometry) the authors assessed structural leaf modifications and by MRI (magnet resonance imaging) the number, distribution and volume of tubers underground.

6.8.5 Conclusions

Assessment of the plants' response to cold stress is important for crops with winter survival or if crops are planted early in the season and experience late frosts (e.g. potato). Due to a low correlation of results between lab and field testings, any assessment of cold tolerance and/or winter survival will need a combination of temperature-controlled climate-chamber experiments and assessments under field conditions, ideally at multiple locations. Currently, standard experimental protocols for testing response to cold stress are lacking, however, methods are available from the scientific literature. For ERA, test procedures including relevant temperature regimes should be developed, specifically relevant for particular crop species (e.g. potato).

For the assessment of drought stress response, the use of targeted drought stress conditions is common practice in plant breeding drought-tolerant crops. For this purpose, field manuals including guidance on how to carry out field experiments under drought conditions, including the management of drought stress and phenotypic trait recording, are available. In addition to the assessment of the survival of the plants (e.g. in the following year) such assessments can provide useful information on the GMPs' ability to survive under drought conditions.

6.9 Phenotyping response to biotic stressors

Currently applied methods to evaluate the response of the GMP to biotic stressors are considered not appropriate to assess potential changes in the GMP's phenotype as compared to the non-GM counterpart. A fundamental analysis and discussion of methods currently applied in GMP applications including the identified shortcomings can be found in Annex A.

In this report, we present state-of-the-art methodological approaches that can be used to characterise the plant phenotype with respect to its response to biotic stressors, i.e. pests and pathogens. Annex F to this report presents criteria applied for the selection of relevant pest species for the examples maize, oilseed rape, soybean, potato and creeping bentgrass under EU conditions. Annex F also summarizes methods suggested for rearing these species, as well as protocols to assess the plants' response under either field or contained conditions.

Due to the economic relevance, the interactions of crops with pests or pathogens are intensively researched both in the field and in the greenhouse. Therefore, methods to assess the plants' response to pests and pathogens (e.g. damage assessment) are available e.g. from variety testing of new crop varieties (see Chapter 6.1). Methods are also available to assess a range of pest species at least for the pest and pathogen species discussed in this report based on internationally recognized protocols (e.g. EPPO) as well as scientific literature (see Annex F). These methods are suitable to support the agronomic and phenotypic characterisation in GMO risk assessment and should be put into practice.

The suggested methodology also includes protocols for artificial infestation of plants (either under contained or field conditions) and includes an evaluation of the practicability of carrying out such experiments together with rearing of the relevant pest species.

It is important to notice that the methodological approaches chosen will depend on the respective pest species. Pre-season or parallel monitoring is required for many pests, while for others (e.g. those with infrequent occurrences) artificial infestation experiments are needed (see Annex F). Most of the methodological approaches are based on manual field assessments. Currently there are no accepted standardized methods for the assessment and quantification of insect or pathogen damage. Some examples for novel methodologies (e.g. remote-sensing or image-based methods) might be useful in the near future and are also outlined in Annex F.

6.9.1 Conclusions

The analysis of the current practice in GMP applications has shown that the assessment of biotic stressors (pests and pathogens) in the agronomic and phenotypic characterisation is carried out according to their occurrence at the respective field site. In addition, the application of the "standard agricultural practice" with respect to the application of pesticides distorts the evaluation of pest and pathogen occurrences and therefore the plants' responses to biotic stressors. Therefore, conclusions on potential differences between the GMP and its comparator cannot be made.

Instead, a targeted selection of sites with relevant pest pressure (including monitoring and reporting) or the use of artificially infestation experiments is needed. The application of the appropriate protocols for selected pests and pathogens as proposed in this report could contribute to important improvements to the current practice, particularly if the scope of authorization includes cultivation of the GMP in the EU.

7 Analysis of plant- and GM trait-specific characteristics

In this chapter, recommendations are made with respect to case-specific phenotypic endpoints relevant for survival, persistence and invasiveness for individual species and GM traits, considering four different crops (potato, maize, soybean, oilseed rape) as well as creeping bentgrass. In a second step, four potential GM traits (herbicide tolerance, insect resistance, drought tolerance and changes in seed composition) are discussed with respect to relevant characteristics if considering persistence and invasiveness.

7.1 Plant-specific characteristics

In the agronomic and phenotypic characterisation of GMP, specific crop types may require the assessment of additional case-specific endpoints. EFSA refers particularly to so-called "persisting species" such as potato and oilseed rape (EFSA 2015). Such additional endpoints should consider the GM plant's ability to survive and persist in or outside agricultural habitats.

7.1.1 Potato

Important phenotypic plant characteristics relevant for the persistence and invasiveness of potato relate to potato tubers and seeds. In Europe, outcrossing to and hybridization of the potato plant with wild relatives is unlikely, while it occurs in its centre of origin (Celis et al. 2004; OECD 2006). The potato plant produces seeds mainly formed by selfing. Potato seedlings can also form from these berries (from true potato seeds TPS) which can then produce tubers and give rise to potato volunteers in the year following cultivation. Such TPS can survive in soil for at least 6 years (Lawson 1983). In addition, potato tubers are often left in the fields after harvest, in particular if they are small sized (Kim et al. 2010) with estimations of up to 500.000 tubers per hectare left in European fields (Boydston et al. 2006). If soil tillage after harvest is too shallow and not all tubers are deposited at surface level in addition to mild winter conditions, which do not kill tubers by frost, then weedy plants can arise. Potato volunteers can derive from small pieces of tubers, if they have one eye, but also from seeds and even from dropped sprouts. Hence, potato volunteers are considered important perennial weeds and are extremely difficult to control by herbicides as they are only partially effective and the plants can recover from tubers.

The following plant characteristics are therefore specifically relevant for potatoes with respect to persistence and invasiveness:

- Tuber characteristics and tuber winter survival including volunteer seedlings from tubers
- Seed germination and dormancy of true potato seeds (derived from berries) and winter survival of volunteers from seeds

7.1.2 Soybean

An important plant characteristic relevant for persistence and invasiveness of soybean is the ability of the seeds to survive winter conditions. Soybean seeds have no dormancy but can occur as volunteers in subsequent crops under favourable conditions or if significant shattering due to wind occurs (OECD 2000, 2006). In general, soybean volunteers are considered weak competitors. Soybean seeds have a good water permeability due to the need for rapid germination (Kuroda et al. 2013). However, for winter survival, water uptake can be disadvantageous but seed viability is not always lost by water uptake. Soybean seed can also survive winter conditions in Japan (Kuroda et al. 2013). In general, soybean is not competitive, neither in fields nor in natural habitats, and in Europe, so far feral populations are not known to exist. However, soybean volunteers can also occur in Europe, e.g. in Italy (Celesti-Grapow et al. 2010 cited in EFSA 2017b). Jhala et al. (2021) report the occurrence of glyphosate-resistant soybean volunteers in rice fields in southern US.

Soybean is a self-pollinated species and has no wild relatives in Europe. In other parts of the world, the weedy wild species *Glycine soja* and *G. gracilis* are present, e.g. in China, Japan, Korea and Russia and hybridization of cultivated soybean with wild soybeans is possible (Kuroda et al. 2013; OECD 2006). In these countries, interspecific soybean hybrids can successfully survive in semi-natural habitats.

Important parameters for survival of soybean are seed production and shattering (e.g. seed number, seed weight, pod number etc.), flowering phenology (e.g. days to first flower) as well as seed dormancy including seed winter survival (e.g. the total number of seeds expected to germinate the following year). In addition, the number of seed-carrying pods (per plant) and pod shattering (ease of shattering of pods) is important for plant fitness and survival (Matsushita et al. 2020).

The following plant characteristics are therefore specifically relevant for soybeans with respect to persistence and invasiveness:

- Seed germination including dormancy and overwintering ability of seeds
- Pod shattering

7.1.3 Maize

Maize has its centre of origin in Central America and is largely cultivated in Europe. It has no wild relatives in Europe. Due to its biology, it is adapted to warmer climates. Volunteer populations in fields but also feral populations outside agricultural fields are known to occur in Europe (Pascher 2016; Pascher et al. 2011; Palaudelmàs et al. 2009; OECD 2006). Maize occurrence in and outside fields results from harvest residues, crops of previous years, transport, handling, storage, game feeding or even import. The persistence of such populations over several years is currently considered unlikely. More volunteer plants are, however, likely to occur in the future in view of milder winter temperatures (AGES, pers. Comm.).

Since a few years, a wild relative of maize, the teosinte has been detected in Spain and France as an invasive weed in agricultural fields (Devos et al. 2018). These European teosintes derived from a weedy Mexican teosinte and show patterns of introgression of Europe-an maize germplasm, including herbicide tolerance and possibly insect resistance Le Corre et al. 2020; Lohn et al. 2021) with implications for risk assessment and management (Devos et al. 2018).

The following plant characteristics are therefore specifically relevant for maize with respect to persistence and invasiveness:

- Seed germination including dormancy and overwintering ability of seeds
- Pollen viability

7.1.4 Oilseed rape

Oilseed rape does not occur as a wild plant but it is able to build up feral plant populations out-side agricultural fields. It is a well characterized plant, specifically regarding its hybridisation ability with wild relatives, the longevity of seeds in the soil seed bank as well its ability to establish plant populations also in semi-natural habitats (see e.g. Squire et al. 2011; Pascher et al. 2017; OECD 2006; Claessen et al. 2005a; Claessen et al. 2005b).

The following plant characteristics are specifically relevant for oilseed rape with respect to persistence and invasiveness:

- Seed germination including dormancy and overwintering ability of seeds
- Pod shattering
- Pollen viability

7.1.5 Creeping bentgrass

Creeping bentgrass is a clonal perennial plant. A peculiarity of this plant is that – in contrast to other (GM) crop plants – it is intended to persist in the environment where it is released (e.g. on golf courses). Phenotypic characteristics most relevant for its risk to persist or become invasive relate to the sexual reproduction (pollen and seed) but also to the vegetative reproduction of the plant, e.g. via aboveground stolons and tillers. Creeping bentgrass is able to establish new plants via stolons or by dispersal of vegetative parts of the plant. Creeping bentgrass occurs naturally in European natural habitats such as wet meadows, but also as a pioneer plant next to waterways, along shores and reeds (Fischer and Adler W. 1994). It also tolerates salty soils. Hybridisation and establishment of transgenic traits in natural populations due to pollen-mediated intraspecific hybridizations and from crop seed dispersal has been shown (Belanger et al. 2003; Reichman et al. 2006; Snow 2012; Zapiola & Mallory-Smith 2017; Watrud et al. 2004). Vegetative spread can be assessed by measuring plant (colony) diameter, plant surface area, leaf height, number of tillers (stolons), number of panicles, and number of flowers or above-ground plant biomass (Ahrens and Auer 2012; Gardner et al. 2003; Garrison & Stier 2010).

The following plant characteristics are therefore specifically relevant for creeping bentgrass with respect to persistence and invasiveness:

- Seed germination including dormancy and overwintering ability of seeds
- Pollen viability
- Vegetative growth and dispersal

7.1.6 Conclusions

Different crop types constitute different levels of risk with respect to their ability to survive and spread in agricultural and non-agricultural habitats (Figure 2). For all crop types addressed in this study, the ability to spread is linked to the seed characteristics germination and dormancy as well as to the viability of pollen. In addition, for perennial species like grasses spread and survival by vegetative dispersal is crucial. For potato, tuber survival but also seed survival and dispersal are relevant. For plants like soybean or oilseed rape also pod shatter ability affects spread and survivability.

For the agronomic and phenotypic characterisation, these plant traits are of particular relevance. Nevertheless, the target environment has also to be considered, in particular if seeds are released into non-agronomic habitats (e.g. during import, harvest or processing activities). This requires assessment of seed characteristics also under non-optimal conditions (see Chapter 6.3).

Level of risk

Plant species	Classification of persistence and ferality	Assessment environment	Plant traits particularly important in the agro-pheno characterization
Soybean	Non-persistent, non-feral	In-field (volunteers)	• seed germination including dormancy • overwintering ability of seeds • pod shattering
Potato	Non-persistent?, non-feral	In-field (volunteers)	• Tuber characteristics • Overwinter survival of tubers/volunteer plants from tubers • Seed germination including dormancy of seeds (TPS) • Overwintering ability of volunteers from seeds
Maize	Non-persistent, non-feral	In-field (volunteers, weed-hybrids) Outside fields	• Seed germination including dormancy • overwintering ability of seeds • Pollen viability
Oilseed rape	persistent, feral, hybridization with wild relatives)	In-field (volunteers) Outside fields (ferals, hybrids)	• Seed germination including dormancy • overwintering ability of seeds • pod shattering • Pollen viability
Creeping bentgrass	persistent, hybridization, perennial (natural occurrence)	In-field, outside fields (wild populations)	• Seed germination including dormancy • overwintering ability of seeds • Pollen viability • Vegetative growth and dispersal

Fig. 2: Differences in the ability to survive and persist in and outside the agronomic context (fields) and phenotypic traits relevant for the agronomic and phenotypic characterisation. For terminology, see Chapter 3).

7.2 Trait-related characteristics

Case-specific endpoints of the agronomic and phenotypic characterisation refer not only to the plant species but also to those relevant for a particular GM trait, which may require the assessment of additional endpoints. For specific GM plant traits, additional plant characteristics may be relevant to characterise the plant's ability to survive, disperse and persist in the environment.

7.2.1 Insect tolerance

For the agronomic and phenotypic characterisation of GM crops with insect tolerance (e.g. *Bt* crops) the response of the GMP to biotic stressors needs to be assessed. This refers not only the target organism (which constitutes the assessment of efficacy of the GMP) but to other biotic stressors, such as non-target pests that may develop into secondary pests in a specific receiving environment where this GMP is grown. The analysis of GMP applications in this study (see Chapter 4.3) as well as in previous studies (Dolezel et al. 2011) have shown a range of methodological shortcomings in the evaluation of biotic stressors in agronomic and phenotypic field trials. A consistent methodology regarding the selection of relevant non-target pest species, monitoring of pest populations and artificial infestation studies as well as quantitative assessments are generally lacking. Specifically, pest species that are to a lesser degree susceptible to the relevant *Bt* toxins (e.g. *Sesamia* spp., *Spodoptera* spp.) should be considered as non-target pests. For example, the *Bt* toxin Cry1Ab is not effective against certain secondary pests in Europe, such as the western corn rootworm and the true armyworm with implications for the sustainability of this GM crop (Catarino et al. 2015). In addition, unintended effects due to the genetic modification resulting in chemical or nutritional changes of the GM crop can trigger higher infestation by secondary pests (Bastos et al. 2007).

Resistance to a herbivorous insect can increase the fitness of a GMP and therefore affect its ability to survive and persist. Many lepidopteran larvae reduce the plant's fitness, e.g. by defoliation, root damage or damage of vascular tissue (summarized in Letourneau et al. 2003). For example, insect-resistant GM *Brassica napus* showed less defoliation, which lead to higher seed output com-pared to non-GM oilseed rape (Stewart et al. 1997). Also for *Bt* sunflower fecundity benefits were shown (Snow et al. 2003). Xia et al. (2016) showed increased fecundity for a *Bt* rice x weedy rice hybrid under high-insect pressure. Such individual fitness benefits of insect-resistance traits may have effects at the population level. Potential fitness effects depend on the level of susceptibility of herbivores, insect pressure in a specific receiving environment and the genetic background of the plants, specifically in the case of crop-wild hybrids. Relevant ecological questions to evaluate the risk of insect resistance transgenes (e.g. *Bt* transgenes) to increase the fitness or the invasiveness of a plant are outlined by Letourneau et al. (2003). Such an assessment requires manipulative experiments to determine the level of control of the respective herbivore on the plant population under specific environmental conditions.

The following improvements are therefore recommended:

- The assessment of the response of the GMP to biotic stressors should consider non-target pests and pathogens, in particular potential secondary pests (with a specific focus on herbivorous pests in case of lepidopteran *Bt* toxins, see also Annex F of this report on the evaluation of biotic stressors for selected crops).
- Assessment of a possible fitness benefit of the insect resistance with and without selection pressure of the target organism, specifically outside agricultural habitats.

7.2.2 Herbicide tolerance

Herbicide tolerance in GM crops refers to tolerance of the GMP to non-selective herbicides such as glyphosate. Potential changes in persistence, survivability or fitness of the GMP may occur through changes in fitness of the GMP or GM crop-wild hybrids (i.e. fitness benefits), which can result in an increased occurrence of volunteers, feral plants or crop-wild hybrids. A selective advantage may be due to the nature of the trait in the respective environment or due to unintended effects of the genetic modification.

If a plant possesses an herbicide tolerance trait, this will provide a fitness advantage in areas where the complementary herbicide is applied (e.g. in fields). Such increases in volunteer occurrence in fields are generally not assessed in the agronomic characterisation of the GMP. However, herbicide tolerant crops occur as volunteers in fields (Jhala et al. 2021). In general, volunteer dynamics are subject to a range of agricultural and biological factors in the respective region (e.g. crop rotation, management measures, occurrence of weeds etc.). These will become evident in the growing season(s) following GMP cultivation. Increased volunteer occurrence can lead to the formation of persistent populations in but also outside agricultural fields. Hence, agronomic field trials should include volunteer studies. In addition, post-market environmental monitoring should complement volunteer assessments to account for the detection of increased volunteer numbers of GMPs

In herbicide-tolerant plants, both negative and positive effects of the respective resistance trait on fitness have been observed. Fitness costs of herbicide-tolerant plants are extensively discussed by Vila-Aiub et al. (2019); Vila-Aiub (2019). Fitness costs have been described in non-GM as well as transgenic plants (see references in Vila-Aiub et al. 2019) and are predicted to occur also under herbicide-free conditions. Specifically, the lack of fitness costs or even fitness benefits of herbicide tolerance traits in plants have been reported (Lu et al. 2014; Achary et al. 2020; Beres et al. 2018; Yang et al. 2017; Fang et al. 2018). The expression of the EP-SPS gene without herbicide application can affect fitness-related traits (Fang et al. 2018). Plant characteristics such as seed germination under normal and stressed (heat and drought) conditions (in the lab), leaf area, plant height, branching, and number of siliques per plant, number of seeds per silique and per plant were positively affected. Such positive fitness effects of glyphosate tolerance (i.e. a yield increase) under absence of herbicide-application have even been considered as beneficial for plant breeding (Achary et al. 2020). These specific fitness effects depend on the particular genetic modification, the genetic background of the plants as well as environmental conditions. The assessment of plant fitness in the absence of the herbicide is therefore crucial. At least for the EPSPS gene, such beneficial fitness effects are considered to be due to increased levels of auxin, its precursors or aromatic amino acids, which regulate plant growth and development (Fang et al. 2018; Li et al. 2021). Experimental methods to estimate fitness costs with herbicide tolerant plants have been suggested by Vila-Aiub et al. (2015).

The following improvements are therefore recommended:

- Assessment of herbicide tolerant volunteers in the context of agronomic field trials occurring in the year(s) following GMP cultivation
- Assessment of a possible (unintended) fitness effect of the herbicide tolerance trait (with and without selective pressure).

7.2.3 Drought tolerance

Similarly to herbicide tolerance or insect resistance, a GM drought tolerance trait provides the crop with a selective advantage in the managed field, if drought conditions occur. A drought tolerant GMP will therefore be cultivated in regions where the GMP is expected to perform better (e.g. in terms of yield) if abiotic stress occurs either regularly or occasionally under field conditions. Therefore the agronomic performance of a drought tolerant GMPs must be improved under the respective stress conditions, but may not necessarily be better than non-GM crops under non-stress conditions.

Drought tolerance can also provide a fitness benefit under non-agricultural conditions, e.g. for ferals or crop-wild hybrids. A drought tolerance trait may increase feral (or crop-wild hybrid) populations, specifically under drought conditions in a (semi-)natural habitat. Specifically abiotic tolerance traits are known to provide a benefit for adaptive introgression in plants (Whitney et al. 2010). However, such fitness effects are strongly dependent on genotype x environment interactions (e.g. the genetic location; Hartman et al. 2012) and must therefore be assessed in and outside the cultivation context. As tolerance to one specific abiotic stress factor can also affect the response to other abiotic stressors, the evaluation of the plant's response to several abiotic stressors is therefore important (see *Chapter 8.9*).

The following improvements are therefore recommended:

- The assessment should include a possible fitness benefit of the drought tolerance (under stress, i.e. drought conditions).

7.2.4 Changes in seed composition

GM crops with changes in seed composition have been developed for nutritional and food-processing purposes, e.g. increases in oleic acid content and decrease in linole-ic acid (Do et al. 2019; Combs and Bilyeu 2019), or as a land-based source of omega-3-fish oils (Napier et al. 2015; Napier et al. 2019) in different plants (soybean, oilseed rape, *Arabidopsis* sp. and *Camelina* sp.). The genetic modifications include expression of genes specifically in seed, in some cases with large transgenic inserts, e.g. 24 transgenes coding for the fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in transgenic GM oilseed rape LBFLFK (Napier et al. 2019).

In GM oilseed rape with altered fatty acid content, delayed and reduced germination rates translated into differences in field emergence and final plant stand count in field trials. These changes in germination characteristics were considered to be associated with the altered trait (Sottosanto 2018). The changes in fatty acid content affected the sensitivity of the GM oilseed rape to cold temperatures. Effects of the fatty acid profile in seeds on seed germination and seedling emergence have also been described (Bhattacharya et al. 2015; Schierholt & Becker 2011; Knutzon et al. 1992). Specifically, germination at low temperatures can be affected, but also effects on vegetative tissues are known for GM oil-producing plants (Miquel et al. 1993; Miquel & Browse 1994). In addition, changes in fatty acid profiles can affect levels and composition of secondary metabolites such as phytohormones and volatile organic compounds that are involved in biotic and abiotic stress regulation (Kawall 2021).

The following improvements are therefore recommended:

- Assessment of seed germination and dormancy characteristics (e.g. secondary seed dormancy assessment) under abiotic stress (particularly cold stress).

7.2.5 Conclusions

GM crops with “input” traits like insect resistance or drought tolerance provide a fitness advantage under field conditions, specifically if the relevant biotic or abiotic stressors occur. These GM traits may therefore provide a selective advantage to the GM crop or GM crop-wild hybrids also under non-agronomic conditions (e.g. unmanaged fields or semi-natural habitats) which may translate into increased survival or persistence in and outside fields. As a possible fitness benefit becomes only apparent in certain growth stages of the plant (e.g. drought-sensitive stage, occurrence of pests), whole plant studies are needed to test whether such a fitness benefit occurs also under sub-optimal conditions. For herbicide tolerant plants, this fitness benefit is expected under field conditions, i.e. during application of the non-selective herbicide, nevertheless, positive fitness effects are also likely without herbicide application. Therefore, favourable fitness-related effects also need to be considered under non-agronomic conditions. For “output” traits like changes in plant composition, unintended effects may occur which require testing of seed characteristics.

8 Recommendations for the improvement of the risk assessment of GMPs

The aim of this study is to provide suggestions for improvement of the agronomic and phenotypic characterisation of GMPs in order to better inform the environmental risk assessment for the risk area "persistence and invasiveness", including plant-to-plant gene flow. So far, the agronomic and phenotypic characterisation is based on the evaluation of agriculturally relevant parameters with little relevance for environmental risks. The agronomic and phenotypic characterisation is part of the "comparative safety assessment" approach by EFSA and therefore a first step when characterising a GMP in comparison to a non-GM counterpart. This basic characterisation of the GMP may also provide important information for environmental risks if relevant test parameters and study designs are selected that are suitable to indicate environmental risks.

In this chapter, suggestions and recommendations are provided how to complement and improve the current practice of agronomic and phenotypic characterisation. These improvements are needed to better inform risk assessors on potential environmental risks regarding persistence and invasiveness of the GMP.

The following recommendations for improvement are grouped into three categories:

- General recommendations
- Recommendations with respect to the ERA approach
- Methodological recommendations

8.1 General recommendations

Definition of terms

The guidance document on the agronomic and phenotypic characterisation of the GMP uses terms like "persistence", "invasiveness" or "feral plant" without providing definitions. For risk assessment purposes, however, these terms need to be defined in order to be able to specify environmental risks and harm scenarios, which should be addressed during ERA. Exact definitions of these terms are also necessary to delineate spatial and temporal scales of environmental processes which are relevant for the agronomic and phenotypic characterisation. In this report, definitions of important terms relevant for this risk area are proposed, based on the scientific literature (Chapter 3).

Assess basic parameters relevant for survival and persistence for all applications

Currently, the assessment of case-by-case endpoints relating to persistence and invasiveness is only suggested for potentially persisting species (potato and oilseed rape) when the scope of application includes cultivation. For the cultivation of other, "non-persisting" species (maize, soybean) or GM crops with a restricted use of import and processing, such assessments are not considered necessary (see Chapter 2).

We recommend rethinking this approach as the aim must be to detect both, intended and unintended, alterations in the crop's ability for survival and spread. Unintended changes in life-history parameters (e.g. germination) can influence the plant's ability to survive or persist in the environment, which can entail environmental risks. This can affect (already) persisting or non-persisting species likewise. Even if the intended GM traits do not affect the overall fitness of the plant during cultivation, impacts on the plant's life history traits due to effects of the genetic modification have been reported (Chapter 3).

With respect to risks due to survival, persistence and invasiveness, all traits related to reproduction of a plant are highly relevant. The analysis in this report has shown that many plant traits contribute to the fitness and therefore survival of a plant. There is no single and exclusive plant trait that determines the risk of a GMP to become persistent or invasive. Different crop traits are predictive for weediness or ferality, but most traits relate to the reproductive output and fecundity of the plant as well as to seed bank survival (Chapter 3). In addition, vegetative traits can be important, e.g. in case of perennial species. For the spread into the environment however, seeds are of high relevance. Therefore, the assessment of seed and vegetative plant traits is of particular importance during the agronomic and phenotypic characterisation. An extended assessment of seed traits (i.e. survival under field conditions and dormancy testing) has already been suggested case-specifically by EFSA (see Table 2). We recommend an extended assessment of seed traits for all GMP applications. In addition, we recommend the assessment of certain vegetative parts of vegetatively propagating plants:

- Seed traits:
    - Seed germination and dormancy assessed in the lab (complemented by additional assessments under sub-optimal conditions).
    - Seed germination assessed under field conditions (under optimal conditions in managed fields in case of cultivation applications, under sub-optimal conditions in unmanaged fields in case of cultivation and import applications)
    - Seeds survival assessed under field conditions (viability of buried seeds over winter, volunteer studies under managed and sub-optimal conditions in un-managed fields)
- Vegetative parts of the plant (e.g. tubers of potatoes, stolons for perennial grass species): growth and survival

Adapt test designs according to the exposure of the GMP

The agronomic and phenotypic characterisation currently focusses on the assessment of plant traits and characteristics that are important for commercial product development and quality control of the product, which is relevant for product users, particularly the farmer. It aims at testing if the product (the GMP) has the intended traits in its targeted environment (the managed field). Therefore field trials are carried out with standard management conditions (e.g. with regard to the application of fertilizers, pesticides and herbicides), also referred to as "good agricultural practice".

Nevertheless, these "optimal" cultivation conditions do not take into consideration that GM plants may also spread to and occur in non-agricultural habitats, which are generally not managed and are less optimal for plant growth, development and reproduction.

We suggest differentiating and adapting the test designs according to the environmental exposure defined by the scope of the application. The GMP will not only be cultivated in agricultural fields but may possibly spread into the environment along transport routes, during harvesting, handling and reloading activities or due to animal dispersal. Therefore, occurrence in non-agricultural habitats (semi-natural, natural habitats) cannot be excluded and may lead to survival and persistence of the GMP in these habitats.

If individual plant characteristics are considered indicative for the plants' behaviour in the respective environment, then these characteristics need to be tested under the relevant environmental conditions, not only in but specifically also outside agricultural fields. Such a spatial differentiation must be reflected in the tests under contained conditions (e.g. germination) and in agronomic and phenotypic field tests. For example, germination characteristics of the GMP should be tested not only under "ideal" germination conditions but also under suboptimal conditions (e.g. under drought conditions) which may occur if the GMP is released into other habitats than those foreseen. This requires different study and test designs to be developed and applied with different biotic and abiotic conditions, depending on the exposure scenario considered.

Therefore, we recommend including to complement agricultural field tests with tests that are carried out under sub-optimal field conditions (e.g. non-managed agricultural fields) in order to address suboptimal growing conditions for the GMP:

- Import: consider environmental exposure due to spillage – field-testing should consider sub-optimal (i.e. non-managed) field conditions
- Cultivation: consider both, cultivation and spillage – field-testing should consider "optimal" (i.e. managed) and sub-optimal (i.e. non-managed) field conditions

Consider fitness assessments case-specifically

An increased risk for survival, persistence and invasiveness of a GMP is due to:

- The plants intrinsic biology (i.e. crop types that already have a history of building up persisting or feral populations or with wild relatives, e.g. oilseed rape, creeping bent-grass)
- Specific GM traits that confer a selective advantage under cultivation conditions (e.g. abiotic stress tolerances)

The GM traits discussed in this report refer mostly to input traits, such as herbicide tolerance, insect resistance or drought tolerance. These GM traits intend to provide the plant with a fitness advantage during cultivation. This fitness advantage will become apparent by improved growth and yield parameters, which indicate the fitness effect in the agronomic context. Particularly for biotic and abiotic stress tolerances, positive effects on fitness of the GM trait may also occur under non-agronomic conditions, e.g. if GMPs grow outside fields or if these traits introgress into crop-wild hybrids. As stress traits affect the survival and performance of the (juvenile or adult) plant, fitness effects will become apparent only when considering the whole plant under the specific environmental conditions. Plant fitness is highly variable, depending on the specific GM trait, the genomic background and the respective environment. Therefore, manipulative experiments with whole plants are necessary in order to test for potential changes in plant fitness under the respective environmental conditions (for methods see Chapter 6.7).

Observed changes in fitness of a plant do not necessarily lead to changes at population level as such changes are also affected by interspecific competition. Such correlations between changes in individual fitness-related parameters and population-level changes have hardly been assessed so far. Results of fitness, competition or survival experiments need to be related to population-level effects (e.g. by population modelling) in order to be able to conclude on risks due to persistence and invasiveness. Such more complex experimental and modelling approaches go beyond the agronomic and phenotypic characterisation of the GMP but need to be linked to the obtained results therein.

For testing fitness of stress-tolerance GMP, test conditions with and without the respective stress (e.g. drought in drought-tolerant crops) should be applied.

This recommendation is relevant for:

- Crops with a history of occurrence as non-GM feral (or wild) plant in the environment (e.g. oilseed rape, creeping bentgrass) and stress-tolerance traits (input traits such as insect resistance, drought tolerance) if intended for import & cultivation purposes
- All other crops with stress-tolerant traits, if intended for cultivation purposes

8.2 Recommendations for the ERA approach

The guidance for agronomic and phenotypic characterisation of GMPs should be complemented with decision-making criteria

The agronomic and phenotypic characterisation is part of the "comparative safety assessment" concept of the ERA of GMP (EFSA 2010, 2015). This concept aims at the identification and characterisation of intended and unintended effects of the GMP compared with its non-GM counterpart, serving as a starting point for the ERA. Statistically significant differences between the GMP and its comparator can indicate unintended effects and need to be evaluated regarding their biological relevance and their environmental implications, thereby providing a link to the Limits of Concern (LoC) concept and the evaluation of environmental harm.

Currently, the focus of the agronomic and phenotypic characterisation of GMPs is on standard agronomic parameters that are assessed in field trials using a comparative approach in which the GMP is compared to its non-GM counterpart or a range of reference varieties. The results of these field trials are then used to conclude on comparability of the GMP with its non-GM counterpart. They also feed into the problem formulation in ERA and the assessment of the different areas of risk. Therefore these differences or non-equivalences observed need to be interpreted and contextualized with potential environmental harm and requires the definition of biologically relevant effects and effect sizes for parameters and endpoints assessed during the agronomic and phenotypic characterisation. Clear and operable criteria for (un)acceptable effects need to be developed in order to decide on the assessment of the consequences of such differences for the environmental safety of the GMP in question. Only if the biological relevance of differences and/or non-equivalences is determined, the results of the agronomic and phenotypic characterisation can feed into the problem formulation of ERA and support the assessment of environmental risks. Observed differences in agronomic or phenotypic endpoints between the GMP and the non-GM counterpart then need to be contextualized with specific risk assessment studies.

EFSA (2010) introduced a concept to derive thresholds for acceptable adverse effects in ERA, the so called Limits of Concern (LoC, see Chapter 4). Although this concept has not yet been implemented in ERA, suggestions on how to operationalise this concept, specifically with respect to risks regarding persistence and invasiveness of the GMP, have been made in the scientific literature (see Chapter 4). In order to implement this concept, definitions are needed for specific protection entities (e.g. species, habitats) and decisions made, if observed changes are considered adverse and exceed a certain level or threshold of acceptability (i.e. the LoC). To date, no LoCs have been defined for any of the phenotypic characteristics.

With respect to parameters and endpoints assessed in the agronomic and phenotypic characterisation, this could be relevant e.g. in case an increase in frost or drought tolerance of a GMP is observed. This could indicate increased survivability of the plant and therefore a higher chance of volunteers to occur in the next crop. However, without defining biological relevance of this increase, it is unclear whether higher-tier tests, e.g. an assessment of volunteer occurrence of the GM crop, are necessary.

Potential environmental effects of GMPs occur through long-term processes (specifically those due to spread, survival and persistence of a GMP) that cannot be directly assessed in short-term assessments such as the agronomic and phenotypic characterisation. Linking changes in phenotypic traits with population-level processes at landscape scale is challenging. Therefore, phenotypic plant traits and endpoints must be selected which can be used as appropriate proxies and are testable in the agronomic and phenotypic characterisation of the GMP. Such “indicative” parameters are typically connected to the survival and dispersal ability of the plant (e.g. germination ability, survivability, or fecundity).

As a starting point, we propose to consider the following issues when defining the biological relevance of differences observed in the agronomic and phenotypic characterisation:

- Type of effect observed, e.g. qualitative or quantitative effect considering also a non-symmetric relevance of observed effects (e.g. lower vs. higher survivability of the GMP compared to the non-GM plant)
- Exposure routes and habitats exposed: specifically the habitat where effects are (tested and) observed
- Likelihood of a non-reversible spread into habitats
- Types of protection goals / specific protection goals (e.g. those aiming at preventing the occurrence of GMO in certain habitats under protection).

Consider per-se assessments for GMP without comparator

In many other regulatory disciplines, the evaluation of environmental harm is carried out by use of a per-se assessment instead of a comparative assessment. This means that risks of an environmental stressor are assessed without the use of a comparator (e.g. plant protection products or chemicals). The comparative assessment as applied for GMOs is based on the concept of “history of safe use” of the conventional non-GM crop with which the GMP is generally compared. As such, this concept is considered useful for the establishment of environmental safety only to a limited extent. In addition, for some GM crops a conventional comparator may not be available or difficult to define, therefore challenging the comparative approach for ERA purposes. Specifically for novel GMPs with complex genetic modifications and GM traits, challenges with respect to the comparative approach are foreseeable. If no conventional comparator can be defined or differences between the GMP and the non-GM comparator are intended (e.g. with respect to their environmental behaviour, as for e.g. stress-tolerant GMPs), then other assessment approaches will be needed.

8.3 Methodological improvements

The assessment of seed traits should be improved and harmonized

Generic endpoints, which are prescriptive for all plant species, include the assessment of seed traits of the GMP. Seed characteristics need to be assessed for GMP applications related to import and processing for food and feed use as well as for cultivation purposes. EFSA (2015) refers to seed germination tests typically performed in laboratory studies, referring to the necessary seed quality needed to perform agricultural field trials. While seed quality may also affect environmental performance of the GMP, it is not sufficient to inform the ERA regarding unintended changes in seed characteristics relevant for the survival of the seed under different environmental conditions.

The informative value of currently applied standard seed testing methods is limited with respect to the performance of seeds under field conditions. Improved testing methods should take into account that e.g. ungerminated seed may germinate later in the field (due to dormancy) or that seeds due to spillage or from harvest losses may disperse into the environment. Methodological improvements based on standardized test systems are available, e.g. the use of seed vigour tests to mimic less than ideal environmental conditions. However, there are no ISTA-validated tests with temperature regimes below the range of 5-10°C (temperature regime depending on the crop type). In order to reflect winter survival of seeds, other test systems should be used, considering temperatures below the freezing point. In addition, standardised and validated methods are currently not available for all crop types. There are also no validated test methods by ISTA or AOSA for testing seed germination *in-situ* under natural conditions by ISTA or AOSA. Seed germination tests for potato seeds are also not available.

When GMP are cultivated or imported, harvested seeds can be introduced into the environment either by seed spillage (during import, transport activities) or after harvest (harvest losses after cultivation). Harvested seed are genetically not uniform, may have different proportions of transgenic DNA and are generally not treated with fungicides or insecticides. In ERA practice, however, seed used for sowing and cultivation rather than harvested seed are used for testing. In addition, there may be unintended changes in the germination characteristics due to different genetic backgrounds or introgression of transgenes into wild relatives, so-called next generation effects.

For GMPs with the scope of application import/processing, seeds may enter the environment due to unintended spillage of the GMP or parts of the GMP, e.g. during transport and processing activities. A range of documented cases of GMPs and their transgenes occurring in unmanaged populations outside the cultivation context shows that dispersal to and survival in non-managed habitats is crucial for these plants. Therefore, testing survival of harvested seeds under sub-optimal conditions (i.e. by mimicking conditions outside the managed agricultural field) is needed. The use of disturbed habitat types (e.g. a site with little weed competition but without management measures) may give a maximum estimate of seed survival of GMP in such habitats. Therefore, not only seeds for sowing should be tested but also harvested seeds to account for survivability of seed losses and due to spillage during transport or harvest. As standardized and validated methods are currently lacking, methods from the literature can be applied.

The following recommendations are made regarding extended seed testing of GM seeds. Specific recommendations to improve laboratory seed germination tests are outlined in Chapter 6.3.1 and Annex D to this report.

- testing seeds used for sowing (cultivation purposes only) and harvested seeds (for cultivation and import scope)
- testing non-harvested seeds (e.g. TPS for potato)
- testing seed in the laboratory under sub-optimal conditions
- testing of seed dormancy (including secondary dormancy)
- assessment of seed viability / survival *in-situ* (both under optimal and sub-optimal conditions)

Develop methods for assessing short-term persistence of the GMP in agricultural fields (volunteers)

For many crop types (e.g. potato, oilseed rape, maize) an increase in volunteer plants could result in an increased use of corresponding control measures, in particular the use of (additional) herbicides. So far, there are no standardized methods to assess volunteer formation, e.g. used in plant variety trials, which could also be applied in the agronomic and phenotypic characterisation of the GMP.

For GMPs intended for cultivation purposes, a standardized protocol for volunteer provocation field trials should be developed, taking into account common practice, like crop rotation and tillage and considering active tilling of a determined number of seeds into a fallow plot around harvest time. Data collection may be supported by modern phenotyping approaches, e.g. for the detection of the presence of unintended plants on the plots (see Chapter 6.3.2). Since volunteer dynamics are highly context-dependent, post-market monitoring of the occurrence and persistence of volunteer plants is also important.

Amend the concept to assess GxE interactions

The assessment of GxE interactions, as currently carried out in the agronomic and phenotypic characterisation of GMPs, is applied as for plant breeding purposes (see Chapter 5). Therefore, as currently implemented, the evaluation of GxE interactions carried out with GM plants grown in agricultural plots, assessing agronomic traits, is of limited usefulness for ERA purposes. In the context of ERA, the purpose of evaluating GxE interactions is different from plant breeding. While in plant breeding multi-environment testing is used to ensure that the GMP has a good performance across a range of environments, the aim in ERA is to assess unintended effects of the GMP due to the genetic modification. Specifically, survival-relevant parameters are subject to strong GxE interactions and certain GM traits can enhance fitness under different environmental conditions. Since agricultural plots are optimal and homogenous environments for crop plants (as compared to growing conditions outside agricultural fields), evaluating GxE interactions in well-managed fields provides limited information on the GMPs ability to survive and persist outside agricultural plots. In order to evaluate unpredicted reactions of the GMP outside the agricultural context, testing GMPs under stressful and suboptimal conditions with respect to a range of biotic and abiotic conditions, is necessary.

Hence, to improve the evaluation of GxE interactions, an approach is required that also considers heterogeneous, non-optimal field conditions during testing the GMP and its ability to sur-vive and persist in and outside the agronomic context.

Focus and improve the assessment of the response of the GMP to biotic stressors

The agronomic and phenotypic characterisation includes also an assessment of GMP responses to biotic stressors. Only GM plant applications for import/processing need to provide such an assessment. For GMPs for cultivation purposes, EFSA refers to the general ERA guidance. In this guidance an explicit assessment of biotic stressors (e.g. non-target pests) is not foreseen, but can be covered by testing for effects on non-target organisms (NTOs). While NTO testing refers to the (random) occurrence of specific functional groups of NTOs in the respective environment, the assessment of the response to biotic stressors needs to assess a specific response to a specific stressor in order to evaluate any intended or unintended effects of the genetic modification. Therefore, a focussed approach with respect to the selection and assessment of biotic stressors is needed for all GMPs. This assessment must be clearly separated from an assessment of other non-target organisms (i.e. other than non-target pests and pathogens) as well as the general agronomic characterisation of the GMP. The methodological approach should ensure a sufficiently high pest and/or pathogen pressure to elicit the plants' response. This includes test designs without standard insecticide applications in order to ensure exposure to the respective biotic stressors, if these are relevant. In addition, guidance is needed on how to assess, monitor and report the occurrence of specific biotic stressors at specific field trial locations. In addition, artificial infestation experiments should be taken into consideration, if sufficient pest/pathogen pressure cannot be guaranteed under field conditions.

Suggestions for improvements in this report comprise the selection of relevant biotic stressors for a range of crop types in Europe, as well as information on rearing and artificial infestation with relevant biotic stressors, either under contained conditions or in the field (see Annex F).

Further guidance is needed for the assessment of the response of the GMP to abiotic stress

The assessment of plant responses to abiotic stress in the context of the agronomic and phenotypic characterisation is required for all GMP applications, independent of its scope. In practice, this assessment lacks a solid scientific methodology due to unspecific recommendations in the EFSA guidance document. Only for GMPs with abiotic stress tolerance as the intended GM trait, a more focussed assessment is currently advised by EFSA.

If locations for field trials are selected where abiotic stress conditions are likely to occur, then criteria are needed to define abiotic stress conditions for a particular crop type. Guidance is also needed for the monitoring and reporting of these conditions (e.g. precipitation, watering conditions and soil moisture/water content) at specific field trial locations.

For example, drought conditions can occur in a range of combinations regarding affected plant growth stages, drought intensities, and durations. Not all these conditions can be covered in ERA studies, hence, a focus and guidance is needed with respect to how to define such conditions for specific crop types. In Europe, summer crops such as maize and soybean, are impacted by heat and drought stress already (Toreti et al. 2022). Under climate change conditions, drought stress for agricultural crops will be increasingly relevant. The response to cold tolerance is also important to assess the ability of the crop (or its vegetative parts such as potato tubers) to survive overwinter, spread and persist in or outside fields. Hence, the assessment of any differences in the response of the GMP to these two types of abiotic stress should be a standard requirement in the agronomic and phenotypic assessment and further guidance to implement such an assessment is needed.

Guidance for focused experiments for stress-tolerant GMPs is needed

For GMPs with stress tolerance (e.g. drought tolerant GMPs), the respective stress condition must occur in order to elicit a response of the stress-tolerant GMP. Hence, manipulative experiments are needed which apply the respective stress condition on the GMP. Methods for artificial induction of abiotic stress conditions together with phenotyping the plants' response are available (see Chapter 8.9). For applicants, specific guidance for experimental approaches with artificial stress conditions for GMPs with abiotic stress tolerances is needed. This includes the definition of stress conditions and phenotyping the response of the GMP to the specific stress condition.

Changes in regulatory pathways can also affect plant hormones (e.g. absicic acid ABA) or transcription factors, which play a crucial role in the response of the plant to other environmental stressors (see Chapter 8.9). A tolerance to one abiotic stressor can therefore also affect the tolerance of the plant to other abiotic stressors. In addition, due to interactions between abiotic and biotic stress signalling pathways also changes in responses to biotic stressors, such as pathogens or pests, are possible in stress-tolerant plants. Therefore, for abiotic stress tolerant GMPs, the assessment of the response to other abiotic stressors should be required and linked with the assessment of the response to biotic stressors.

Explore modern phenotypic approaches for improved agronomic and phenotypic assessments

Modern (high-throughput) phenotyping approaches are available for a range of research questions with respect to the agronomic and phenotypic characterisation of plants and already used in plant breeding. These range from simple digital image applications to complex remote sensing techniques. Although no standardized methods or applications are available so far, certain approaches could already be applied for phenotyping specific traits or characteristics of GMPs. For example, methods for high throughput phenotyping for seed germination, seed vigour testing or potato tuber characterisation are readily available (see Chapter 8). Modern phenotyping approaches can measure morphological changes for a range of crop species, are able to discriminate between genotypes and can even identify associations between genomic regions and differences in plant traits (e.g. germination). In addition, high-throughput phenotyping approaches could be used when assessing and identifying plant responses to abiotic stress conditions. Up to now, phenotyping of GMPs is based on methods derived from classical plant breeding, such as visual inspection of plants in the field and qualitative scoring of parameters. While these methods are familiar and suitable for the general agricultural characterisation of the GMP they are not sufficient for ERA purposes. This may also be the case with novel GM plants with fundamental changes in physiology, morphology or plant architecture. With the application of new genomic techniques, novel plant phenotypes can be achieved, e.g. by de novo domestication or due to an increasing depth of intervention, i.e. by simultaneous modifications of several alleles, gene families or functional genes. Therefore new functional phenotypes with no familiarity regarding their behaviour in agro-environments can be expected, e.g. if fundamental crop- or species-specific characteristics are modified, such as morphological or reproductive plant characteristics, altered composition or tolerance to abiotic stress. Consequently, differences or non-equivalences are likely to be observed if a comparative agronomic and phenotypic assessment is performed. As our knowledge and experience with regard to both agricultural and environmental effects of such plants is limited, a novel assessment approach will be needed also for the agronomic and phenotypic assessment of these plants. This should, however, not only cover an improved molecular characterisation, or the use of omics-technologies to detect off-target and unintended on-target effects but also the use of improved and modern phenotyping methods, e.g. whole plant phenotyping at high spatial and temporal resolution, considering different environmental contexts. Nevertheless, also for novel phenotyping methodologies decision criteria are important, in order to determine the biological relevance of observed differences between the GMP and its comparator.

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List of abbreviations

AOSA	Association of Official Seed Analysts
EFSA	European Food Safety Authority
EU	European Union
EPPO	European and Mediterranean Plant Protection Organization
GMO	Genetically modified organism
GMP	Genetically modified plant
GM	Genetically modified
GxE	Genotype by Environment
ISTA	International Seed Testing Association
NTO	Non-target organism
SCST	Society of Commercial Seed Technologists
VCU	Value for Cultivation and Use

A Annex: Agronomic and phenotypic assessment in GMP applications

In this Annex, the studies contained in GMP applications, which evaluated agronomic and phenotypic plant traits with relevance for persistence and invasiveness are presented.

A.1 Selection of GMP applications

In a first step, we conducted a search in the database of the Federal Agency for Nature Conservation (on Feb 16th, 2021). This database includes all reports submitted by applicants in the course of an application procedure according to EU legislation (Regulation (EC) 1829/2003 and Directive 2001/18/EC, respectively). We used the following search terms: *ecol*, *ecol and environ*, *volunteer*, *germination*, *pollen* and *fitness*. Except for the search for *fitness*, which did not retrieve any results, a varying number of reports was found. Most reports were retrieved with the use of the terms *ecol and environ*. In certain GMP applications, the assessment of ecological, i.e. biotic and abiotic, interactions such as the assessment of pests and diseases, was not presented in a separate report, but within the main text of the applications, the comparative assessment or the agronomic and phenotypic assessment of the GMP and was therefore not retrieved by the database search. In these cases, we retrieved the respective reports from the document management system (DMS) of EFSA.

We focused on the following plant species of relevance for European agriculture: maize, oilseed rape, potato and soybean. We excluded all dossiers submitted before Regulation (EC) No 1829/2003 came into effect (i.e. C-Dossiers submitted under Directive 2001/18/EC). We aimed at selecting five applications per plant species for the analysis. However, for some plant species, we identified less than five applications, which were relevant. For example, only three potato dossiers, which were all withdrawn from the authorization procedure, were available for the analysis (Table 6). In the selection of applications, the following criteria were applied:

- Representation of different applicants within a group of plant species
- Representation of different traits within a group of plant species Inclusion of traits with high relevance for persistence, fitness or invasiveness of a plant (e.g. drought tolerant maize)
- Inclusion of stacked event GMPs
- Application submitted before and after publication of the EFSA Guidance (EFSA 2015)

Tab. 6: Overview of 17 GMP applications selected for the evaluation of studies assessing studies submitted to evaluate the fitness, persistence and invasiveness of a GMP in the context of the agronomic and phenotypic evaluation (HT = herbicide tolerance, MS = male sterility, FA = fatty acid, RHS = Roundup Hybridization System, IR = insect resistance, gly = glyphosate, glu = glufosinate); A = adopted, P = pending, W = withdrawn.

No.	GMP	Application	Event	Trait	Status
1	Oilseed rape	NL-2010-87	GT73	HT (gly)	A
2		BE-2011-101	MON88302	HT (gly)	A
3		NL-2013-119	MON88302x Ms8Rf3	HT, MS (glu, gly)	A

No.	GMP	Application	Event	Trait	Status
4		DE-2019-157	LBFLFK	FA	P
5		NL-2019-158	Rf3 CQ (*B. juncea*)	HT, MS (glu)	P
6	Potato	NL-2009-69	AV43-6-G7	Increased Amylopektin	W
7		SE-2010-88	AM04-1020	Increased Amylopektin	W
8		UK-2011-102	PH05-026-0048	*Phytophthora*- resistant	W
9	Soybean	NL-2010-78	MON87705	FA	A
10		NL-2012-106	DAS-444Ø6-6	HT (gly, glu, 2,4-D)	A
11		NL-2018-148	DP305423xMON87 708xMON89788	FA, HT (ALS-inhibit. herb. dicamba, gly)	W
12		NL-2018-153	GMB151	Cyst nematode re-sistant., HT (HPPD-in-hibit. herb)	A
13	Maize	NL-2006-31	LY038	Increased Lysine content	W
14		NL-2009-70	MON87460	drought resistant	A
15		NL-2019-161	MON87429	HT, RHS[3] (glu, dicamba, quizalofop, 2,4 D)	P
16		NL-2015-127	1507xMON810 xMIR162xNK603	IR, HT (glu, gly)	A
17		NL-2016-133	MZHG0JG	HT (glu, gly)	A

A.2 Categorization of studies

We use the term "report" for all studies compiled by the applicant in one report, which is clearly identifiable by a study or report number. In some cases, such reports covered several studies or experiments, which differed in their experimental designs. For example, some reports included several assessments of biotic interactions, e.g. with different methodology. If the experimental design (e.g. inoculated with pathogen or not) or the scale of the study (lab, greenhouse, field) differed, we considered the respective experiment as a separate study in the analysis. Thus, the number of studies reviewed in our analysis exceeds the number of reports identified in the applications.

A.3 Studies submitted for agronomic and phenotypic assessment of GMPs with relevance for persistence and invasiveness

Overall, we identified 82 studies of relevance for the assessment of the persistence and invasiveness of a GMP from 17 selected notifications (Table 7). We categorized the studies according to their purpose as indicated by the applicants and identified the following four categories:

[3] RHS: tissue-specific expression of CP4 EPSPS protein allows to induce a non-viable pollen phenotype by well-timed glyphosate application (Roundup Hybridization System)

Studies on germination and dormancy, studies on pollen viability, volunteer studies and studies on ecological and environmental interactions. The latter included various assessments of the response of the GMP to biotic (e.g. pest, disease) and abiotic stressors (e.g. cold, drought).

Studies on germination & dormancy and pollen viability were contained in all applications except potato (24.4 % and 8.5%, respectively). The potato applications did not contain studies on germination of seeds or pollen viability. Studies on the survival of the GMP as volunteers (7.3%) were contained in oilseed rape and maize applications only. Most studies submitted assessed ecological or environmental interactions of the GMP (59.7%) and were contained in all types of GM crops.

Tab. 7: Studies submitted in GMP applications with relevance for the assessment of persistence and invasiveness of the GMP (numbers refer to the number of studies submitted, numbers in brackets to the number of applications in which a study was presented).

GMP	Dormancy & Germination	Pollen Viability	Volunteers	Ecological & Environmental Interactions
OSR (5)	10 (5)	1 (1)	4 (1)	8 (5)
Potato (3)	-	-	-	17 (3)
Soybean (4)	5 (4)	1 (1)	-	7 (4)
Maize (5)	5 (5)	5 (5)	2 (1)	17 (5)
Σ	20	7	6	49

The studies analysed were carried out in three different levels of containment: studies conducted in the laboratory or a climate chamber, e.g. when using different parts of the GMP (e.g. seeds, pollen, and tubers), studies conducted with whole plants grown in pots in the greenhouse and studies conducted in field trials (Table 8). While half of the studies were conducted in field trials (53.6%), and more than a third in the laboratory (40.2%), only 6.1 % of the assessments were carried out in the greenhouse. Studies on germination and pollen viability took place in climate chambers (20 and 7 studies, respectively), while studies on ecological interactions were predominantly conducted in agronomic field trials (44 studies). Only few studies (5) were conducted under greenhouse conditions.

Tab. 8: Containment level of studies for the agronomic and phenotypic assessment of GMPs with relevance for persistence and invasiveness. OSR = oilseed rape, P = potato, S = soybean, M = maize. LC = lab/climate chamber, GH = greenhouse, FT = field trials, S = sum

1.1	LC				GH				FT				S
Crop	OSR	P	S	M	OSR	P	S	M	OSR	P	S	M	
Germination % dormancy	10	-	5	5	-	-	-	-	-	-	-	-	20
Pollen viability	1	-	1	5	-	-	-	-	-	-	-	-	7
Volunteers	-	-	-	-	-	-	-	-	4	-	-	2	6

1.1	LC				GH				FT				S
Ecol/env interactions	-	4	-	2	-	2	-	3	8	11	7	12	49
Σ of studies	11	4	6	12	-	2	-	3	12	11	7	14	82

A.4 Assessment of seed viability and dormancy

We identified 20 studies investigating seed dormancy and germination, 10 in oilseed rape dossiers and 5 in maize and soybean dossiers, respectively. No seed assessments were made for potatoes (for overview of the study designs see Table 9). All studies on seed dormancy and germination were conducted in the laboratory.

The purpose of the studies, as indicated by the applicants, was the assessment of seed quality (germination, viability and health), the comparison of germination characteristics with the non-GM comparator, or, in some cases, the evaluation of dormancy. Applicants used standard protocols for seed testing according to various institutions, e.g. Association of Official Seed Analysts (AOSA), the International Seed Testing Association (ISTA) and the Society of Commercial Seed Technologists (SCST). However, they often adapted the protocols and complemented them with additional temperature regimes.

A standard germination assessment comprised a constant temperature regime (i.e. under optimal conditions). Additional temperature regimes were included in the submitted studies, e.g. warm and cold germination tests (i.e. germination under suboptimal conditions) or additional diurnal temperature regimes, including daily fluctuations in temperatures. In general, the assessment of germination took place after incubation of the seeds for several days (at least 4 days, up to 28 days). Applicants generally used a tetrazolium test to determine the viability of the non-germinated seeds at the end of the experiments.

Study designs varied in the number of seeds assessed, the variation in the number and duration of observations, the number of replications and the number of reference varieties used (Table 9). Seed treatment included fungicides (in the case of oilseed rape seeds also insecticides), seed sterilization or no treatment at all.

Tab. 9: Overview of the study designs of studies on germination & dormancy of GM crop seeds. Information was derived from four GM OSR, five GM soybean and five GM maize applications. No in brackets refer to no of assessments.

	Oilseed Rape	Soybean	Maize
Total no. of studies	10	5	5
germination tests conducted	warm germination test, cold germination test, seed cold tolerance test, secondary/dark dormancy germination test	warm germination test, cold germination test, warm, cold and diurnal growing conditions, constant and alternating temperature regimes	constant and alternating temperature regimes, warm, cold and diurnal growing conditions
Seed treatment (no. of studies with treatment)	fungicides & insecticides (2) sterilized (2)	fungicides (1), sterilized (1)	fungicides (3)

	Oilseed Rape	Soybean	Maize
Protocols used	AOSA 2009, AOSA 2013, AOSA 2016	AOSA 2000, 2006, 2007, 2009, 2010, 2013, ISTA 2014, ISU Seed Laboratory 2016, SCTS 2010	AOSA 1983, 1998, 1999, 2000, 2002, 2005, 2006, 2009, 2010, 2013, 2017, SCTS 2010,
no. of seeds assessed	200-400	200-400	200-400
no. of seeds per replicate	50-100	100	50-100
no. of replications	2-8	4-8	4-8
no. of ref. varieties	0-4	0-17	2-9
no. of observations	2-4	1-2	1-3
Duration of observation in days[4]	6-28	4-14	4-12

In the standard germination assay, seeds are placed on a moistened germination towel or filter paper, rolled up, placed in a bucket and put in a germination chamber. Sometimes the filter paper is wrapped in a wax paper or a ventilated plastic bag in order to retain the humidity. In some cases, filter papers are placed in petri dishes. In one study, oilseed rape seeds were placed in deep-well plates and covered with water. In another study, soybean seeds were placed on cafeteria plates and covered with soil. The conditions in the chamber vary usually from 25°C in daylight for eight hours followed by 15°C in the dark for 16 hours. After the first assessment of germination, seeds are remoistened and returned to the germination chamber. In general, the assessment of germination gathers the numbers of normal, abnormal and non-germinated seedlings. After varying rounds of incubation and assessment, the final evaluation of seed germination is made. Sometimes the assessment of non-germinated seeds differentiates dead seeds from hard seeds and firm swollen seeds (for a specific description of the assessment categories see Table 10). Usually only for the seed viability assessment under optimal conditions, normal germinated seed were distinguished from abnormally germinated seeds. For alternating or additional temperature regimes, often no distinction was made between normally and abnormally germinated seeds, instead total germination was assessed as the sum of normal and abnormal germinated seeds. At the final evaluation, hard and form swollen seeds are subject to the viability test with tetrazolium chloride. The application of triphenyl tetrazolium chloride (TTC) allows the assessment of viability via mitochondrial respiration (dehydrogenase activity). With this test, non-germinated dead seeds can be distinguished from non-germinated viable seed and thus serves to identify dormant seeds. The percentage of dead seed is calculated from the sum of dead seeds and hard or firm swollen seeds with negative TTC test results. The percentage of dormant seed is determined as the sum of ungerminated seeds with positive TTC test. Thus resulting in total numbers of viable and dead seeds.

[4] Sometimes germination was assessed repeatedly and thus also before the end of the experiment, e.g. after 2-3 days. Here only the maximum duration of observations is indicated.

Tab. 10: Assessment categories for germination studies of oilseed rape, soybean and maize (according to GMO application numbers 101, 78 and 70).

Assessment categories in germination studies
germinated seeds
Normal germinated seed: Seedlings that exhibited normal developmental characteristics and possessed both a root and a shoot.
Abnormal germinated seed: Seedlings that could not be classified as normal germinated (e.g., insufficient root and shoot development, lacked a shoot, shoot with deep cracks or lesions, or exhibited mechanical damage).
ungerminated seeds
Dead seed: Seeds that had visibly deteriorated and had become soft; or negative result for the Tetrazolium (Tz) test.
Dormant seed: positive results for the Tetrazolium (Tz) test.
Hard seed: Seeds that did not imbibe water and remained hard.
Firm/swollen seed: Seeds that had visibly swollen (imbibed water) and were firm but lacked any evidence of growth.

This basic experimental design varies with respect to the temperature regimes applied (see Table 11). For example in so-called warm germination assays, the temperature in the germination chamber is at optimal constant 25°C. For cold germination assays, usually seeds are put in the germination chamber for ten days at constant ten degrees followed by three days at constant 25°C. Cold germination assays sometimes also combine constant and alternating temperature regimes. For example, after incubations at low temperature (10° for 7 days), seeds are exposed to either constant warm (e.g. 20° or 25°) or alternating conditions (e.g. 20°/30°). In one study with oilseed rapeseeds were subjected freezing temperature (10 days at -5°C followed by 7 days at alternating temperatures). The constant and alternating temperature regimes vary among germination studies and the crop plant studied. Usually, germination studies take place without light. However, for oilseed rapeseeds various light/darkness conditions are used. In most but not all studies, viable non-germinated seeds, i.e. dormant seeds, are identified at the end of the experiment by the Tetrazolium (Tz) test.

In one study with oilseed rapeseeds, however, a specific experimental design was applied deliberately inducing dormancy. In this case, the moistened seeds were subject to 24-hour darkness and at constant 18°C for 14 days. After dormancy induction, the seeds were remoistened and put back to the germination chamber for another two days before the first evaluation of germinated seeds. The number of germinated seeds was determined again after two and ten additional days under these conditions. After the 28-day count, the remaining non-germinated seeds were remoistened and subjected to alternating dark/light conditions (12 hours dark at 5°C, 12 hours light at 25°C). After three and seven days, the counts were repeated. At the end of the testing (35 days in total), non-germinated seeds were tested for viability using a Tz test. In one oilseed rape application, studies specifically assessing the influence of storage time or conditions, the applied sterilization method and the applied seed treatment on seed germination was assessed.

Tab. 11: Study designs of assessments of germination & dormancy of the GMP. °= degree Celsius, d = days, n. i. = not indicated. No in brackets refer to no of assessments.

	Oilseed Rape	Soybean	Maize
Total no. of studies	10	5	5
Germination tests	Standard/warm germination, cold germination, seed cold tolerance, germination after induction of secondary dormancy	Warm/optimal germination, cold germination	Warm/optimal germination, cold germination
Parameters assessed	Germinated/ungerminated seeds	Germinated/ungerminated seeds	Germinated/ungerminated seeds
	Seed viability (Tz-test) (3)	Seed viability (Tz-test) (4)	Seed viability (Tz-test) (5)
Dormancy test	Secondary dormancy induced (1)	-	-
Seed treatment	fungicides, insecticides (2), sterilized (1), n. i. (5), untreated (2)	fungicides (1), sterilized (1), n. i. (3)	fungicides (3), n. i. (2)
Light conditions	alternating light/darkness (5), without light (3), constant dim light (1)	without light (4), n. i. (1)	without light (4), n. i. (1)
Temp. regimes	Constant: 5°, 10°, 15°, 18°, 30° Alternating/diurnal: 4°/20°, 5°/25°, 15°/25°, 20°/30° (for 16/8 hrs) for max. 10 d or 13 d Cold: 7 or 10 d at 10° followed by 3 d at constant 25° or 7 d at alternating temp. Cold tolerance: -5° (1)	Constant: 10°, 20°, 30° Alternating/diurnal: 10°/20°, 10°/30°, 10°/25°, 20°/30° Warm: 7 or 8 d at 25° Cold: 7 or 10 d at 10° followed by 3, 5, 6 or 7 d at 25°	Constant: 5°, 10°, 15° 20°, 25°, 30°, 35° Alternating/diurnal: 10°/20°, 10°/25°, 10°/30°, 20°/30° Suboptimum: 7 d at 10° followed by 4 d at 25° Cold/stress: 10 d at 10° followed by 3 d at 25°

Regarding the statistical analysis of the germination data, applicants usually provided an analysis of variance. A statistical comparison was made between the test and the control entry.

In potato applications, no potato seeds were assessed for the viability of their seeds. Instead, applicants assessed the frost hardiness of potato tubers, as vegetative propagation units (Table 12).

Tab. 12: Studies evaluating frost hardiness or freezing tolerance of potato tubers in GM potato applications.

Potato studies evaluated	Results
No. of studies	2
Purpose of the study as indicated by applicant	Comparison of frost hardiness/freezing tolerance
Protocols used	Chen et al. 1980, Irzykowski et al. 1996
Temperature regimes used	tests start at 4°C, three days gradual lowering to the minimum temperature, two days at minimal temperature (climate chamber set to -1.5°C, -2.5°C, or -3.5°C), three days gradual thawing, tests start at 4°C, five days gradual lowering to the minimum temperature, two days at minimal temperature (climate chamber set to -2.5°C or -7.0°C), five days gradual thawing.
parameter/endpoints assessed	survival rate (surviving tubers developing sprouts were assessed after 2-3 weeks at 18°C)
Experimental design	Tubers placed on soil surface, Tubers covered with soil (10 and 20 cm depth)
No. of tubers per replicate	8
No. of replications	4-6
No. of ref. varieties	2-4
No. of observations	1
Duration of frost regimes in days	8-12 (time for lowering the temperature, keeping the minimum temperature and gradual thawing)

A.5 Pollen viability assessment

Pollen viability studies were contained in seven studies of seven applications (1 oilseed rape, 1 soybean and 5 maize applications, Table 13). Pollen were generally taken from plants grown in field trials, in one case they were taken from plants raised in the growth chamber.

Applicants applied different study designs: numbers of plants taken and flowers per plant collected, numbers of grains assessed as well as temperature regimes used differ between applications and studies. No standardized protocols were used and no standardized experimental conditions were applied. The assessed parameters were grain diameter, percentage viable pollen and general morphology.

Only staining techniques were used, mostly Alexander's stain (Alexander 1980), together with microscopic examination. In two cases, digital imaging software for pollen counts was used. Alexander's stain evaluates sterility rather than viability, as it differentiates mature from immature pollen grains (Dreccer et al. 2019). Although Alexander`s stain can distinguish aborted from non-aborted pollen, it also frequently stains old or dead pollen and shows no correlation with germination (see Dafni & Firmage 2000).

Specific stress conditions were not applied to the plants from which pollen were collected, however, the water and temperature regimes during pollen production and dehiscence in the plant before and during the experiments remain unknown. Stress conditions can affect pollen viability, for example, potato pollen grains are more resistant to temperature stress, while others are not (Bots & Mariani 2005).

Tab. 13: Pollen viability studies in GMP applications. n. i. = not indicated

	Oilseed rape	Soybean	Maize
No of studies	1	1	5
Plants derived from	Growth chamber	Field trial	Field trial
Environmental conditions	21°/18°C (day/night), 16 hrs photoperiod	n. i.	well-watered and water-limited conditions[5]
Purpose of the study	Assessment of pollen viability and morphology	Assessment of pollen viability and morphology	Assessment of pollen viability and morphology
Parameters assessed	Pollen grain diameter, % viable pollen, morphology	Pollen grain diameter, % viable pollen, morphology	Pollen grain diameter, % viable/non-viable pollen (Luna et al. 2001), general morphology
Methodology/protocol	Alexander's stain, micrographs	Alexander's stain	Alexander's stain, Lugol staining, microscopic/digital imaging examination
Replication	5	3	3-4
No. of plants/flowers sampled	5 plants (3 flowers of each plant collected)	5 plants/20 flowers per plot	3-5 plants per plot
No. of pollen grains assessed	75 pollen grains per sample	10 pollen grains per replication	100-150 pollen grains per sample for viability, 10 pollen grains for diameter & morphology
No of ref. varieties	4	5	3-4

A.6 Volunteer studies

We identified six studies from two applications, one GM oilseed rape (no. 87) and one GM maize (no. 70), in which applicants assessed volunteer plants of the respective crop. Two studies were conducted for GM maize and four for GM oilseed rape (Table 14).

Oilseed rape

Two of the oilseed rape studies were pooled for evaluation due to their methodological similarity. The aim of the studies was to assess different management options for GM oilseed rape volunteers by use of different herbicide and mechanical control options. The applications discussed various aspects of GM volunteer control as well as resistance development of GM oilseed rape. The applicant presented an overview of all field trials conducted in different countries between 1990 and 1996. In addition, information on the field trials conducted in

[5] Reduced soil moisture conditions (30-40 %) during first reproductive stages (from V10-R3 growth stage).

Europe between 1990 and 1994 was contained. Information regarding the trial designs, number of sites and assessment methods is limited. All studies included the assessment of GM volunteers, assessing GM volunteer control measures within agricultural fields. In these studies, chemicals (e.g. herbicides against dicotyledonous plants) and/or mechanical practices (e.g. shallow cultivation, ploughing) were applied to control the development of GM oilseed rape volunteers in the subsequent crop.

The two studies (pooled for the purpose of this study) simulated the occurrence of volunteers, either by planting GM and non-GM winter oilseed rape concurrently with various cover crops (e.g. winter barley, winter wheat and sugar beet) or without any cover crops. The purpose of these trials was the assessment of chemical control options and potential differences in the response of GM and non-GM oilseed rape to non-complementary herbicides (i.e. other herbicides than glyphosate). In another study, seeds were deliberately shattered on the ground at harvest of GM oilseed rape. Shallow cultivation incorporated the seeds at various depths into the soil and volunteers germinated continuously, thus simulating real farm situations. The applicant applied herbicides to assess GM volunteer control. In the fourth study, the occurrence of volunteers was assessed in the course of plant variety trials with GM oilseed rape. In this case, GM volunteer emergence in the following cop (winter wheat and linseed) was assessed, but again control practices were applied. None of these studies assessed volunteers beyond the season following the GM oilseed rape cultivation or applied no management measures.

Maize

In the drought resistant maize application, two studies were included, which specifically examined the occurrence of GM volunteer maize plants (Table 14. In both studies, a specific number of maize seeds was deliberately scattered in the study area. In one study, the study area was uncultivated agricultural land and in the other case unmanaged land. The experiments were conducted without volunteer control.

Tab. 14: Field studies assessing volunteers in GM oilseed rape and GM maize notifications, n. a. = not applicable, n. i. = not indicated

	Oilseed rape	Oilseed rape	Oilseed rape	Maize	Maize
Methodology/study design	Simulation of volunteers by sowing GM and non-GM oilseed rape together with the respective cover crop[6]	Deliberate scattering of GM OSR seeds after harvest followed by shallow cultivation[7]	Assessment of occurrence of GM volunteers in the course of a variety trial	Maize seeds scattered in fall on agricultural sites not used for maize cultivation8	Maize planted in unmanaged areas without plot preparation (i.e. natural grass lands & pastures)9
No. of sites	1	1	2	3	4
Management/control measures applied	Various herbicide treatments	Shallow cultivation followed by herbicide treatment	Two shallow cultivations followed by herbicide treatment or ploughing in fall and tillage in spring	Herbicides applied for weed control	No agricultural management applied
Type of following crop/crop rotation	winter barley, winter wheat, sugar beet and without crop	none	wheat, linseed	none	n. a.
Parameters assessed	no. of volunteers/m^2	% inhibition (parameter not defined)	Volunteer emergence (parameter not defined)	volunteer plants	replacement values calculated (i.e. ratio of no. of seeds produced by volunteers to no. of seeds sown)10
No. of observations	7-8	5	2	6-7	1

[6] various densities: >30 plants/m^2 in winter wheat and in winter barley respectively, 70 plants/m^2 in sugar beet, and 80-100 plants/m^2 without cover crop.
[7] Seeds were incorporated into the soil at various depths (0-10 cm) and volunteers germinated continuously.
[8] 200 seeds scattered per plot by hand, replication = 3, no. of reference varieties = 6.
[9] approx. 100 (min. of 50) seeds planted per plot, replication = 3, no. of ref. varieties = 7, ground cover in unmanaged areas varied from 25 % to 98 %.
[10] Additional parameters assessed 5-7 times: early stand count, growth stage and plant vigour monitoring, late vegetative plant height, beginning and ending dates for pollen shed and silking intervals, final stand count, plant height at maturity, number of ears produced, number of seeds produced, and average number of ears per plant.

	Oilseed rape	Oilseed rape	Oilseed rape	Maize	Maize
Duration of observa-tion	7-9 months	2 months	2-4 months	6-9 months	n. i. (assessments were made in the following year)

A.7 Assessment of ecological and environmental interactions – biotic stressors

All applicants assessed ecological and environmental interactions of the GMP during field trials carried out for agronomic, phenotypic or compositional assessments of the GMP. Ecological and or environmental interactions comprise the assessment of pest and disease stressors as well as the response of the GMP to abiotic stressors (for discussion of abiotic stressors see A.8).

Maize

Applicants assessed the occurrence and severity of damage of pest (and disease) stressors, which occur during the growing season at individual locations/sites of field trials (but not necessarily at all field trial sites, Table 15). In most cases, applicants did not indicate how pest species were selected. Stressors were chosen that caused plant injury in the plots or were likely to occur during the growing period. Therefore, types of pests and pathogens assessed varied among sites in all applications. In some cases five or six of the most abundant pest arthropods were assessed at each site.

In general, GM maize applicants applied pesticides, so called "maintenance pesticides" (insecticides, fungicides) during field experiments when evaluating pests and pathogens. In one application, pests that controlled with theses pesticides were indicated, but this was not generally the case. Even for an insect resistant (stacked) GMP, insect pressure was controlled by pesticide applications. The applicant noted that "….*damage data were evaluated in the context of overall plant health and were not meant to be an indicator of insect efficacy*". In no application examined, applicants used artificial infestations or manipulative experiments in order to assess the response of the GMP to a specific pest or pathogen species.

Pests and diseases assessed in the field trials were usually mentioned with their common names (sometimes only in results tables), without citing their scientific names.

Pests usually assessed in GM maize were: aphids, thrips, armyworms, cutworms, (Northern/Western) corn rootworm beetles, corn earworms, western bean cutworms, southwestern Corn Borer, European corn borer, wireworm, Corn flea beetles, white grubs, Billbugs (weevil), spider mites (*Tetranychus* spp.), stink bugs (Pentatomidae), grasshoppers (*Melanoplus* spp.), Japanese beetles (*Popillia japonica*), Leafhoppers, leafroller, seed corn maggot.

Diseases commonly assessed in GM maize were: Anthracnose, dwarf mosaic virus, ear rot, root rot, stalk rot (*Pythium*), *Fusarium*, leaf blight, leaf rust, grey leaf spot, northern/southern corn leaf blight, *Penicillium*, *Pythium*, *Rhizoctonia*, seedling blight, stalk rot, smut (head and ear), corn stunt, eyespot, Goss's bacterial wilt, Stewart's wilt.

Pests were usually assessed by a qualitative, visual estimate of plant damage or occurrence using a qualitative 0-9 scale where 0 corresponds to no insect feeding damage, and 9 corresponds to a very high feeding damage. In other cases, only four categories were used to indicate the severity of each stressor: none (no symptoms), slight (symptoms not damaging to plant development, mitigation not likely required), moderate (likely requires mitigation), severe (symptoms damaging to plant development, mitigation unlikely to be effective). In one case, the severity scale 0-9 was compacted into four categories (0, 1-3, 4-6, 7-9).

Assessment was usually done in plants of two rows per plot, four times per season at specific growth stages of maize or at R5 growth stage only.

Only for drought resistant maize pest abundance was assessed by using yellow sticky traps which were placed at the approximate midpoint between the ground level and the top of the plant canopy. Once the main ear was visible, the sticky traps were deployed at the approximate corn ear level for the remainder of the arthropod collections. The sticky traps were deployed for approximately seven days. Up to six of the most abundant pest arthropods were determined for each collection at each site. These arthropods were then enumerated separately for each collection at each site. For this maize, European corn borer ECB and corn earworm CEW assessments followed a different methodology, counting the number of living larvae, the number of wholes and the number and length of feeding galleries in each stalk or ear.

Corn earworm (CEW) damage was evaluated by examining five non-systematically selected ears using a rating scale adapted from Widstrom (1967): 0 = No visible corn earworm damage, 1 = Silk shows evidence of feeding, feeding on the ear is < 0.5 in, 2 = corn earworm feeding to 0.5 in beyond the ear tip, 3 = corn earworm feeding to 1.0 in beyond the ear tip, 4 = corn earworm feeding to 1.5 in beyond the ear tip, 5 = corn earworm feeding to 2.0 in. beyond the ear tip, 6 = corn earworm feeding to 2.5 in. beyond the ear tip, 7 = corn earworm feeding to 3.0 in. beyond the ear tip, 8 = corn ear-worm feeding to 3.5 in beyond the ear tip, and 9 = corn earworm feeding to 4.0 in or greater beyond the ear tip.

European corn borer (ECB) damage was evaluated by examining five non-systematically selected plants. Damage was assessed by counting the number of entry/exit holes, number of galleries, and length of galleries in the ear shank and the stalk.

Results are usually presented in tables for each location/site, either by indicating the qualitative score per site or by providing a “summary”, i.e. indicating the stress severity (mild, minimal, severe). Categorical data are sometimes summarized across sites or observation times.

Oilseed Rape

A range of pests and diseases are commonly assessed in GM oilseed rape applications (Table 15). In most cases, diseases and arthropod pests were chosen that were either actively causing plant injury in the study area or were likely to occur in canola during a given observation period. In some cases, only three types of pests or diseases, respectively, were chosen for assessment at one site, according to their biological and economic importance.

In most studies, herbicides, pesticides or fungicides were applied as needed at the respective field trial sites. In two out of five studies of herbicide tolerant oilseed rape the respective complementary herbicide was not applied.

The assessment of pests and arthropods was carried out at four observation times, e.g. at development stages BBCH 11-14/12-16, 31-39, 60-61/61-67, 69, 71-89 or 85-86 or indicated as follows: 1: seedling to rosette stage 2: bud to first flowering stage 3: full flowering to flower completion stage 4: pod development stage.

In three studies, the observation scale of symptoms caused by the pest and/or disease was qualitatively assessed on a 1-4 scale (none, slight, moderate, severe symptoms). In two studies, an assessment of susceptibility to the respective pest/pathogen via a yes/no classification was done. In one study, the number of plants with aphids was counted although this was not reflected in the results, where only a qualitative statement regarding the aphid infestation was given (“no significant aphid infestation”). In one study, plant damage was rated according to the extent of damage with 0 = no damage, 1 = mild damage with < 10% visible damage, 2 = moderate damage with 10–30% visible damage, 3 = severe damage with > 30% visible damage.

In two studies, the number of observations across sites and the number of observations where no differences were observed between the GMP and the control were indicated.

Pests commonly assessed in oilseed rape were: aphids, thrips (Thysanoptera), flea beetle, cabbage worm, cabbage seedpod weevils (Curculionidae), painted lady, diamond-back moth (*Plutella xylostella* larvae), grasshopper, seedpot weevil, cutworm, clover cutworms (Noctuidae), armyworm, blister beetle, alfalfa looper, red turnip beetle (Chrysomelidae), *Meligethes aeneus* (pollen beetle), crucifer flea beetles (Chrysomelidae), (bertha) armyworms (*Mamestra configurata*), grasshoppers, pea leaf minors (*Liriomyza huidobrensis*), Leafminers (Lepidoptera), Pieridae, Cutworms (Noctuidae), Diamondback moth (*Plutella xylostella*), grasshoppers (*Melanopus* spp.), Loopers (Noctuidae), *Lygus* bugs (Miridae), red turnip beetles, slugs (Gastropoda), swede midge (Cecidomyiidae).

Diseases commonly assessed in oilseed rape were: *Alternaria* black spot, black Leg, *Aster* yellows, powdery mildew, *Sclerotinia* stem rot, bacterial leaf spot, *Rhizoctonia*, downey mildew, *Fusarium*, *Pythium*, black rot, white rust, CMV, clubroot, anthracnose, *Cercospera* leaf spot, gray mold, *Phomopsis viticola*, *Phytophthora*, *Puccinia striiformis*, white leaf spot, gray mold, root rot complex, white leaf spot, seedling blight, seedling disease complex.

Potato

The studies on the ecological interactions of the GM potatoes with pests and diseases were carried out in the context of studies to assess effects of the GMP on non-target arthropods. In the case of the *Phytophtora*-resistant potato, a specific performance and resistance test was additionally included in the application (see Table 15).

The assessed diseases were mostly fungus infections, such as *Phytophtora infestans* (in all three notifications), *Alternaria* sp. (fungus, two applications) and wart disease (*Synchytrium endobioticum*, two applications), virus infections such as potato virus X and Y (one application), or bacterial infections, e.g. potato blackleg (two applications).

Fungal infections such as *Phytophtora* were assessed by infection tests in the field in which the plants were artificially infected with a suspension according to protocols of plant variety testing. In other cases, the percentage of the crop with typical symptoms or the percentage of infected plants per plot was assessed. In one application, in addition plants were sprayed with a mix of European isolates classified as resistant or susceptible according to an EPPO guideline. For other fungal infections like *Alternaria*, the percentage of crop or plants per plot with typical symptoms was assessed. For wart disease, either an artificial inoculation was used (according to the Plant Protection Protocol) or a test in the growth chamber using the Glynne-Lemmerzahl Method was applied, assessing the number of sensitive tubers. Virus diseases (potato virus X and Y) were assessed by artificially infected plants between healthy plants according to a plant variety testing protocol. Bacterial infections like potato blackleg were assessed by counting the numbers of infested plants per plot or per m^2.

Commonly assessed pests of potato were: potato cyst nematodes (*Globodera* sp., one application), Colorado potato beetle (*Leptinotarsa decemlineata*, two applications) and aphids (two applications). Nematodes were assessed by artificial inoculation with a suspension of nematode larvae/eggs and calculating the relative susceptibility of the plants according to a national Plant Protection protocol. Colorado potato beetle was assessed in field studies by assessing the abundance, e.g. counting number of larvae and adults on plants, in one case according to an EPPO guideline (EPPO standard PP1/12 for *L. decemlineata*, EPPO 1999). Aphids were also assessed by counting individuals per leaf (abundance) and the number of species per site (diversity) referring to regulatory testing principles with non-target arthropods (Candolfi et al. 2000) and the EPPO Standard PP1/230 for aphids on potatoes (EPPO 2005).

Soybean

For soybean, studies on pests and diseases were contained in reports on the agronomic, phenotypic and/or compositional characteristics of the GMP or the assessment of ecological interactions. Pests and disease were assessed in field trials, in no case a field trial or glasshouse test with artificial infestation was carried out (Table 15).

The selection of pests and diseases varied between sites, with not all pest species and diseases assessed at each site. The selection was mostly based on organisms either actively causing plant injury in the study area or likely to occur in soybeans during a given observation period.

Pests commonly assessed included defoliating arthropods and pod feeding arthropods such as: aphids, bean leaf beetle, corn flea beetle, green cloverworm, Japanese beetle, potato leafhopper, stink bug, thrips, black cutworms, grasshoppers, Mexican bean beetles, soybean loopers, stink bugs, whitefly, caterpillars, armyworms, leafrollers, spider mites, wireworms, yellow wooly bear caterpillars. In one application, the species assessed were not indicated, because their severity never exceeded 30 % in the different sites.

Generally, pest arthropods were assessed visually on (mostly 10) plants from two rows in a plot using a qualitative rating of the damage severity or presence of aphids which was transformed into a severity scale (1-4 scale, e.g. none = no symptoms observed; slight = symptoms not damaging to plant development, moderate = intermediate between slight and severe, severe = symptoms damaging to plant development). In another application, the applicant estimated the percentage plant tissue or leaf area diseased or damaged over all plants in plot where 0% = no disease/damage; 100% = all plant tissues in the plot. In one application, the applicant used a beat sheet sampling method (plants shaken from rows 5-7 to collect arthropods) and determined the six most abundant pests and the six most abundant beneficial arthropods. They used four samples per plot with the sheet.

Assessments were generally carried out during four growth stages of the soybean plant (one application) or at R6 growth stage (one application).

Diseases commonly assessed include: Anthracnose, *Alternaria* (leaf spot), charcoal rot, *Phytophthora*, *Rhizoctonia solani*, *Fusarium*, downy mildew, powdery mildew, *Septoria* (brown spot), bacterial blight, bean pod mottle virus, *Cercospora* leaf spot, Asian rust, brown stem rot, brown stem rust, charcoal rot, frogeye leaf spot, Pythium, *Sclerotinia*, seedling blight, soybean cyst nematode, sudden death, soybean mosaic virus, white mold, soybean rust.

Disease incidence was assessed by a qualitative 1-9 scale (classified into four categories) or 1-4 scale (none-slight-moderate-severe) of plants from two rows. In one application, the visual estimate was carried out estimating the percentage of plant tissue/leaf area diseased over all plants in plot with 0%= no disease/damage; 100% = all plant tissues diseased in plot.

Tab. 15: Studies on plant response to biotic stress in GMP applications. The number in brackets indicates the number of studies, which included the respective aspect of the study design except for the first line, n. i. = not indicated

	Potato	**Oilseed rape**	**Soybean**	**Maize**
No. of studies (no. of dossiers)	15 (3)	6 (5)	7 (4)	10 (5)
Level of containment	Studies in climate chamber or greenhouse (4), studies in field trials (11)	Studies in field trials (6)	Studies in field trials (7)	Studies in field trials (10)
Study on	agronomic properties, pest and disease incidence, NTO studies, resistance testing (*Phytophtora*-resistant potato)	agronomic and phenotypic characteristics, environmental interactions, agronomic performance and composition, observations from field tests	agronomic & phenotypic characterisation, ecological interactions, agronomic performance and composition	phenotypic evaluations, ecological interactions, agronomic characteristics
Pests/diseases assessed	Pests: CPB (field) Aphids (field) *Globodera* sp. (glasshouse) Diseases: *Phytophtora* (field, greenhouse) *Synchytrium endobioticum* (growth chamber) *Erwinia* (field) *Alternaria* (field) PVX, PVY (field) Unspecified virus (field)	arthropod pests and diseases: Varied among observations and field sites *“Those that were actively causing plant injury in the study area or were likely to occur in canola during a given observation period”*	arthropod pests and diseases: varied among observations and between sites *“Those that were actively causing plant injury in the study area or were likely to occur in soybeans during a given observation period”*	arthropod pests and diseases: Varied among observations and field sites *“Those that were causing plant injury in the plots or likely to occur during the observation period”*
Artificial inoculation	Yes (*Phytophtora* (2) PVX (1), PVY (1) *S. endobioticum* (2) *Globodera* sp. (1) N (for all other)	No	No	No

	Potato	Oilseed rape	Soybean	Maize
Application of plant protection products (pesticides, herbicides, fungicides) in field trials	Yes (4), no (3), n. i. 82)	Yes (5), n.i. (1)	Yes (3), n.i. (4)	Yes (9), n.i. (1)
Type of assessment/endpoints used	diseases: % infected leaf area or plants or crop canopy, qualitative 1-10 scale, % resistant plants pests: relative susceptibility (*Globodera*), abundance (CPB) species diversity, abundance (aphids)	pests and diseases: visual rating of symptoms or damage on a 0-3, 1-4 or 1-9 scale, yes/no classification, no. of plants with aphids,	Pest and diseases: Visual rating 1-9/1-4 scale, 0-5 scale (e.g. aphids, leafhoppers), 4 categories for pest damage Visual estimate 0-100% or % plant tissue or leaf area affected Abundance of 6 most abundant pest arthropods (beat sheet sampling) (1 study) disease/damage type recorded if incidence greater than 30% (1 study)	Pests and diseases: Visual estimation on qualitative scale (6) Yellow sticky trap for 6 most abundant pest arthropods (1 study) Quantitative assessment (3): ECB and CEW For ECB: No. of live larvae, no. of holes, no. of feeding galleries, length of feeding galleries in stalks (10 plants) For CEW: Abundance or 0-9 rating of damage
Method/protocol	protocol for official tests for entrance on the Dutch Variety List, protocol of the Dutch Plant Protection Service susceptible cultivar as control, Glynne-Lemmerzahl-Method EPPO Standard PP1/12 (3) (*L. decemlineata*, EPPO 1999) EPPO Standard PP 1/230 (1) (aphids on potatoes, EPPO 2005) EPPO PP1/213(2) guideline for efficacy evaluation of PPPs	no protocol cited	no protocols cited	no protocols cited for CEW: Method adapted from Widstrom (1967) with a qualitative 0-9 scale

	Potato	Oilseed rape	Soybean	Maize
Locations/Seasons	1-7/1-2	3-13/1-2	4-21/1-2	3-12/1-2

A.8 Assessment of ecological interactions – abiotic stressors

The response of the GMP to abiotic stressors was generally assessed in all GMP applications. Plant response to abiotic stressors was evaluated in the course of field trials conducted for the comparative assessment of the GMP with its non-GM counterpart. In these field trials, abiotic stressors were generally assessed together with biotic stressors (see A.7). Applicants evaluated abiotic stress response for GM oilseed rape, GM soybean and GM maize in field trials (Table 16). In contrast, none of the analysed studies conducted with GM potatoes evaluated plant response to abiotic stress under field conditions.

Abiotic stressors reported in these field studies comprised hail injury, heat stress, drought, excess moisture, cold stress, nutrient/nitrogen deficiency, soil compaction, flooding, wind damage, sunscald and mineral toxicity. Usually, three abiotic stressors were assessed per site. The abiotic stressors often vary between observations at a specific site, but also among the various sites within a specific field trial. According to the applicants, criteria for selection of specific abiotic stressors were their incidence at the specific site or selection according to biological and economic importance. In addition, applicants stated that stressors were chosen if they "*either actively cause plant injury in the plots or are likely to occur in the crop during a given observation period*".

For the field trial assessments of abiotic stress of GM oilseed rape, GM soybean and GM maize, the methodology applied was comparable across GM plants and studies. The assessment was generally based on visual observations and qualitative assessments of the different abiotic stressors. The qualitative data were not subject to a statistical analysis.

The applicants presented the results as categorical parameters of each stressor per site, observation date and entry. In some cases, data for abiotic stressors were also presented in an aggregated form. In some studies, several abiotic stressors were indicated per site while in other studies stressors were aggregated across sites or observations dates. In this context, applicants stated that test and control substances "*…were considered different in susceptibility or tolerance to abiotic stressors … if the severity of injury to the GM plant did not overlap with the severity of injury to the control across replications*".

Tab. 16: Field studies assessing plant response to abiotic stress in GMP applications. n. a. = not applicable

Plant	Oilseed rape	Soybean	Maize	Maize
No of studies (no. of applications)	4 (5)	4 (3)	11 (4)	
Abiotic stressors	Incidence	Incidence	incidence[11]	Water limitation
Artificially induced abiotic stress	No	No	No (6)	Yes (5)

[11] All sites managed to well-watered conditions either by rainfall or irrigation.

Plant	Oilseed rape	Soybean	Maize	Maize
Assessment	Symptoms: visual observation	Symptoms: visual observation	Symptoms: visual observation	Symptoms: visual observation Growth & physiol. parameters
Parameters	Plant damage, stressor symptoms (ordinal scale)	Plant damage, stressor symptoms (ordinal scale)	Plant damage, stressor symptoms (ordinal scale)	Studies 1, 2: growth parameters, physiology[12], yield[13] Studies 3, 4, 5: Plant damage, stressor symptoms (ordinal scale)
No. of observations of plant damage	4 per season	4 per season	4 per season	max. 4 per season (at V2-V4, V10-V15, VT-R3, R6)
No. of seasons/sites per season (no. of studies)	1/8, 1/9, 1/12, 1/13	1/4, 1/11, 1/14, 1/17	1/5, 1/8 (3), 1/10 (2)	1/1 (2), 1/3 (2), 1/4
Stress conditions	n. a.	n. a.	n. a.	**Study 1**: 14 days of drought stress at V8 **Study 2**: well-watered treatment (soil moisture at 80% of field capacity); water-limited treatment (reduction of water by 25 %, V7-R2) Studies 3+4+5: well-watered treatment (for optimal grain yield); water-limited (no irrigation during late vegetative growth and early grain filling growth stage)

[12] Photosynthesis, stomatal conductance, transpiration rate and leaf extension rate (LER) or plant height, LER and plant biomass increase.
[13] Yield, kernels per ear and 200 or 50 kernel weight.

Tab. 17: Studies under containment assessing plant response to abiotic stress in GMP applications (Numbers in brackets refer to numbers of studies).

Plant	**Potato**	**Maize**	**Maize**
No of studies (applications)	2 (2)	4 (1)[14]	1 (1)[14]
Abiotic stress assessed	Cold stress	Cold stress, heat stress, drought stress, salt stress	Drought stress
Stress artificially induced	Yes	Yes	Yes
Type of containment	climate chamber (2)	growth chamber (2), greenhouse (2)	greenhouse (1)
Experimental design	tubers placed on soil surface and tubers covered with soil (10 cm & 20 cm depth)	Plants grown in pots (V3 or V4 growth stage)	Plants grown in pots (V4/V5 growth stage)
Test regimes used	Experiment I: tests start at 4°C, 3 days gradually lowering to three minimum temperatures (1.5°C, -2.5°C and -3.5°C), 2 days at minimum temperature, 3 days gradual thawing Experiment II: tests start at 4°C, 5 days gradually lowering to two minimum temperatures (2.5°C & -7.0°C), 2 days at minimum temperature, 5 days gradual thawing	Cold stress: optimal temperature (30°/22 °C) and 3 cold treatments (mild 20/15 °C, moderate 15/10 °C and severe 4°/4 °C) at V3 for 8 days Heat stress: optimal temperature (30/22°C), and 3 heat treatments (mild 40/35 °C, moderate 43/35 °C and severe 47/35 °C) at V3 for 5 days Drought stress: well-watered and 3 drought levels at V4 for 15 days: Target pot weight (g): Well-watered: 4700 - 4800 Mild 3500 - 3900 Moderate 2700 - 2900 Severe 2160 - 2400 Salt stress: 3 salt levels (mild, moderate and severe) at V4 for 12 days	1 drought cycle in the greenhouse: starting at V5, plants exposed for 6 days to drought (target pot weight) and then re-watered

[14] All studies were included in the application of drought resistant maize.

Plant	Potato	Maize	Maize
Parameters assessed	survival rate (surviving tubers developing sprouts were assessed after 2-3 weeks at 18°C)	Different parameters of growth and development: Plant height, growth stage, Chlorophyll Content, plant vigour, fresh weight, dry weight of above-ground biomass, necrosis (heat), leaf rolling (drought), electrical conductivity (salt)	Physiological parameters: photosynthesis, stomatal conduct-ance, leaf extension rate (LER), ion leakage and relative water content (RWC)
No. of observations	1	3[15]	4-8
Duration of test in days	8-12[16]	Cold: 8, Heat: 5, Drought: 15, Salt: 12	6

[15] One evaluation was conducted before the beginning of the experiment, followed by day 4 and 8 after treatment (DAT) for cold stress, 3 and 5 DAT for heat stress, 7 and 14 DAT for drought stress and 9 and 12 DAT for salt stress.

[16] Duration of frost regimes indicates the time used for lowering the temperature, keeping the respective minimum temperature and gradual thawing.

B Annex: Assessment frameworks for alien species

The negative impact of an invasive alien species might become apparent only long time after the introduction into a certain area. At that time, the species could have already spread considerably, making eradication or mitigation measures challenging. Thus, the aim of respective assessment frameworks is to identify alien species that could have a negative impact as early as possible. The identification of invasive alien species and their ranking/classification is a prerequisite for the prioritisation and implementation of respective management measures (Blackburn et al. 2014, Hawkins et al. 2015). Different assessment schemes were developed worldwide and as highlighted by Blackburn et al. (2014), those are seldom based on quantitative evaluations, presumably due to data limitations. Accordingly, results can vary across protocols (depending e.g. on their scope) but also between assessors as examined by Gonzales-Moreno et al. (2019). Consistency can be improved by selecting assessors with high expertise regarding the assessed species, and providing clear guidance and adequate training. In addition, assessments by expert groups could improve the results.

As described by Essl et al. (2011) most risk assessment approaches for invasive alien species developed in various European countries focus on the spatial distribution, the capacity to spread and – in line with the CBD definition – on the impact of the introduced species on biodiversity (sometimes also economic impacts are considered). The risk assessment systems are designed either to predict whether a species might become an invasive alien species in the future, or to prioritise invasive alien species already present.

In the following, three examples for assessment frameworks were analysed: The IUCN Environmental Impact Classification for Alien Taxa (EICAT), the European Union approach to define so called “Invasive Alien Species of Union concern” and the German-Austrian Black List Information System (GABLIS). The aim was to analyse whether information on phenotypic characteristics of the organisms are collected and if so which methods are foreseen.

B.1 IUCN Environmental Impact Classification for Alien Taxa (EICAT)

The IUCN Environmental Impact Classification for Alien Taxa (EICAT) is the IUCN standard for the classification of alien species depending on the impact to the environment and more specifically to native species (IUCN 2020a). Its aim is not only to identify alien species that have negative impacts e.g. on a local population, but amongst others also to facilitate the prediction of future impacts of a species in other regions. EICAT was developed based on Blackburn et al. (2014) and Hawkins et al. (2015) and comprises twelve impact mechanisms (e.g. hybridisation) and five impact categories (e.g. major) linked by respective criteria to be used for classification. Although it was developed for the assessment of alien species on a global level, it can also applied to other geographic scales. Global assessments of species may be published in the IUCN Global Invasive Species Database following a respective review and according to the procedures laid down in IUCN (2020b).

The twelve impact mechanisms considered are the following: competition, predation, hybridisation, transmission of disease, parasitism, poisoning/toxicity, bio fouling or other direct physical disturbance, grazing/herbivory/browsing, chemical impact on ecosystem, physical impact on ecosystem, structural impact on ecosystem, indirect impacts through interactions with other species.

Species are classified into five impact categories: minimal concern, minor, moderate, major, and massive. Since EICAT is based on available data, additional categories for species with data deficiencies are included. Those comprise: data deficient, no alien populations and not evaluated. “No alien population” means, that there is no evidence that the respective species is existing in a wild state outside its natural territory. This includes also species cultivated in an area where it is not native. Species are classified as “data deficient” when they are in fact considered as alien, but it is not possible to classify the species according to its impact (e.g. due to lack of adequate information).

IUCN (2020a) not only includes a general definition of the impact categories, but also specific descriptions of the criteria linking the impact categories to the respective impact mechanisms. A species is e.g. generally considered having a massive impact when “it causes naturally irreversible community changes through local, sub-populations or global extinction (or presumed extinction) of at least one native taxon”. The criterion for moderate impact specifically for competition is e.g. “competition resulting in a decline of population size of at least on native taxon, but no local population extinction”.

EICAT is complemented by respective guidelines for using the EICAT system (IUCN 2020b). It includes amongst others information on how to deal with uncertainties in the assigned classification. Every classification in one of the impact categories needs to be accompanied by a respective level of confidence (high, medium, low). The level of confidence considers the following aspects: presence of confounding effects, study design, data quality and type, spatial and temporal scale.

According to the definition of invasiveness in the context of alien species, EICAT aims at assessing the impact of a species. Some of the impact mechanisms assessed, e.g. competition and hybridisation, are also of relevance in the context of persistence and invasiveness of GMOs. The assessment is conducted based on available information on the species and a respective literature review. No additional data are generated, e.g. in field experiments. The documentation of the assessment needs to include a summary of the ecology of the species. However, the guidelines do not specify specific phenotypic characteristics that need to be considered in the assessment.

B.2 Regulation and assessment of invasive alien species in the EU

In the EU, a selection of invasive alien species is regulated since 2015 by Regulation (EU) No 1143/2014. A core element of this Regulation is to define so called “Invasive Alien Species of Union concern”. In order to determine those species, several criteria in line with the EU definition of invasive alien species have to be met. This includes, amongst others that they are capable of establishing viable populations, spread in the environment, and are likely to have adverse impacts on biodiversity, related ecosystem services, human health or economy. In addition, actions on the EU level are needed to prevent their introduction, establishment or spread. This has to be demonstrated by a respective risk assessment.

The Regulation also includes measures for those species that need to be taken in order to prevent the introduction in the EU, measures for early detection and rapid eradication in order to prevent their establishment as well as management measures to prevent spreading of those invasive alien species that are already established in the EU.

Common elements of the above mentioned risk assessment are defined in Article 5 of Regulation (EU) No 1143/2014 and further described in Commission Delegated Regulation (EU) 2018/968. It includes e.g. the description of the species with its natural and potential range, its current (and likely future) distribution in the EU and neighbouring countries and its invasion history. Also information on reproduction and spread patterns (including e.g. number of seeds) need to be provided and an assessment of the risks of establishment and spread in the EU. In this regard also climate change conditions should be taken into account. The risk assessment should also provide a description of the adverse impacts.

The Commission Delegate Regulation in its Article 2 also includes minimum standards on the methodology to be applied. The basis for the risk assessment should be the 'best available scientific evidence'. However, it could also include information from other sources like expert opinions. In case of no or incomplete knowledge this should be addressed accordingly. Every answer in the risk assessment should also include an assessment of the level of uncertainty or confidence. In addition, quality control is foreseen which includes at least one peer-review by two independent reviewers.

In order to meet the requirements, a risk assessment template (EC 2020), based on the Great Britain non-native species risk assessment scheme (GBNNRA), was amended to ensure compliance with Regulation (EU) 1143/2014 and relevant legislation, including the Delegated Regulation (EU) 2018/968, and is currently used in developing risk assessments according to Article 5 of Regulation . Member States are free, however, to select any other template as long as it is compliant with the Regulation 1143/2014. The template includes guidance for information to be provided (e.g. on the species and its distribution as well as on already existing risk assessments) and a set of questions to be answered (e.g. to assess the probability of establishment or probability of spread). Some examples are provided in the following:

- How likely are the biological characteristics of the organism to facilitate its establishment in the risk assessment area? (Information to be included comprises e.g. reproduction mechanism, number of seeds)
- How likely is it that a number of individuals sufficient to originate a viable population will spread along this pathway from the point(s) of origin over the course of one year? (Information to be included comprises e.g. propagule pressure)
- How important is the impact of the organism on biodiversity at all levels of organisation caused by the organism in its non-native range excluding the risk assessment area?

In addition to impacts on biodiversity, also economic, social and human health impacts are considered in these risk assessments. The template includes also definitions and detailed descriptions on the information to be provided. To determine the level of confidence, one of three categories- low, medium, high- have to be selected. This rating depends on the information available or in case information is unavailable or contradictory. In addition, lack of information needs to be marked.

Like the EICAT assessment, also the risk assessment to tackle priority species in the EU is based on available information with no experiments foreseen. The assessment template as described above includes a variety of information to be documented and answers to be provided by the assessor. Some of the questions are also of relevance for the risk assessment of GMOs, e.g. regarding the probability for establishment and spread. In that respect relevant information on the biology of the plant need to be provided. However, the template includes only examples (e.g. reproduction mechanism, number of seeds) but no list of phenotypic characteristics or parameters for which information is required.

B.3 The German-Austrian Black List Information System (GABLIS)

The "German-Austrian Black List Information System" (GABLIS) is a risk assessment tool for invasive alien species, applicable to various taxonomic groups. Its aim is to classify alien species according to their invasiveness, focussing on negative impacts to native biodiversity (Nehring et al. 2010, Essl et al. 2011).

Based on general information on the species and applying respective criteria to assess invasiveness, alien species are assigned to three main list categories. The "White List" comprises alien species that are not considered invasive, the "Black List" alien species whose negative impact on biodiversity has been confirmed and thus are considered invasive. The data basis for the assessment may not only include scientific reports and publications, but also expert opinion. However, data may be limited especially for those alien species not yet present. Thus, the assessment of possible future impact is associated with respective uncertainties, reflected by the establishment of an additional "Grey List" for alien species whose risk to biodiversity remains uncertain.

The classification to these lists is based on five main criteria that assess risks to biodiversity: inter-specific competition, predation and herbivory, hybridisation, transfer of pathogens or organisms and negative effects on ecosystem functioning. In order to estimate e.g. whether hybridisation poses a threat to native species, the risk assessor has not only the options "yes" (negative impact confirmed) or "no", but can also state that there is "evidence-based assumption" for the threat to native species (evidence contradictory or less clear) or that an assessment is not possible ("unknown"). The damage threshold for these criteria is qualitatively determined: "if at least one population of a native species is locally endangered by an alien species and if invasion into new areas or similar habitats is likely to increase the risk of extinction of the native species in large parts of its range, so that, eventually, its inclusion in the Red List of endangered species is expected" (Essl et al. 2011).

The Black List is subdivided into three lists. Invasive alien species are assigned according to their distribution and depending on combat and eradication measures available. The "Black List – Warning List" comprises invasive alien species that are not present yet, the "Black List – Action List" covers those species that occur only in small areas with eradication measures available. Those invasive alien species that occur in small areas with no eradication measures available or species occurring in large areas and measures for their eradication not feasible are assigned to the "Black List – Management List". This sub-classification is also based on respective criteria.

Additional biological-ecological criteria are included in the GABLIS tool in order to distinguish between Grey List and White List. Those are:

- Occurrence in natural, semi-natural or other high nature value habitats (this excludes anthropogenically modified habitats like fields, field margins or ruderal sites)
- Reproductive capacity (i.e. reproduction rate in short time under favourable conditions)
- Spread capacity (i.e. potential for rapid spread due to the potential for long-distance dispersal)

- Current spread history (in the area under assessment and neighbouring areas)
- Monopolisation of resources (e.g. fast increase in biomass due to monopolisation of nutrients or space)
- Facilitation by climate change (i.e. whether species benefits from climate change)

Also for species assigned to the Grey List, sub-lists can be selected according to the level of uncertainty. The "Grey List-Watch List" covers those alien species with higher certainty of being invasive ("probable"), the others are assigned to the "Grey List-Operation List" ("unlikely").

GABLIS is currently (July 2021) under revision taking into account the compliance with Regulation (EU) 1143/2014 (Wolfgang Rabitsch, pers. communication).

In addition, GABLIS is an expert assessment based on already available information. In line with the respective definition, invasiveness is considered as negative impact on biodiversity and not only the spread of an alien organism. Some of the five criteria assessed are also of relevance for the risk assessment of GMOs, e.g. hybridisation. As in the other assessment frameworks an assessment protocol covers the aspects to be considered as well as information to be provided. Reproduction and the capacity to spread are covered by the biological-ecological criteria as described above. However, the assessment protocol includes no predefined list of phenotypic parameters related to establishment and spread for which information has to be provided.

C Annex: Phenotyping in plant variety testing (VCU)

C.1 Plant variety testing – Introduction

Before a new crop variety can be listed on a national level in the European Union, it has to, among other conditions, successfully pass a mandatory variety testing system for agricultural crops, called Value for Cultivation and Use (VCU) test.

The VCU tests differ on a national level by complexity, as well as by the specific traits that have to be looked at for each crop. However, each protocol is based on rateable or measurable phenotypic traits that have to be assessed during multiple (two to three) growing seasons.

When placing a GMO on the EU market, current assessment of the environmental behaviour is based on EFSA guidelines published in 2015, which primarily focus on agricultural parameters that play a role predominantly in product development. Part of the project at hand was to investigate if and in what form existing VCU protocols can be used to more efficiently assess the environmental behaviour of GM crops for the environmental risk assessment.

For this purpose, the five exemplary crops potato (*Solanum tuberosum*), maize (*Zea mays*), oilseed rape (Brassica napus), soybean (*Glycine max*) and creeping bentgrass (*Agrostis stolonifera*) were chosen, based on the fact that GMOs of each of these five species are already available on the international market, i.e. they are highly relevant for GM crops. For each of these five crops, the VCU protocols from five selected European countries were sourced from each respective variety testing offices (Table 18). These included Austria (with its national variety testing office BAES), Belgium (ILVO), Czech Republic (ÚKZÚZ), France (GEVES) and Germany (BSA). Once procured, the Belgium, French and Czech protocols were translated from their original language into German by the online translator DeepL.

Methods from the above-mentioned sourced VCU protocols were searched for ecologically and agronomically important plant characteristic of these five crop types. If none of the VCU protocols of any of the five countries provided a suitable method, scientific literature was searched for alternatively applicable methods.

C.2 Aim of the VCU tests

VCU test protocols aim at evaluating candidate varieties as comprehensively as possible in terms of cultivation including yield, disease resistance and utilization properties.

In many cases, the test protocols contain approaches that are suitable for further development to cover aspects of invasiveness and environmental interaction, partly also of persistence in the context of an environmental risk assessment.

C.3 VCU tests and invasiveness, persistence and environmental interactions

Invasiveness and Persistence

Parameters describing rapidness of field emergence, crop stand establishment and further crop development up to the end of flowering may give valuable information for assessing varietal differences in invasiveness. Other invasiveness-related surveys carried out later in the growing season address the risk of crop losses, described by parameters such as the loss of grains per unit area, the occurrence of pod bursting or, other variety characteristics that potentially influence the risk of unintended seed loss into the environment.

The assessment of persistence plays a minor role in VCU testing programs, except for perennial crops, for which annual trial settings are foreseen, with the trial and assessment activities finalized at the time of harvest. VCU standard procedures usually do not include surveys to detect differences in a crop´s persistence potential such as forms of dormancy, seed longevity in the soil, or volunteers in subsequent crops.

Interactions with biotic and abiotic stress factors

The definition of the value of cultivation and use calls for, among other things, the improvement of new varieties in their cultivation and disease characteristics. Therefore, surveys in variety value tests focus on traits that help to avoid or reduce biotic or abiotic stress-induced risks in cultivation. In variety comparisons, therefore, differences in the extent of infestation by pests and diseases, the resistance to abiotic stresses such as drought, frost, cool temperatures, or variety performance under, for instance, extensive farming practices are important. In contrast, the observation of the interaction between varieties and beneficial insect occurrence hardly plays a role (yet). Furthermore, pests, diseases or suboptimal growing conditions cause plant reactions and symptoms. These, in many cases, can be assigned specifically and, upon adequate documentation, be used to assess plant behaviour under environmental stress conditions.

Tab. 18: Test protocols of the variety testing offices from Austria (BAES), Belgium (ILVO), Czech Republic (ÚKZÚZ), France (GEVES) and Germany (BSA), used to analyse the scope of examinations and assessments within national list trialling regarding VCU.

Crop species	Country	VCU protocols
Potato *Solanum tuberosum*	Austria	BAES, Bundesamt für Ernährungssicherheit (2015a): Methoden für Saatgut und Sorten - Richtlinien für die Sortenwertprüfung, Sorten- und Saatgutblatt, 23 Jg. Sondernr. 44, Wien, 4.1.9 Kartoffel
	Belgium	ILVO, Instituut voor Landbouw-, Visserij- en Voedingsondzoek, Technische interregionale werkgroep voor de samenstelling van de nationale rassenlijst voor landbouwgewassen (2017a): Criteria voor het onderzoek van de rassen met het oog op hun toelating tot de rassenlijst, Aardappel, 18/09/2017 ILVO, Instituut voor Landbouw-, Visserij- en Voedingsondzoek (2017b): Beoordelingsmethodes voor de onderzoekscriteria voor rassen met het oog op hun toelating tot de rassenlijst, Methodologie Aardappel – GTIW87 – 18/09/2017, Versie 2.1
	Czech Republic	ÚKZÚZ, Ústřední kontrolní a zkušební ústav zemědělský (2019a): Metodika zkoušek užitné hodnoty, Brambor, Zuh/3-2019

Crop species	Country	VCU protocols
	France	GEVES, Groupe d´étude et de contrôle des variétiés et des semences (2021a): Protocole d'Expérimentation Pomme de Terre, Essais de Valeur Agronomique, Technologique et Environne-mentale, Version en vigneur pour la campagne 2021, DOCVAT/PAT/PROTO/001/IND15 GEVES, Groupe d´étude et de contrôle des variétiés et des semences (2021b): Protocole d'Expérimentation Pomme de Terre, Études des bioagresseurs et études des critères de qualité, Version en vigneur pour la campagne 2021, DOCVAT/PAT/PROTO/002/IND12
	Germany	Bundessortenamt (2019): Richtlinien für die Durchführung von landwirtschaftlichen Wertprüfungen und Sortenversuchen, 4.3 Kartoffel
Soybean *Glycine max*	Austria	BAES, Bundesamt für Ernährungssicherheit (2015b): Methoden für Saatgut und Sorten - Richtlinien für die Sortenwertprüfung, Sorten- und Saatgutblatt, 23 Jg. Sondernr. 44, Wien, 4.1.4 Mittel- und großsamige Leguminosen
	Belgium	ILVO, Instituut voor Landbouw-, Visserij- en Voedingsondzoek, Technische interregionale werkgroep voor de samenstelling van de nationale rassenlijst voor landbouwgewassen (2019): Criteria voor het onderzoek van de rassen met het oog op hun toelating tot de rassenlijst, Soja, Versie 15 maart 2019
	Czech Republic	ÚKZÚZ, Ústřední kontrolní a zkušební ústav zemědělský (2019b): Metodika zkoušek užitné hodnoty, Sója, Zuh/25-2019
	France	GEVES, Groupe d´étude et de contrôle des variétiés et des semences (2021c): Protocole d'Expérimentation Soja, Essais de Valeur Agronomique, Technologique et Environnementale, Version en vigneur pour la campagne 2021, DOCVAT/SOY/PROTO/001/IND18
	Germany	Bundessortenamt (2021): Richtlinien für die Durchführung von landwirtschaftlichen Wertprüfungen und Sortenversuchen, 4.13 Sojabohne (Körnernutzung)
Maize *Zea mays*	Austria	BAES, Bundesamt für Ernährungssicherheit (2015c): Methoden für Saatgut und Sorten - Richtlinien für die Sortenwertprüfung, Sorten- und Saatgtublatt, 23 Jg. Sondernr. 44, Wien, 4.1.2 Mais und Hirsearten
	Belgium (Flandern)	ILVO, Instituut voor Landbouw-, Visserij- en Voedingsondzoek, Technisch interregionale werkgroep voor de samenstelling van de nationale rassencatalogus voor landbouwgewassen (2020): Criteria voor het onderzoek van de rassen met het oog op hun toelating tot de catalogus, Korrelmaïs, 25 februari 2020
	Czech Republic	ÚKZÚZ, Ústřední kontrolní a zkušební ústav zemědělský (2019c): Metodika zkoušek užitné hodnoty, Kukuřice (na zrno a na siláž), Zuh/15-2019

Crop species	Country	VCU protocols
	France	GEVES, Groupe d´étude et de contrôle des variétiés et des semences (2020a): Protocole d'Expérimentation Maïs Grain, Essais de Valeur Agronomique, Technologique et Environnementale, Version en vigneur pour la campagne 2020, DOCVAT/MAT/PROTO/001/IND17
	Germany	Bundessortenamt (2008a): Richtlinien für die Durchführung von landwirtschaftlichen Wertprüfungen und Sortenversuchen, 4.2 Mais
Oilseed rape *Brassica napus*	Austria	BAES, Bundesamt für Ernährungssicherheit (2015d): Methoden für Saatgut und Sorten - Richtlinien für die Sortenwertprüfung, Sorten- und Saatgutblatt, 23 Jg. Sondernr. 44, Wien, 4.1.6 Ölfrüchte (Rübsen, Raps, Sonnenblume, Öllein, Mohn, Ölkürbis)
	Czech Republic	ÚKZÚZ, Ústřední kontrolní a zkušební ústav zemědělský (2017): Metodika zkoušek užitné hodnoty, Brukvovité olejniny, Repka, Zuh/4-2019
	France	GEVES, Groupe d´étude et de contrôle des variétiés et des semences (2020b): Protocole d'Expérimentation Colza Oléagineux d'Hiver, Essais de Valeur Agronomique Technologique et Environnementale, Version en vigueur pour la campagne 2020-2021, DOCVAT/COLH/PROTO/001/IND18 GEVES, Groupe d´étude et de contrôle des variétiés et des semences (2020c): Protocole d'Expérimentation Colza Oléagineux d'Hiver, Essais de caractérisation des variétés au virus de la jaunisse TuYV, Version en vigueur pour la campagne 2020-2021, DOCVAT/COLH/PROTO/004/IND3 GEVES, Groupe d´étude et de contrôle des variétiés et des semences (2020d): Protocole d'Expérimentation Colza Oléagineux d'Hiver, Essais maladie Phoma, Version en vigueur pour la campagne 2020-2021, DOCVAT/COLH/PROTO/003/IND15 GEVES, Groupe d´étude et de contrôle des variétiés et des semences (2020e): Protocole d'Expérimentation Colza Oléagineux d'Hiver, Essais maladie Cylindrosporiose, Version en vigueur pour la campagne 2020-2021, DOCVAT/COLH/PROTO/002/IND15
	Germany	Bundessortenamt (2000): Richtlinien für die Durchführung von landwirtschaftlichen Wertprüfungen und Sortenversuchen, 4.11 Kruziferen (Körnernutzung)
Creeping bentgras *Agrostis stolonifera*	Austria	BAES, Bundesamt für Ernährungssicherheit (2015e): Methoden für Saatgut und Sorten - Richtlinien für die Sortenwertprüfung, Sorten- und Saatgutblatt, 23 Jg. Sondernr. 44, Wien, 4.1.3 Futtergräser
	Belgium	ILVO, Instituut voor Landbouw-, Visserij- en Voedingsondzoek, Technische interregionale werkgroep voor de samenstelling van de nationale rassenlijst voor landbouwgewassen (2014): Criteria cultuuren gebruikswaarde voor het onderzoek van rassen met het oog op hun toelating tot de catalogus, Grassen, 14 maart 2014
	Czech Republic	ÚKZÚZ Ústřední kontrolní a zkušební ústav zemědělský (2019d): Metodika zkoušek užitné hodnoty, Trávy, Zuh/27-2019

Crop species	Country	VCU protocols
	France	GEVES, Groupe d´étude et de contrôle des variétiés et des semences (2020f): Protocole d'Expérimentation Graminées à Gazon, Essais de Valeur d'Utilisation, Version en vigueur pour la campagne 2020, DOCVAT/GAZ/PROTO/002/IND17
	Germany	Bundessortenamt (2008b): Richtlinien für die Durchführung von landwirtschaftlichen Wertprüfungen und Sortenversuchen, 4.18 Gräser- und Kleearten einschließlich Luzerne, Esparsette

C.4 Potato (*Solanum tuberosum*)

Phenotypic assessment in potato based on the Value of Cultivation and Use (VCU) protocols from Austria, Belgium, Czech Republic, France and Germany refers to plant establishment, the rapidity of further crop development, susceptibility to viral diseases, phytoplasma, bacterial and fungal diseases on the plant and tuber, maturity behaviour, yield and yield structure, dry matter or starch content, and processing characteristics.

Depending on the test protocol and parameters, data are collected by visual assessment on a 1-to-9 scale for each plot or sample, often also by counting plants in the crop due to the low number of plants per plot, and by determining the weight of the yield according to size fractions of the tubers. Testing for suitability for certain processing procedures and for eating quality follows specific protocols. Hereinafter, characteristics and methods for their assessment are considered with a focus on the potential for invasiveness and persistence of potato plants.

Seedling

A parameter of interest at the seedling stage is plant establishment. Variety performance in this characteristic is recorded by indicating the date on which half (BE, FR), two thirds (AT) or three quarters (CZ, DE) of the plants have emerged, depending on the protocol. The emergence score is supplemented by counting stunted plants in each plot about 3 weeks after emergence (AT, CZ and DE).

Mature plant

When plants outgrow the seedling stage, multiple surveys are conducted to estimate the growth rate/duration (period)/development, a characteristic that is deemed to be ecologically and agronomically relevant for both the invasiveness and persistence of a plant. The VCU protocols of AT, FR and CZ require the assessment of the rapidity of young plant development after emergence, or deficiencies in canopy closure between rows (DE, AT), both based on the nine-step-scale. The Belgian protocol asks for the period in days between emergence and complete stand closure between rows, and the Czech protocol for the date of row closure as well as for a further assessment of the crop development status at the full flowering stage.

Concerning the plant characteristics related to flower biology / time to flowering or maturity /flowering period, not all VCU protocols include the observation of the beginning of flowering. In contrast, the maturity behaviour, as an agriculturally relevant parameter, is usually observed in several ways. Information on that characteristic is provided as maturity date at which a certain percentage of leaves have died off: for FR 50% of the leaves, for CZ and DE 80%, and for AT all leaves. Another maturity score results from a rating on the 1-to-9 scale by evaluating when the standard varieties have reached a maturity status „7“, which means more than 80% of dead leaves. Earlier maturity may reduce the potential for invasiveness as, in addition to lower yields, growth factors such as light, water and nutrients would become more available to competing weeds earlier in the season.

In potato, plant size/height/biomass/yield/dry matter are ecologically and agronomically important and indicative for invasiveness and persistence.

After reaching row closure, usually no further surveys of aboveground biomass growth or plant development are included in the VCU test.

From then on, regarding biomass production, the focus is increasingly on tuber formation. In FR, the speed of tuber formation is determined in separate trials by determining the weight of the tubers after 80 days of vegetation period in defined size fractions: smaller or larger than 35, and larger than 50 mm. According to the Czech VCU protocol, the total weight of tubers is determined at regular intervals. In DE and AT, early harvest (60-70 days after emergence) and harvest at maturity are foreseen for varieties of the very early maturity group.

A key criterion for plant size/height/biomass/yield/dry matter is tuber yield at maturity, which is measured according to all VCU protocols. Likewise, the determination of dry matter content or starch content is provided in all five countries.

Within the mature plant cycle, seed dispersal ability/seed shatter ability is considered relevant for invasiveness and persistence. Potato plants may show a tendency to form berries with high seed production. Each berry may contain up to hundred small seeds with a thousand seed mass of about 0.75 g.

The berries remain in the field. Some varieties also drop the berries before the tuber harvest. Under favourable weather conditions, volunteer plantlets may emerge in the next season. In general, however, the emergence of potato volunteers from residual tubers is considered much more likely than from seeds. Plants from tubers are more competitive. Although the occurrence and number of berries are cultivar-dependent, these characteristics are not surveyed in the VCU tests with view on their limited agricultural relevance. However, the CPVO-protocol for the Distinctness, Uniformity and Stability (DUS-test) includes the characteristics "frequency of inflorescences” and “size inflorescences", visually assessed and rated on a 5-step scale (1,3,5,7,9) or 3-step scale (3,5,7).

Tuber survival characteristics are deemed ecologically and agriculturally critical for invasiveness and persistence of potato plants. Due to market and processing industry requirements, often a minimum tuber size is demanded. Undersized potatoes are often left in the field. Mechanical precautions on the potato harvesters such as pinch rolling are implemented to reduce the survivability of these residual tubers and the occurrence of volunteers in the following crop. However, their effectiveness is influenced by the amount of stones in the field. In addition, mild winters counteract natural elimination through frost damage. At least 50 hours of frost (product of sub-zero temperatures and duration of exposure) are required to eliminate tubers during the winter months. The occurrence of volunteers in next season is not assessed within the VCU.

Toward the end of the life cycle stage of „mature plant“, determination of the tuber yield, and investigations on tuber size/morphology and grading are planned in all five countries. Corresponding parameters of agronomical importance are total yield and partial yields of the various tuber size classes, the hundred-tuber mass, or ultimately the number of tubers per plant. Potato varieties can be described as being more likely to form large tubers or small tubers. The production of higher amounts of small tuber could promote their invasiveness.

Another parameter influencing tuber survival can also be seen in the susceptibility to mechanical damage. The assessment of this variety-dependent characteristic is requested in all analysed VCU protocols. Damaged tubers are potentially exposed to a higher risk of spoilage. Therefore, susceptibility to mechanical damage is undesirable in potato production but is expected to have a reducing effect on the invasion potential and persistence.

In AT, susceptibility to mechanical damage is assessed on a sample of 50 tubers after a six-week storage period by classifying tubers as undamaged, slightly or severely damaged based on the depth of the injuries.

The CZ protocol differentiates in a 10-kg sample of tubers between undamaged, slightly, medium and severely damaged tubers similarly by the depth of the injuries. The tuber weights in the respective classes are determined.

In DE and FR, provocation methods are applied to determine the susceptibility to mechanical damage of potato varieties. Additional sieve chain passes (DE), a standardized drop test or pendulum blows against the tubers at different temperatures and storage times (FR) provide detailed results on variety differences in this characteristic. The number of corked injuries on a 50-tuber sample are counted after peeling.

Primary and secondary dormancy behaviour of tubers is rated as one of the major factors influencing invasiveness and persistence from an ecological and agronomic perspective. Varieties the tubers of which do not germinate during storage are desirable, since a pre-growth of potato tubers produces too long shoots before a targeted pre-sprouting phase. The shoots often break off during the cultivation process and can thus impair the sprouting power of the freshly laid tubers in the field. The VCU protocols of all 5 countries provide for the observation of premature shoot formation in the potato store. For this purpose, the assessment focusses on the appearance or the length of the shoots after a certain storage period, partly supplemented by the survey of respiration losses.

Assessment of tubers that are undersized and remain in the field

High proportions of small tubers increase the number of residual tubers, which, as undersizes, are not recorded at harvest or are returned to the ground by the separating devices of the harvesting machine. They thus increase the potential for volunteer plants in the following year. Variety-specific size class distribution provides an important indication of the occurrence of undersizes. In plant breeding (VCU) methods are available for the determination of tuber size class distribution (mostly large - medium – small). The determination of the size class proportions by means of square sieves (e.g. in Austria: Large >60 mm, Medium >35 mm, Small <35 mm) can be determined on the entire plot yield or on a sufficiently large subset (at least 10 kg/plot). For varieties with long-oval or long-shaped tubers, the large sieve is designed with a narrower mesh (55 mm).

Other parameters directly or indirectly related to tuber size are the hundred-tuber mass of the variety or the number of tubers per plant. To determine the hundred-tuber mass, an amount of about 6 kg of tubers is taken from each of the harvested material from at least two plots, taking into account the size class distribution. The samples are weighed exactly and the tubers are counted for the calculation of the hundred tuber mass (BAES 2015b).

Possibilities to improve assessments based on state-of-the art methods

A new methodological approach for determining size class distribution is the use of RGB cameras with measurement of tuber length and width (Si et al., 2017; Si et al., 2018; Neilson et al., 2021). The image-based data achieved satisfactory alignment with conventionally determined tuber dimensions and calculated length/width ratios. These methods can be used for breeding for tuber shape or for rapid and sufficiently accurate determination of tuber dimensions. However, the transformation of these results to shares of specific tuber size classes has not (yet) been figured out. Long et al. (2018) used a RGB-D technique to determine tuber volume with about 3-fold smaller prediction error for regular tuber shapes. Hassankhani & Navid (2012) used an image processing system based on CCD cameras for sorting by size classes and quality aspects (tuber health). The image processing system was tested on pre-sorted samples and provided results that were 100% consistent with the test sample data in terms of size distribution and 89 to 100% consistent in terms of diseased tubers.

Environmental interactions at all life cycle stages

Biotic stress (naturally occurring insects and pathogens)

Pathogen response is estimated ecologically and agronomically relevant to GMP invasiveness and persistence across all life cycle stages. The potato plant has a large number of biotic antagonists. In literature, phytoplasmas and wide range of viral, bacterial, and fungal diseases affecting potato are described.

Among virus diseases, potato leaf roll virus (PLRV), potato virus X (PVX), potato virus Y (PVY) and potato virus YNTN are listed for observation in all VCU protocols. In addition, potato virus A (PVA), potato virus M (PVM) and potato virus S (PVS) are mentioned according to the Belgian and Czech VCU protocols.

Field surveys focus on visual observation of the symptoms and the number of infested plants per plot for the relevant viruses. The Czech and Austrian protocols also provide for the survey of mixed infections. Verifications of virus occurrence demand laboratory work.

In FR, resistance against potato virus Y (PVY) infestation is observed in small plot trials with 15 plants per plot in two replications, the border rows of which are planted with virus-infected tubers. To analyse virus attack, three tubers are harvested from each plant. The tubers are pre-germinated from mid-December, and a piece of tissue containing one germinated eye is removed and individually placed in horticultural germination frames. The thus single-shoot plants are visually inspected for virus symptoms or analyzed by an ELISA test. As a result, the number of diseased plants, their total number and the percentage of virus infection are recorded. To test for PMA and PMX virus infections, shoots are grafted onto healthy plants. The reaction of the variety is assessed visually based on virus symptoms and in the laboratory by means of an ELISA test.

Among the bacterial diseases, the occurrence of blacklegged plants (Pectobacterium spp. and Dickeya spp.) and common scab (Streptomyces scabies) on the tubers play are of agronomical importance. Blacklegged plants are counted in the field plots. After harvest, the scab infestation areas of the tubers are rated on a 9-step scale in AT and CZ. In DE, the infested area is visually assessed in a 50-tuber sample per plot or in a 100-tuber sample per variety. FR calculates an index using a 100-tuber sample/plot for assessing the numbers for of not, slightly, medium, heavy and very heavy infested tubers, respectively.

The investigated VCU protocols include numerous fungal diseases. Quite predominantly, the surveys are focussed on observations of Phytophthora infestans on leaves as the late blight disease or on tubers as tuber brown rot.

Fungal diseases of the potato plant: According to the Austrian VCU protocol, visual assessment of the late blight infested plants in the field plots is mandatory from the first occurrence on the 1 to 9 scale with 1=slightly and 9=highly infested.

In the German protocol, late blight infestation is to be recorded on the scale from 1 to 9 upon the very first occurrence of symptoms in the trial, and a second time when susceptible varieties show the highest levels of infestation.

Similarly in CZ, field observation of late blight infestation (9 to 1 scale, here 1=highly infested!) starts with the first occurrence followed by further ratings at the beginning of each monthly decade until the shoots have died.

In the Belgian VCU protocol, leaf infestation is visually scored on a scale of 0 to 10 on untreated plots, with 0 representing complete destruction of foliage. The survey is carried out several times. The area included under an infestation curve obtained in this way is ultimately decisive for the evaluation.

According to the French protocol, the untreated candidate observation plots are surrounded by a susceptible cultivar to increase the infection pressure. From the first appearance of late blight spots, weekly surveys are conducted until complete death of the leaf apparatus. The increasing percentage of dead foliage is assessed by visual observations. The area under an infestation curve resulting from these scores and the corresponding observation times (in days) indicates the varieties' resistance behaviour.

Other relevant diseases observed on the potato plant are early blight (Alternaria solani), black dot (Colletotrichum coccodes), Verticillium-wilt (Verticillium alboatrum) or violet root rot (Rhizoctonia solani). The plant reaction in the field towards these fungi is rated on the 9-step scale or by counting diseased plants per plot.

Fungal tuber diseases: In AT, brown rot infestation on tubers is assessed based on a sample of 50 tubers after a six week storage by rating on the 1-to-9 (9 = highly infested) scale.

In BE, samples of 50 tubers from plots of untreated trials are stored for a period of three weeks under favourable conditions for infection and are then checked for the number of brown rot-infested tubers. These assessments are repeated after some days. Following a further fortnight period, the total number of diseased tubers is recorded.

In CZ, tuber samples are analysed for brown rot after harvest, after an interim storage and after storage over winter. Parameters recorded are the weight of infected tubers and the total sample weight.

In FR, the number of tubers infested by late blight is recorded in plots specially planted for this purpose (2-rowed with 10 plants per row). Obviously non-infested tubers of these plots are stored for further three weeks and the assessment for brown rot infestation is then repeated.

The occurrence of other fungi on the tubers is observed by rating on a 1-to 9-scale (AT), or by weighing the infested tubers, the total sample (CZ) and the total plot yield. These assessments include black pit (Alternaria alternata), gangrene (Phoma foveta), fusarium dry rot (Fusarium spp.), powdery scab (Spongospora subterranea), silver scurf (Helminthosporium solani), black scurf (Rhizoctonia solani) or watery wound rot (Pythium ultimum). In this way, relative infestation values can be calculated.

Concerning pests, the VCU protocols provide for control measures in accordance with the recommendations of good agricultural practice. Aphids are usually not treated, especially not in trials for a specific assessment of the susceptibility towards virus diseases.

In general, damage to the plants is recorded on the 9-step scale, if necessary. These surveys are necessary in order to have sufficient data on pests for the evaluation and plausibility check of the other trial results.

Abiotic stress

Abiotic stress factors such as late frost, cold temperatures, water scarcity or hail are relevant from an agronomical and ecological point of view. Corresponding observations often follow the 9-step scale. However, the reaction of varieties to abiotic stress strongly depends on the physiological stage, in which the adverse environmental conditions occurred.

The above-mentioned VCU protocols currently do not include a specific survey for drought tolerance. However, VCU tests usually have to cover all relevant cultivation regions in which a certain crop species is potentially cultivated in practice. Following this precondition may result in the need to include drier sites in the test network. Thus, conclusions about the drought tolerance of potato varieties can be drawn at least to some extent from their yield performance at drought-prone locations – keeping in mind the dependency of the variety reaction from the timing of the drought stress impact.

Further experimental approaches could be trial designs with unirrigated and irrigated plots at locations with expectable shortage in natural water supply or the use of phenotyping platforms. In the latter case, it is possible to control the amount of natural precipitation by trial design. One of the main criteria for drought tolerance should be the variety specific decline in yielding capacity due to lack of water supply.

Conclusions

- In potato, the visually performed VCU methods capture very well the plant establishment and the subsequent plant development phases (growth rate/duration (period)/development) until row closure.
- The rapidity of canopy closure or in potato row closure is assessed either by indicating the length of time required for achieving this status or by assessing the extent of canopy closure at a given time. In the second case, repeated surveys can provide good documentation of growth progress.
- On a phenological basis, flowering data are recorded in some, and ripening data are recorded by all protocols. Yield build-up is also well documented by methods that track tuber development, and provide for tuber size analysis in addition to total harvest (plant size/height/biomass yield/dry mater).
- An extensive range of methods is available for assessing disease incidence. Viral, bacterial and fungal diseases are observed, visually or with laboratory confirmation (viroses). Visual

surveys are based on specific infestation criteria such as diseased plants, infestation percentages, volume percentages (tubers), or assignment of symptom expression on the 9-level scale (not just 5 levels!).

- Importantly, protocol regulations exist that provide for provocation of symptom occurrence in separate trial plots (e.g. viroses, common scab, late blight, cyst nematodes, mechanical damage susceptibility, premature germination on storage).
- The following characteristics are not examined in standard VCU tests:
  - floral biology (pollen production, attractivity for pollinators)
  - surviving ability of residual tubers, mostly undersized, but for which tuber size analysis at least allows some conclusions on the relevance of this issue
  - surviving ability of seeds
  - plant emergence from seeds
  - dormancy behaviour of seeds

For further details, please refer to Table 19.

Tab. 19: Potato – VCU methods and assessment of invasiveness, persistence and environmental interaction.

Life cycle stage	Characteristics	Suitable methods and parameters from VCU protocols
Seedling	plant establishment	Crop stand after emergence (visually, 1-to-9 scale)
	growth rate/duration	Youth development (visually, 1-to-9 scale)
	early ground cover	Percentage of ground cover between rows (visually, %)
Mature plant	plant vigour	Youth development (visually, 1-to-9 scale)
	growth rate/duration (period)/development	Youth development (visually, 1-to-9 scale) Extent of canopy closure, (visually, 1-to-9 scale)
	plant size/height/biomass/yield/dry matter	Tuber yield (kg/plot), including grading
	flower biology/time to flowering or maturity/flowering period	Beginning of flowering and maturity (dates) Days to flowering, days to maturity, (days since planting)
	fertility/vernalisation requirement	No VCU method
	attractiveness to pollinators	No VCU method
	pollen shed/ viability/ compatibility/morphology	No VCU method
	Seed dispersal ability/seed shatter ability	No VCU method Proposal: Assessment of occurrence and number of berries (DUS method)

Life cycle stage	Characteristics	Suitable methods and parameters from VCU protocols
	Tuber survival and characteristics (number/size/bud number	Some VCU parameters give information on these parameters: e.g., share of small sized tubers
	Vegetative (horizontal) growth (plant/colony diameter)	-
Seed	seed size/morphology/moisture	Hundred tuber mass (kg),
	seed number/weight	Tuber yield, tubers per plant
	seed longevity/survival	No VCU method
	seed germination characteristics	No VCU method
	primary/secondary dormancy	Appearance and length of shoots during storage
	volunteers in subsequent crops	No specific VCU method VCU assesses tuber size distribution as a characteristic potentially facilitating the occurrence of volunteers
All stages	response to naturally occurring insects and pathogens (biotic)	Pests: insects, sucking, feeding damage, visually, 1-to-9 scale Diseases (viruses, phytoplasmas, bacteria, fungi: Occurrence of symptoms, visually, 1-to-9 scale. Provocation of infestation for some very important viruses (PVY) and fungal diseases (late blight)
	response to abiotic stress (heat, drought, and excess of water)	Late frost damage: frost symptoms, visually, 1-to-9 scale Hail: extent of canopy damage, visually, 1-to-9 scale Drought stress: yield figures and size grading, kg/plot, tuber size grading (%)

C.5 Soybean (*Glycine max*)

In soybean, the phenotypic assessment within the VCU test focusses on plant establishment, rapidness of juvenile growth, susceptibility to diseases, standing ability, maturity behaviour, yield, yield structure, and relevant quality parameters. Protocols for VCU testing in soybean were available from Austria, Belgium, Czech Republic, France and Germany, for corresponding references see Table 18.

Seedling

In the seedling life cycle stage, plant establishment is highly indicative from the ecological and agronomical perspective for invasiveness and persistence.

All investigated VCU protocols ask for a description of variety performance in this early stage. Characteristics noted down are the „date of emergence“(CZ, DE and FR), the „status of the crop stand after emergence“ (all five countries), “early stand counts” (FR), or calculating the total plant number per plot on the basis of counting on subplot areas 6 to 8 weeks after sowing (BE). Another significant parameter is the extent of ground coverage (AT, BE, DE and FR) by the growing crop or similar assessments such as “growth density and evenness”. For more in-depth tracking of the growth progress, these surveys can be repeated at appropriate time intervals.

Rapidly emerged and gapless soybean stands have better competitive ability against weeds. Assuming the same high seed quality, genotypes with a faster germination process generally have advantages in establishing stands outside the cultivated areas, although the establishment potential of soybeans outside agricultural fields must be considered low.

Mature plant

At the beginning of the life cycle stage mature plant, the characteristic growth rate/duration/(period)/development is deemed ecologically and agronomically essential for supporting invasiveness and persistence of soybean plants.

Parameters in VCU tests that describe the response of a variety at this growth stage are juvenile development, focussing on the height of the crop and the above-ground plant mass (visually assessed on the 9-step scale), the extent of row closure on a given date (9-step scale), or the date of canopy closure between rows. The German protocol asks to assess "deficiencies in the crop stand at flowering time" (visually, 1 to 9 scale).

Here, repeated data collection, carried out for all varieties (plots) at the same time, allows a more detailed monitoring of the crop development dynamics.

Good juvenile development forms the basis for a good plant constitution when entering the generative phase, signalling the beginning of the growth period when soybean plants are very sensitive to water deficiency. VCU protocols provide for monitoring of plant characteristics flower biology/time to flower or maturity/flowering by requiring to observe the "beginning of flowering" and, in some cases, the "end of flowering", the "time of maturity" (dates), or a contemporaneous survey of the "maturity status of all varieties" (rating on the 9-step scale) during the ripening phase. The German protocol requires to record "deficiencies in the crops before harvest" (visually, 1 to 9 scale). Again, fast-growing genotypes may have advantages in terms of competitiveness against other plants in the given environment.

Monitoring of pollen production capacity, pollen shed, wind dispersal or frequency of pollinator visits is not part of a VCU testing program. Similarly, further pollen characteristics such as viability/compatibility/morphology are not analysed in the VCU.

As a characteristic indicative of plant size/height/biomass/yield/dry matter, the measurement of "growth height" is a survey consistent in all analysed VCU protocols. This rating is usually carried out only once after length growth is completed. Throughout the growing season, varieties with at least average or higher growth rates are seen as more competitive against weeds than short-grown cultivars. This fact is especially important for organic farming. Repeated measurements of stand height at regular intervals, starting around the onset of flowering until the end of longitudinal growth, are not included in VCU protocols. These measurements would be technically easy to implement and would provide useful information on the dynamics in aboveground mass formation. The Belgian protocol calls for plant height measurement already at the beginning of flowering and a second time before harvest.

Yield formation is the most relevant parameter for describing crop productivity. As the core feature of performance testing, "grain yield" is measured on all trial sites, including the standardisation to a defined moisture content. Basically, high capacities in kernel yield or in dry matter production may also be seen as factors contributing to invasiveness in terms of the number of kernels per plant or generally of assertiveness of the whole crop, e.g. in case of plant emergence on feral sites.

Regarding seed dispersal, the parameter “pod shattering” is well established in VCU testing with the parameter “percentage of opened pods”, observed by visual assessment. Differences in “pod firmness” have an influence on the occurrence of seed losses and thus on the amount of seeds left on the field. Early varieties, in particular, have their ripening period still in the warm season. Dry and very warm periods favour bursting of mature pods.

Severe “lodging” may also contribute to grain losses during harvest and thus potentially increases the residual seed quantities on the field. The assessment of “standing ability” (9-step scale) is a general rule in VCU-soybean trialling. Usually, lodging is to be observed at flowering time and at harvest time, or generally from the first occurrence onwards.

Seed

The determination of “seed moisture at harvest” and grain size differences in the form of “thousand grain mass” are collected in all VCU protocols.

However, investigations of dormancy, seed longevity or seed germination characteristics are not within the scope of VCU testing.

Root

By default, plant root studies are not performed in VCU due to resource constraints.

All stages

Varieties with tolerances to biotic and abiotic stressors can be expected to have advantages in terms of invasiveness and persistence.

Biotic stress (naturally occurring insects and pathogens)

Soybean can be attacked by a number of relevant pests, viral, bacterial and fungal pathogens (Hartman et al. 2015). Response to naturally occurring insects and pathogens (biotic stress) is estimated to be ecologically and agronomically important for invasiveness and persistence of soybean plants.

The VCU protocols refer to pests occurring in Central Europe and provide for the survey of viral, bacterial and fungal diseases using a 9-step scale.

Pests

Among the pests mentioned in the VCU protocols are wireworms (*Agriotes* spp.), bean seed fly (*Delia platura*), aphid species, crane flies (*Tipula* spp.), leaf beetle species (*Sitona* spp.) or spider mites (*Tetranychus urticae*).

In soybean VCU testing, field trials usually are not treated with insecticides, provided that a certain level of damage is not exceeded. The natural occurrence of the pests is surveyed and rated on the 9-step scale by focussing on the plant damages. Monitoring of pests themselves is only foreseen for low fugitive species (e.g. aphids).

Impairments due to birds or wildlife are surveyed in a similar manner. Some VCU protocols provide for instructions in order to avoid areas with expected higher frequency of wildlife.

Diseases

The following diseases are mentioned in the VCU protocols:

Viruses: Soybean mosaic virus (SMV): This virus appears from the bud stage onwards. Its occurrence is assessed according to the 9-step scale (all five countries, if applicable)

Bacterial diseases: Bacterial blight (*Pseudomonas savastanoi* pv. *glycinea*), wild fire (*Pseudomonas syringae* pv. *tabaci*) or bacterial pustule (*Xanthomonas axonopodis* pv. *glycines*).

Fungal diseases: Anthracnose (*Colletotrichum truncatum*), *Phomopsis* seed decay (*Diaporthe*/*Phomopsis* complex with *Diaporthe longicolla* often prevailing), *Phylosticta* leaf spot (*Phylosticta sojicola*), *Phytophthora* root and stem rot (*Phytophthora sojae*), downy mildew (*Peronospora manshurica*), *Sclerotinia* stem rot (*Sclerotinia sclerotiorum*), brown spot (*Septoria glycines*), *Tielaviopsis* root rot (*Thielaviopsis basicola*).

Disease surveys get importance from the time of occurrence of relevant infestation levels, or at significant differentiation between varieties. The recording is usually repeated when the degree of infestation changes during the course of the vegetation period. The natural occurrence of the diseases is surveyed and rated on the 9-step-scale. Specific experimental settings to provoke disease occurrence are not included in the soybean VCU protocols.

Abiotic stressors

For abiotic stressors, surveys for damage due to late frost, responses to cool temperatures or herbicide damage are described on the 9-step scale.

Another variety characteristic with regard to harvest losses is the height of the lowest set pods, the survey of which is provided for in most VCU protocols. If length growth is hampered, e.g. by cool weather conditions in early plant growth or by drought in general, the first pods may be set so low that they are almost touching the ground. Grain losses are practically unavoidable here, despite technical improvements to harvesting machinery.

Yield growth in soybean takes place during the warmest months of the year. This fact underlines the importance of drought tolerance. Drought-tolerant genotypes are still able to realize a better yield performance under water scarcity – inside and outside the field. Drought tolerance thus would also contribute to both invasiveness and persistence.

The above-mentioned VCU protocols do not currently include a specific (artificially induced) survey for cold or drought tolerance.

Conclusions

- In soybean, VCU methods provide good coverage of plant establishment, including surveys on emergence, completeness or incompleteness of the stand, rapidity of juvenile growth, and stand or row closure. Repeated surveys would provide good documentation of growth progress.
- Concerning phenological characteristics, flowering data are recorded according to some protocols, whereas maturity data are recorded according to all protocols.
- Biomass production is also well documented by measuring growth height from flowering and grain yield based on a standardized crop moisture, grain size, actual plant numbers (plant size/ height/ biomass/yield/dry mater).
- Premature grain losses are recorded as grain failure or pod burst according to the 9-step scale, based on visually assessed losses.
- In the case of pests, the symptoms of damage are recorded if necessary.

- Viral, bacterial and fungal diseases are observed on the basis of assignment of symptom manifestations following the 9-level scale (not only 5 levels).
- Among abiotic stress factors, the effects of cold weather phases or herbicide applications are observed.
- Not studied are
  - floral biology (pollen production, attractiveness to pollinators)
  - survivability of volunteer grains under field conditions or
  - occurrence of volunteer plants
  - yield performance under artificially induced drought stress

For further details, please refer to Table 20.

Tab. 20: Soybean – VCU methods and assessment of invasiveness, persistence and environmental interaction.

Life cycle stage	Characteristics	Suitable methods and parameters from VCU protocols
Seedling	plant establishment	Date of seedling emergence (date), early stand counts, crop stand after emergence (visually, 1 to 9 scale), number of plants/plot calculated 6 to 8 weeks after sowing on the basis of central plot counting
	growth rate/duration	Extent of early ground cover (visually, %)
	early ground cover	Extent of ground coverage (visually, %)
Mature Plant	plant vigour	Youth development, focussing on height of the crop and aboveground plant mass (visually, 1 to 9 scale), youth development (visually, 1 to 9 scale)
	growth rate/duration (period)/development	Youth development (visually, 1 to 9 scale), deficiencies in crops stand flowering (visually, 1 to 9 scale),
	plant size/height/ yield/ dry matter	Plant height at beginning of flowering (cm), plant height before harvest (cm), rapidness of plant height growth (visually, 1 to 9 scale), kernel yield (kg/plot)
	flower biology/ time to flowering or maturity/ flowering period	Beginning and end of flowering and date of maturity (dates), days to flowering, days to maturity (since planting), maturity behaviour (visually, 1 to 9 scale), deficiencies in the crops just before harvest (visually, 1 to 9 scale)
	fertility/vernalisation requirement	No fitting VCU method in investigated protocols
	attractiveness to pollinators	No fitting VCU method in investigated protocols
	seed dispersal ability/seed shatter	Seed losses per area (visually, 1 to 9 scale), occurrence of broken pod on the plants (visually, 1 to 9 scale), height of the lowest set pods (cm)
Seed	seed size/morphology/ moisture	Thousand seed mass (g), moisture content at harvest (%)

Life cycle stage	Characteristics	Suitable methods and parameters from VCU protocols
	seed number/weight	Thousand seed mass (g), calculations based on yield, plant numbers and thousand seed mass possible
	seed longevity/survival	No fitting method in investigated protocols
	seed germination characteristics	Seed germination test, (%)
	primary/secondary dormancy	No fitting method in investigated protocols
	volunteers in subsequent crops	Parameters influencing the occurrence of volunteers: Seed losses per area (visually, 1 to 9 scale), occurrence of broken pod on the plants (visually, 1 to 9 scale), height of the lowest set pods (cm) No fitting VCU method in investigated protocols for establishment of volunteers
Root		No fitting VCU method in investigated protocols
All stages	Biotic stress, response to naturally occurring insects and pathogens (biotic)	Insects: Sucking, feeding damage, visually, 1-9 scale wireworms, bean seed fly, aphid species, crane flies, leaf beetle species, spider mites, Diseases: Occurrence of symptoms, visually, 1-9 scale, soybean mosaic virus, bacterial blight, bacterial pustule, anthracnose, *Phomopsis* seed decay, *Phylosticta* leaf spot, *Phytophthora* root and stem rot, downy mildew *Sclerotinia* stem rot, brown spot, *Thielaviopsis* root rot
All stages	Abiotic stress (heat, drought, excess of water)	Observations visually, 1-9 scale: Frost symptoms, reaction to cool temperatures, reaction to herbicide applications

C.6 Oilseed rape (*Brassica napus*)

In terms of Value of Cultivation and Use (VCU) testing of oilseed rape, the phenotypic assessment focusses on plant establishment, plant development in autumn, winter survival, the rapidity of spring growth, susceptibility to viral and fungal diseases, standing ability, maturity behaviour, yield, yield structure, and relevant quality parameters. The analysed protocols for VCU testing for oilseed rape are from Austria, Czech Republic, France and Germany, for corresponding references see Table 18.

Seedling

Within the seedling life cycle stage, plant establishment is regarded as of high agronomical relevance for a crop´s invasiveness and persistence.

All investigated VCU protocols ask for a description of variety performance in this early stage. Characteristics noted down are the „date of emergence"(CZ, DE), and the „status of the crop stand after emergence"(AT, CZ, DE, FR), respectively. Later on in autumn observations target „missing crops at the five-leaf stage"(percentage; CZ) or „plant density in autumn"(FR). Further investigations towards the end of the vegetation period aim at „deficiencies in the crop stand before winter", rated on the 1-to-9 scale (AT, DE). Winter oilseed rape varieties showing sufficient plant establishment and development in autumn provide good preconditions for high yield performance and consequently for higher invasiveness and persistence. This would also hold true for feral plants from these varieties.

After the winter months, resilience towards winter damages and recovering ability are seen most relevant indicators for invasiveness and persistence. In VCU testing, corresponding assessments are tried for the extent of leaves or even plants lost due to frost impact by ratings following a 1-to-9 scale (AT, DE), percentages (CZ), or assessing vigour at winter end (FR, 1-to-9 scale).

Mature plants

The growth rate prior to the onset of flowering potentially promotes invasiveness and, from the agronomical point of view, lays the foundation for good yield establishment. The characteristic growth rate/duration/(period)/development is captured by parameters describing "earliness and rapidness of shooting" or "vigour after winter" in AT by assessment of „Sprouting" (1-to-9 scale) and later on in the growth period by measurement of the final plant height (in all investigated VCU protocols).

Oilseed rape forms about 70% of its aboveground plant mass from the time it emerges from the rosette stage to the end of flowering. Thus, plant height is very suitable to describe the characteristic plant size/height/biomass/yield/dry matter potential, which is rated agronomical and ecologically highly indicative for invasiveness and persistence

However, in the VCU protocols growth height measurement is usually a one-time survey after the end of flowering. In contrast, regularly repeated measurements of plant height from the onset of longitudinal growth on until the end of flowering would provide accurate information about the course of crop development and allow conclusions that are indicate for the invasiveness potential.

Grain yield is a key characteristic to describe productivity. Good yield performance with consequently very high numbers of seeds per unit area may potentially also result in higher grain losses – as shown in absolute terms. High values in both parameters are considered relevant for persistence and invasiveness.

Regarding flower biology/time to flowering or maturity/flowering period, oilseed rape with its innumerable blossoms over a period of several weeks, is a partial cross-pollinator species and thus prone to vertical gene flow either to plants of other oilseed rape varieties, feral rape plants or compatible wild relatives and weeds. Besides wind pollination, also insect pollination is frequently observed. Therefore, characteristics describing flower biology are deemed highly indicative for both invasiveness and persistence of oilseed rape.

Following the investigated VCU protocols, the date of flowering is recorded as the beginning of the generative phase. Some protocols also ask for the end date of flowering enabling the calculation of the flowering period. However, pollen production, pollen shedding, wind dispersal or pollinator insect frequency studies are not planned within the VCU tests.

The characteristic fertility/vernalisation requirement is classified important and relevant for persistence and invasiveness. Winter oilseed rape requires a certain degree of vernalisation to become sufficiently fertile. The extent of vernalisation needed also depends on the rape variety; however, it is not evaluated in the VCU test.

Oilseed rape flowers exert a strong attractiveness to pollinators, which is a key feature for the interaction of the plant with organisms of the environment and highly indicative for the invasiveness and persistence of the plant. However, observation of this trait is extremely time-consuming if it is to be reliably quantified at the cultivar level as well. VCU tests do not measure differences in the attractiveness of varieties to pollinators.

Pollinators may contribute to a further reasonable pathway for a gene transfer into neighbouring crop stands or when considering possible the flight distances also into cultivated areas far away.

This should be considered in particular as oilseed rape plants do have a reasonable number of wild relatives with which outcrossing is possible. Therefore, characteristics related to pollen shed/viability/compatibility/morphology are important in terms of invasiveness and persistence meaningful parameters that are also indicative of the possibility of wind pollination. Methods from literature should be used and adapted to study these plant characteristics. In the settings of small plot trials, with pollen entering from other varieties from all sides, investigations are tricky and need elaborate precautions. These pollen specific traits are not analysed in a regular VCU testing program.

Herbicide-, insecticide- or drought tolerance and maybe modifications in quality profiles could enhance the competitiveness of GMP in their natural surroundings.

Pod firmness counteracts seed dispersal ability/seed shatter ability, both characteristics that can contribute significantly to unintended seed loss into the field and thus to invasiveness and persistence. Pod burst resistance is determined by the genotype and has been continuously improved in recent years. VCU protocols ask for an assessment of pod firmness either indirectly by visual observation of grain losses per area or in some cases by applying mechanical stresses to the mature pods.

Similarly, differences in standing ability can influence the amount of seed loss, too. Severely lodging plants are prone to incomplete grain pickup by the harvester. Assessment of stand ability is a regular issue in VCU testing.

Seed

In the “seed” life cycle stage seed size/morphology/moisture and seed number/weight are deemed relevant regarding invasiveness and persistence. VCU protocols regularly require to measure seed moisture for yield comparison on a standardized moisture level and of seed size in the form of thousand kernel mass. Seed numbers per plant can be recalculated from the plot yield and the numbers of plant per plot.

Further very indicative seed traits for the invasiveness and persistence potential of oilseed rape crops are seed longevity/survival, seed germination characteristics, primary/secondary dormancy leading to volunteers in the following crops.

Regular VCU trialling deals with none of these characteristics. Variety registration procedures usually take two or three years. Under natural preconditions, this period is much too short for an exhaustive investigation of the above mentioned seed characters. Special methods and treatments are necessary to elaborate conclusive, variety specific results on these topics.

Root

In the VCU test, no investigations are done on the root system.

Environmental interactions at all stages

The response to naturally occurring insects and pathogens (biotic stress) is a plant characteristic ecologically and agronomically important for all life cycle stages to assess the crop's invasiveness and persistence.

Biotic stressors for oilseed rape are numerous. Rapeseed plants interact intensively with pests and can be attacked as early as seedlings have emerged (e.g. by slugs or ground fleas) and throughout the season until harvest (e.g. cabbage pod midge). The insect pests mentioned in the VCU protocols include: cabbage stem flea beetle (*Psylliodes chrysocephalus*), turnip flea beetles (*Phyllotreta atra*, *P*. *cruciferae*), turnip saw fly (*Athalia rosae*), rape stem weevil (*Ceutorhynchus napi*), cabbage stem weevil (*Ceutorhynchus pallidactylus*), turnip gall weevil (*Ceutrohynchus pleurostigma*), cabbage seed weevil (*Ceutorhynchus assimmilis*), rape (pollen) beetle (*Brassicogethes aeneus*), brassica pod midge (*Dasineura brassicae*) and cabbage aphids (*Brevicoryne brassicae*).

In most protocols, eventual occurrence is recorded visually using a 1-to-9 scale, based on the extent of plant damage or on the number of pests present per plant. The usage of insecticides is called for when the interpretability of the trials is jeopardized by the damaging effect. The Austrian and French protocol refer to the relevant damage threshold concepts.

The following oilseed rape diseases are mentioned in the VCU protocols, depending on the country: viruses such as the turnip yellow virus (TuVY), many mycoses like black spot (*Alternaria brassicae*), grey mould (*Botrytis cinerea*), cylindrosporium disease (*Cylindrosporium concentricum*), blackleg disease (*Phoma lingam*), Sclerotinia stem rot (*Sclerotinia sclerotiorum*), verticillium wilt (*Verticillium longisporum*, *V*. *dahliae*) or club root (*Plasmodiophora brassicae*). Blackleg disease, sclerotinia stem rot and verticillium wilt are explicitly mentioned in all investigated VCU protocols.

Unless specified in detail, disease infestations are observed as they occur. In most cases, the assessment uses the nine-step scale. The rating levels are assigned to specific proportions of symptomatic plants with specific degrees of infestation, depending on the disease. Some VCU-protocols line out approaches that are more detailed for the assessment of important stressors:

Viruses

The French protocol defines the procedure for assessment of susceptibility for turnip yellows virus (TuYV). In specific trials, one leave from each of 15 plants per plot is sampled in late autumn and again in spring. Results display the number of virus-infested plants and the virus load.

Fungi

The German protocol asks for infestation surveys for *Phoma lingam* and *Verticillium dahliae*, respectively. Twenty-five stubbles per plot are dug out, grouped according to infestation severity on the 9-step scale and counted.

Other protocols (CZ, FR) distinguish between an infestation with Phoma lingam on the leaves noted down in autumn and an infestation of the plant stems before harvest. In both cases, the number of infested plants and the severity of their infestation form the basis for classifications. The French protocol provides for contamination of the experimental plots with infected stubbles of plants from the season before and infested canola stalks are also used to contaminate experimental plots when testing for susceptibility to cylindrosporiosis.

Abiotic stressors

In winter oilseed rape, cold and frost effects are a commonly surveyed stress factor. Depending on VCU protocols, observations on winter hardiness can be based on plant counts in fall and spring, number of dead plants per plot, and assessment of leaf damage due to cold (1-to-9 scale). The survey of winter damage may also be an integrative assessment of the spring plant stand using ratings from 1 to 9, which includes the percentages of damaged plants and the extent of each type of damage (frostbitten plants and foliar damage). In the Austrian VCU test, additional small plot trials are sown on a site with high sea level altitude for this purpose.

Varietal reactions to other limiting environmental factors, such as drought stress or heat, are covered, e.g. in Austria, by trial locations in the drier growing regions and a region-specific assessment of performance traits (grain yield, oil yield).

Conclusions

- In winter oilseed rape, VCU methods record the course of plant establishment from emergence (surveys of emergence date, condition of the stand after emergence) to before the end of vegetation (plant number before winter, stand deficiencies before the onset of winter).
- Continuation after winter by observation of plant number in spring, vigour after winter, rapidity of pre-flowering development.
- Concerning phenological traits, flowering and maturation data are required in all protocols.
- Biomass production is also well documented by measuring growth height from flowering and by determining grain yield based on standardized crop moisture, grain size, plant numbers (plant size/ height/ biomass/yield/dry mater).
- Premature grain losses are recorded as grain failure or pod burst according to the 9-step scale, based on visually assessed losses.
- Pests are assessed based on natural occurrence using damage symptoms or the number of individuals.
- Diseases are observed based on infestations with assignment of symptom expression levels on the 9-level scale (not just 5 levels!). Specific protocols for susceptibility, Phoma disease and Cylindrosporiosis (with provocation measures) and viruses.
- Abiotic stress monitoring focuses on winter damage.

- Not examined are
    - vernalization requirements (these will be met under local climatic conditions).
    - floral biology (pollen production, pollen viability, pollinator attractiveness).
    - surviving ability of drop-out grains under field conditions
    - occurrence of volunteer plants
    - dormant behaviour of volunteer oilseed rape

For further details, please refer to Table 21.

Tab. 21: Oilseed rape – VCU methods and assessment of invasiveness, persistence and environmental interaction.

Life cycle stage	Characteristics	Suitable methods and parameters from VCU protocols
Seedling	plant establishment	Early stand count (n) Crop stand after emergence (visually, 1-to-9 scale)
	growth rate/duration	Percentage ground cover (visually, %)
	early ground cover	Percentage ground cover (visually, %)
Mature Plant	plant vigour	Percentage ground cover (visually, %) Youth development (visually, 1-to-9 scale)
	growth rate/duration (period)/development	Youth development (visually, 1–to-9 scale) Rapidness of plant height growth (visually, 1-to-9 scale)
	plant size/height/biomass/yield/dry matter	Measurement of plant height not before BBCH 69 (cm)
	flower biology/time to flowering or maturity/ flowering period	Beginning of flowering and date of maturity, (dates) Days to flowering, days to maturity, (days since planting)
	fertility/vernalisation requirement	No VCU method
	attractiveness to pollinators	No VCU method
	pollen shed/viability/compatibility/morphology	No VCU method
Seed	seed size/ morphology/moisture	Thousand seed mass (g), Moisture content at harvest (%)
	seed number/weight	Calculated value form plot yields, plant counts, thousand seed masses (g)
	seed longevity/survival	No VCU method
	seed germination characteristics	Seed germination test (%)
	primary/secondary dormancy	No VCU method

Life cycle stage	Characteristics	Suitable methods and parameters from VCU protocols
	volunteers in subsequent crops	No VCU method
Root	fine root diameter, specific root length (density)/root area, root angel, rooting depth	No VCU method
All stages	biotic stress, response to naturally occurring insects and pathogens (biotic)	Insects: Sucking, feeding damage, visually, 1-to-9 scale Diseases: Occurrence of symptoms, visually, 1-to-9 scale
	abiotic stress (heat, drought, excess of water)	Winter damage: Frost symptoms, visually, countings, 1-to-9 scale Drought: performance test on trial sites with expectable scarcity of water supply

C.7 Maize (*Zea mays*)

In maize, the phenotypic assessment within the VCU test focusses on plant establishment, rapidness of juvenile growth, susceptibility to diseases, standing ability, maturity behaviour, yield, moisture content at harvest and relevant quality parameters. VCU protocols for maize were available from Austria, Belgium, Czech Republic, France and Germany, for corresponding references see Table 18.

Seedling

In the seedling life cycle stage, plant establishment is highly indicative for the crop's invasiveness and persistence potential from the ecological and agronomical perspective.

All investigated VCU protocols require to assess variety performance in this early stage by collecting either the „date of emergence" (75% of plants have emerged, CZ, DE and FR), the „status of the crop stand after emergence"(rating on the 1 to 9 scale; AT, DE) or the number of plants per plot (AT, CZ, DE, FR). Provided that all seeds are of equal quality, genotypes with a faster germination process generally show advantages in establishing feral populations outside the cultivated areas. However, the establishment potential of maize on such areas is considered rather low.

Maize as a thermophile crop is sensitive to cool temperatures in the juvenile stage. Symptoms like leaf brightening, growth stagnation or anthocyanin discolorations reveal such negative influence. VCU protocols of Czech Republic, Germany or France explicitly ask to assess variety reactions to the cold (ratings on the 9-step scale). Low susceptibility to cold stress potentially constitutes an advantage in terms of the crop's invasiveness and persistence.

Mature plant

In the weeks following the seedling stage, the growth rate/duration/(period)/development is deemed ecologically and agronomically important for the invasiveness and persistence of maize plants. The crop observations in all five VCU-protocols focus on the rapidness in development of the young plants, which is rated on the 1 to 9 scale at the 6 to 7 leaves stage. These visual youth development surveys comprise plant height, leaf number and differences in aboveground plant mass in a rating on the 9-step scale. For a more detailed observation of early growth over a longer period, existing observation methods may be applied repeatedly. Rapid juvenile growth causes a higher competitive potential also against weedy plants, and contributes to higher invasiveness.

Monitoring the growth rate/duration/(period)/development is continued by recording deficiencies in the crop stand after the end of the flowering period (DE).

A VCU parameter associated with plant size/height/biomass/yield/dry matter is the tendency to form side shoots, a characteristic that is rated on the 1 to 9 scale (AT, CZ and DE). Particularly frequent when plants have more space (e.g. marginal plants) and good nutrient supply. Side shoots are undesirable in cultivated crop stands as they hardly contribute to yield performance. In addition, plants with side shoots require more space in their immediate surroundings than those without.

Plant height is a key parameter in maize VCU testing and, together with grain yield, provides good indications of genotypic differences in plant size, biomass and yield potential. Surveys for both parameters are standard routine in VCU testing, and pronounced expressions in these parameters contribute to the crop's persistence and invasiveness potential.

In VCU tests, measuring growth height is a standard observation. However, this survey is usually carried out only once in the season, ideally in the period from the end of flowering until harvest. Repeated measurements at regular intervals beginning, e.g., before the onset of flowering until the end of length growth would provide additional information on the dynamics of aboveground mass formation.

In maize, yield formation is the core feature in performance testing and well suited to describe crop productivity. Thus, grain yield – standardized to a defined moisture content – is measured on all trial sites. High yield capacity may also contribute to persistence and invasiveness, e.g. in case of plants on feral sites.

Crop stand monitoring is completed towards the end of the vegetation period of maize by noting down the ratings (9-step scale) for health status of the crop before harvest (FR).

The relevant characteristic flower biology/time to flowering or maturity/flowering period can be assessed by noting down the dates for the appearance of male and female flowers (silking). Recording the date of silking is a standard observation in all investigated VCU-protocols. Date of silking is a first indication of a variety's ripening time requirement. In all considered protocols, data collection to assess ripening behaviour includes the harvest date of the trial and the variety-specific harvest moisture as an essential criterion for estimating the ripening time requirement. In addition, leaf maturity surveys (AT, CZ) are used to describe the stay green effect.

However, monitoring of pollen production capacity, pollen shed, wind dispersal or frequency of pollinator visits are beyond the scope of VCU-testing. Similarly, further pollen characteristics such as viability/compatibility/morphology are not analysed in the VCU tests.

Seed

The determination of moisture at harvest is a standard measurement in in all investigated VCU protocols, as this characteristic is crucial for an assessment of the ripening time requirement.

Seed size differences are assessed by the differences in the thousand kernel mass and are asked for in the Czech and German VCU protocol.

However, as already mentioned in the general comments above, no surveys concerning dormancy, seed longevity, seed germination characteristics are conducted in VCU-testing. Similarly, no recording of the occurrence of maize volunteers in subsequent crop is included in the VCU test programs.

Root

By default, plant root studies are not performed in VCU tests due to resource constraints.

All stages

Biotic stress (naturally occurring insects and pathogens)

A reasonable number of pests and, in particular, fungal pathogens can occur on maize plants The response to naturally occurring insects and pathogens (biotic stress) is estimated to be ecologically and agronomically important for the invasiveness and persistence potential of maize plants.

With the exception of the Belgian VCU protocol, all other protocols studied provide for control measures when necessary, following the damage threshold concept. For maize, seed dressing is common and a standard routine for the seeds used for establishing the VCU trials. Nevertheless, approaches to observe damages by pests are available in VCU protocols for frit fly (*Oscinella frit*), corn borer (*Ostrinia nubialis*), seed corn maggot or bean seed fly (*Dehlia platura*), wireworm (*Agriotes* spp.) corn rootworm (*Diabrotica virgifera*) and aphids.

The Czech VCU protocol provides for rather detailed information for pest monitoring, based either on counts per plot (or plant row) or classifications on the 9-step scale. However, also the latter ratings are based mostly on the proportions of infested plants. The Austrian protocol stipulates that the presence of pests should be recorded, if applicable. Upon occurrence, the extent of damage is observed and assessed on the 1-9 scale. In the case of the European corn borer, the infested cobs in the left border row of the rour-rowed plots are counted. The Austrian protocol explicitly mentions wireworms, corn borers or frit flies in maize. In DE and FR, pest surveys focus on corn borer infestations and, if applicable, frit fly infestations. The surveys for corn borer infestation on the cob are usually carried out together with the observation of Fusarium spp. infection on the defoliated cobs.

The examined VCU protocols mention the following maize diseases:

Viruses: Barley yellow dwarf virus (BYDV), maize dwarf mosaic virus (MDMV), maize rough dwarf virus (MRDV). Only the Czech VCU protocol explicitly requests to observe the occurrences of virus diseases following the 9-step scale.

Bacterial diseases: Bacterial stock rot (*Pectobacterium carotovorum*, *Dickeya zeae*), Holcus spot (*Pseudomonas syringae* pv. *syringae*) and Stewart´s bacterial wilt (*Pantoea stewartii*). Observation of bacterial diseases is explicitly requested only in the Czech VCU protocol (infested plants per plot).

Fungi: Corn smut (*Ustilago maydis*), head smut (*Sphacelotheca reiliana*), northern corn leaf blight (*Helminthosporium turcicum*), maize rust (*Puccinia sorghi*), stem and ear fusariosis (*Fusarium* spp.).

The observation of corn smut is demanded in all VCU protocols by counting out the diseased plants per plot. The absolute infestation numbers are partly converted into relative values (BE). Only the Czech protocol explicitly mentions head smut, which is assessed by counting diseased plants. In contrast, all VCU protocols ask for investigations of the occurrence of northern corn leaf blight by ratings following the 9-step scale. In Belgium, infestation is recorded every two weeks from flowering on using a score from 1 to 5, depending on the percentage of leaf area infested. The final score for a variety is based on an “area under disease progress curve”. The recording of an infestation by maize rust (AT and CZ) follows the 9-step-scale.

Considerable effort goes into surveying for *Fusarium* infestation on corncobs as it may result in the production of mycotoxins, a potential risk to human and animal health. For the assessment of natural infestation, the corncobs of a significant number of plants need to be defoliated and analysed in the trial plots. The Fusarium surveys aim at the number of infested cobs per plot or per analysed plant sample of the plot and the extent of infestation. In this regard, he protocols also provide for monitoring of corn borer infestation based on visible feeding damage.

In Austria, corn varieties are artificially infected with a spore suspension in two ways each year in extra trials:

Depending on the flowering time of the varieties, the spore suspension is injected directly into the cobs. To evaluate the variety-specific resistance to the spread of Fusarium in the cobs, the extent of the infested areas (grain number) around the puncture hole is observed on all infested plants before harvest time. In another trial setting, the resistance to infection via the silk is tested by spraying the silk with the spore suspension using a spray bottle. A fogging system supports the infection development. In autumn, the diseased area on the defoliated cobs is evaluated.

Depending on the specific situation, plants resistant against pests or diseases may show an additional advantage concerning competitive potential.

Abiotic stressors

Some VCU protocols provide for the evaluation of the reactions due to cold stress during juvenile growth (CZ, DE and FR) by ratings on the 9-step scale. For Austria and Belgium, these aspects may be seen as at least partly captured by observing differences in the youth development.

Further detrimental growth effects such as damages by late frost events can also be recorded on the 9-step scale in various VCU protocols (explicitly mentioned in AT and DE).

The German protocol asks for the variety reaction towards early stem breaking (green snapping) caused by heavy weather events (broken plants per plot).

Just as in soybean, yield production in maize is going on during the warmest months of the year. Drought-tolerant genotypes would be able to perform better and ultimately yield higher under conditions of water scarcity. Drought tolerance thus would also contribute to both invasiveness and persistence.

Regarding variety tolerance to drought stress, the Czech and French protocols provide for recording drought stress symptoms using the 9-step scale. However, the significance of these observations depends on the seasonal growing conditions in terms of temperature and water supply.

Conclusions

- Similar to soybean, the VCU methods for maize also cover the course of plant establishment in grain maize with surveys of the date of emergence, the number of plants or of the completeness or patchiness of the stand after emergence.
- Further development (growth rate/duration (period)/development) is well covered by surveys on the rapidity of juvenile growth and crop stand observation at the beginning of flowering. Repeated surveys would provide a tighter documentation of growth progress.
- Concerning phenological characteristics, date of cob flowering is covered in most protocols; maturing behaviour is observed by leaf maturity and moisture content at harvest.
- The documentation of biomass production focusses on measuring growth height from the end of flowering, cob set height and grain yield based on a standardized harvest moisture (plant size/height/biomass/yield/dry mater).
- Concerning premature grain losses, broken plants per plot (stems broken below cob set) are counted.
- For pests, the survey is based on the natural infestation via the damage symptoms (number or share of infested plants or assignment rules of the infestation patterns to the 9-step scale, not a 5-step scale).
- The same applies to the survey of disease observations. Provocation tests are used to assess susceptibility to cob fusariosis.
- Regarding the reaction to abiotic stresses, the effects of cool weather conditions, drought stress or herbicide applications are observed by symptom expression (9-step scale).
- Not studied are:
    - surviving ability of volunteer grains under field conditions
    - occurrence of volunteer plants
    - occurrence of primary and secondary dormancy

For further details, please refer to Table 22.

Tab. 22: Maize – VCU methods and assessment of invasiveness, persistence and environmental interaction.

Life cycle stage	**Characteristics**	**Suitable methods and parameters from VCU protocols**
Seedling	plant establishment	Date of emergence (date), crop stand after emergence (visually, 1 to 9 scale)
	growth rate/duration	Tolerance toward cold stress (visually, 1 to 9 scale)
	early ground cover	Youth development (visually, 1 to 9 scale)
Mature Plant	plant vigour	Youth development (visually, 1 to 9 scale)
	growth rate/duration (period)/development	Youth development (visually, 1 to 9 scale), tendency to form side shoots, (visually, 1 to 9 scale)

Life cycle stage	Characteristics	Suitable methods and parameters from VCU protocols
	plant size/height/ biomass/yield/dry matter	Measurement of plant height (cm), cob setting height (cm)
	flower biology/time to flowering or maturity/ flowering period	Beginning of flowering separately for male and female flowers (date), date of harvest (date), days to flowering, days to harvest (days since planting), crop stand after flowering stage (visually, 1 to 9 scale). For maturity: moisture content at harvest (%)
	fertility/vernalisation requirement	No fitting methods in investigated VCU protocols
	attractiveness to pollinators	No fitting methods in investigated VCU protocols
	pollen shed/viability/ compatibility/morphology	No fitting methods in investigated VCU protocols
Seed	seed size/morphology/ moisture	Thousand seed mass (g), moisture content at harvest (%)
	seed number/weight	Thousand seed mass (g)
	seed longevity/survival	No fitting methods in investigated VCU protocols
	seed germination characteristics	Seed germination test, (%)
	primary/secondary dormancy	No fitting methods in investigated VCU protocols
	volunteers in subsequent crops	No fitting methods in investigated VCU protocols
Root	fine root diameter, specific root length (density)/root area, root angle, rooting depth	No fitting methods in investigated VCU protocols
All stages	biotic stress, response to naturally occurring insects and pathogens (biotic)	Insects: Sucking, feeding damage, visually, 1-9 scale Diseases: Occurrence of symptoms (visually, 1-9 scale, numbers of infested plants/plot, in case of repeated recordings area under disease progress curve): Corn smut (*Ustilago maydis*), head smut (*Sphacelotheca reiliana*), northern corn leaf blight (*Helminthosporium turcicum*), maize rust *(Puccinia sorghi*), stem and ear fusariosis (*Fusarium* spp.).
	Abiotic stress (heat, drought, excess of water)	Tolerance toward low temperatures in juvenile growth (visually, 1-9 scale), tolerance towards drought stress symptoms (visually, 1-9 scale), reaction to frost damage(visually, 1-9 scale), greensnapping (broken plants / plot)

C.8 Creeping bentgrass (*Agrostis stolonifera*)

In regards of creeping bentgrass (*Agrostis stolonifera*), the phenotypic assessment of feed grasses as described in Austria, Germany, Belgium, Czech Republic and France follows a mostly similar Value of Cultivation and Use (VCU) protocol across the five countries, with a strong focus being on plant establishment, plant development, plant vigour and biomass formation. However, the assessment of any flowering, seed or root properties is not defined in any of the five investigated protocols. The following paragraphs give a short overview of the described methods in the VCU protocols in regards to feed grasses. Here, the focus lies on characteristics that have been deemed important from an ecological and an agronomical perspective to indicate the persistence and invasiveness of *Agrostis stolonifera*.

In general, collection of VCU data occurs either by means of visual assessment of a trait (where often a rating from 1 to 9 is assigned to the plot), or by measurements of specific phenotypic traits (for example plant height and weighing of the fresh or dried yield). For grasses, due to the high sowing density and therefore high number of shoots per plot, counting of individual plants is not part of the data collection. As it stands, collection of data in regards to the chemical compositions of the seeds via laboratory analysis is not part of any of the five investigated VCU protocols. Solely measuring the crude protein content of the harvested biomass is part of the Austrian VCU protocol.

Most investigated protocols put an emphasis on the “seedling” life cycle stage of the grasses, where multiple assessments applicable for rating plant establishment can be found. This agronomic characteristic is generally assessed by noting down the date of seedling emergence and/or the date of the beginning of vegetation (AT and DE). In the VCU protocols of BE, CZ and FR, the “completeness of emergence” is rated on a scale from 1 to 9, while “deficiency after emergence” (where a rating from 1 to 9 is also given) could potentially also be used for the phenotypic assessment of Agrostis stolonifera plant establishment.

Once the plants have grown out of the “seedling” stage and entered the life cycle stage of “mature plant”, multiple measurements have to be taken for the VCU, which could consequently also be applied to assess the plant’s growth rate/duration (period)/development. In this case, tried methods from VCU protocols could be applied to assess these characteristic, which has been rated as highly ecologically and agronomically relevant concerning a crops’ invasiveness and persistence. One assessment which can be found in the VCU protocols of Austria, Germany and Belgium and which can be used to test for growth rate/duration (period)/development is the “date of ear emergence”, while in the Austrian and Czech protocols methods that specifically assess “youth development” and “rate of growth”, respectively, can be found. Additionally, the plant’s development could potentially also be rated in an indirect way by applying the methods “deficiency at harvest” and “mass formation after cutting” which can be found in the German VCU protocol.

In all investigated VCU protocols, multiple assessments are linked to and have to be taken before or after the cutting of the grass, which generally occurs multiple times over the growing season. Hence, multiple methods which are tied to the cutting of the grass could be used to track the plant characteristic of growth rate/duration (period)/development. Among these, “plant height at harvest/cutting of the grass”, which can be found in the Austrian, German, Belgian and Czech VCU protocol, is the most obvious to use.

The plant characteristic of flower biology/time to flowering or maturity/flowering period, albeit deemed very important from an ecological and agronomical viewpoint to estimate the persistence and invasiveness of a grass, is not very well covered by any methods in the VCU protocols. Here, only the method “date of ear emergence”, which can be found in the Austrian, German and Belgian VCU protocols might be applicable, as none of the five investigated protocols specifically assesses flower or flowering characteristics of grasses.

From an ecological and agronomical viewpoint, two other typical plant characteristics, which can be considered highly indicative for the persistence and invasiveness of a plant like Agrostis stolonifera, are pollen shed/viability/compatibility/morphology as well as seed dispersal ability/seed shatter ability. However, no matching methods were found in any of the five investigated VCU protocols of grasses, which could be applied to reliably assess these specific plant characteristics.

Vegetative (horizontal) growth (plant/colony diameter) is one other plant characteristic in the mature plant life cycle stage which is deemed ecologically as well as agronomically relevant and indicative for the persistence and invasiveness of genetically modified creeping bentgrass. Here, multiple possible methods to assess and rate this plant characteristics were found. For instance, “density of grass cover” has to be assessed when following the Austrian, German and French VCU protocol, while the German protocol additionally and specifically asks for “density of grass cover at the end of vegetation” as well. In all instances, “density of grass cover” includes rating the spread of the grass between the sowing lines, and hence could be used as an indicator for vegetative growth. In the Czech protocol, “tillering-density after each cut except for the last one” is one described method that could be applied here to test for the same plant characteristic. Finally, “sparsity of the stand at the end of vegetation” is one further method, found in the German VCU protocol for grasses, which potentially could be taken into account to specifically test for the plant characteristic of vegetative (horizontal) growth (plant/colony diameter).

Since seeds of any member of the family Poaceae are small, lightweight, and often distributed via the means of anemochorie, hydrochorie and/or zoochorie, multiple plant characteristics of the seed life cycle stage have been categorized as highly ecologically and agronimcally relevant to assess the invasiveness and the persistence of a genetically modified creeping bentgrass. Specifically, these include seed size/morphology/moisture, seed number/weight, seed longevity/survival and seed germination characteristics. Generally, a low seed weight of about 13500 seeds g-1, and a multiple-year seed longevity of buried Agrostis stolonifera seeds in the soil seed bank further show the importance of assessing the seed characteristics of this plant. However, in none of the five investigated VCU protocols for feed grasses, no method at all could be found, which directly or indirectly could be applied to assess or rate the Agrostis stolonifera seed life cycle. As it stands, adapted methods from different protocols and/or scientific literature would be needed to fill this gap.

Finally, a plant characteristic which has been deemed both ecologically and agronomically important for all life cycle stages to assess a crop’s invasiveness and persistence is its response to naturally occurring insects and pathogens (biotic stress). None of the investigated VCU protocols calls for an assessment of insects or other pests. However, the German and Austrian VCU protocols explicitly instruct to use chemical/industrial pesticides against any naturally occurring insects to allow a uniform assessment of the plots.

Environment interactions at all life stages

According to the literature, *Agrostis stolonifera* is susceptible to multiple diseases, among which are *Fusarium* patch (*Microdochium nivale*), dollar spot disease (*Slerotinia homeocarpa*), Fusarium blight (*Fusarium roseum* and *Fusarium tricinctum*), *Phytium* blight (*Phytium* spp.) and *Typhula*. With regard to the plant's response to naturally occurring pathogens, only slightly different approaches to test for this plant characteristic can be found in the five investigated VCU protocols. The German and Belgian protocol call for the rating of any occurring disease (thereby exemplifying fungal rust in the German, and crown rust and leaf spot diseases in the Belgian protocol) on a scale from 1 to 9 at the time of highest differentiation, without specifying a list of wide-spread feed grass diseases. The Austrian protocol defines the assessment of diseases as optional i.e. that is if they occur in the plot. Here, a specific list of diseases is given which includes fusarium patch (*Microdochium nivale*), Typhula, yellow rust, black rust, crown rust, powdery mildew (Erysiphales), downy mildew, *Mastigosporium*, bacteriosis and any plant specific viruses. Similarly, the French VCU protocols for grasses asks for the mandatory rating of resistance to common plant diseases like rust, *Helminthosporium*, *Microdochium nivale*, *fusarium* and *Corticium fuciforme* for all varieties of feed grasses, if they are noted in the assessed plots. Additionally, in the French VCU protocol, diseases specifically occurring in lawn grasses like Agrostis stolonifera, notably *Sclerotinia* or Dollar spot disease (*Slerotinia homeocarpa*), Anthracnose (*Colletotrichum* spp.), Myxomycose and Curvulariose are specified and have to be written down by the assessor on the assessment sheet. The Czech protocol mandates an assessment of for example *Fusarium*, leaf rust and leaf rot before each cutting of the grass. In this protocol, *Microdochium nivale* is specifically mentioned to be assessed each spring after melting of the snow. The VCU approach of assessing the susceptibility of a grass towards any disease by assigning a rating to the plot is similar to methods used in published research studies, where each plot is rated on a scale from 0-100, representing percentage of plot area affected by disease.

In general, the paragraphs above should give a short overview of which methods, found in five investigated European VCU protocols, could be applicable to evaluate phenotypic characters relevant for invasiveness and persistence of genetically modified *Agrostis stolonifera* plants. However, since all VCU protocols are only published in each countries respective language, the authors would like to point out that some assessments / described methods might have slightly different remarks and annotations in their original language than in the procured translations.

Conclusions

- In general, multiple methods to cover several aspects of the two life cycle stages "Seedling" and "Mature plant" can be found in all five investigated VCU protocols
- However, since *Agrostis stolonifera* relies on wind pollination for sexual reproduction, any methods covering pollen distribution would be very important to indicate the persistence and invasiveness of this plant during its "Mature plant" life cycle stage. None of the investigated VCU protocols assesses this characteristic.
- Seeds of *Agrostis stolonifera* are very lightweight and small, promoting a long-range distribution either by wind or by bodies of flowing water. The VCU protocols have no methods to describe any seed characteristics, however, multiple different protocols can be found in the scientific literature.

- Multiple plant diseases for *Agrostis stolonifera* are noted in the scientific literature. The most common ones have to be assessed on a scale from 1 to 9 during the VCU trials of all five investigated protocols, however the specific diseases that have to be assessed differ from country to country.

For further details, please refer to Table 23.

Multiple typical plant characteristics that affect the vegetative or reproductive phenotype of the plant and could potentially be indicative for the persistence and invasiveness of *Agrostis stolonifera* are not covered by the investigated VCU protocols. Among these, especially the characteristics linked to sexual reproduction like pollen shed/viability/compatibility/morphology, seed dispersal ability/seed shatter ability, seed number/weight and seed germination characteristics have been deemed especially important to investigate when assessing plants of *Agrostis stolonifera*. Here, additional scientific literature research has been conducted to potentially cover the gaps in the VCU protocols with derived methods from scientific publications.

A couple of research studies have shown the possibility and occurrence of transgene flow from genetically modified *Agrostis stolonifera* plants to feral populations. In these instances, gene flow was considered pollen-mediated, while also highlighting the occurrence of gene flow via seeds, where the wind and irrigation canals facilitate the spreading of the seeds. In addition to dispersing panicles and seeds, waterways have also been discussed as a potential mechanism of facilitating gene flow via stolons. Hence, methods described in alternative protocols should be used to assess pollen shed/viability/compatibility/morphology as well as seed dispersal ability/seed shatter ability of *Agrostis stolonifera*. It is important to point out, that due to its perennial nature, *Agrostis stolonifera*, is regularly maintained at one site for up to five years, thereby further increasing the possibility of gene flow or hybridization into feral plant populations.

As a starting point, multiple research studies on *Agrostis stolonifera* assess seed traits like seed number/weight and seed germination characteristics by applying seed assessment protocols as defined in Rules for Seed Testing by the Association of Official Seed Analysts.

Tab. 23: Creeping bentgrass – VCU methods and assessment of invasiveness, persistence and environmental interaction.

Life Cycle Stage	Characteristics	Suitable methods and parameters in the VCU protocols
Seedling	plant establishment	Date of seedling emergence and/or the date of the beginning of vegetation (date); completeness of emergence, deficiency after emergence (visually, 1 to 9 scale)
	growth rate/duration	Youth development (visually, 1 to 9 scale)
	early ground cover	Completeness of emergence, deficiency after emergence (visually, 1 to 9 scale)
Mature plant	plant vigour	Regrowth after cutting, density of grass cover, density of grass cover after cutting, defects in crop stand before winter, defects in crop stand after winter (visually, 1 to 9 scale); gaps in crop stand (visually, in %)

Life Cycle Stage	Characteristics	Suitable methods and parameters in the VCU protocols
	growth rate/duration (period)/development	Date of ear emergence (date); youth development, rate of growth, deficiency at harvest, mass formation after cutting (visually, 1 to 9 scale); plant height at harvest/cutting of the grass (measurement, cm)
	plant size/height/ biomass/yield/dry matter	Plant height before cutting (measurement, cm); green matter yield (measurement, kg); dry matter content (measurement, %); dry matter yield (measurement, kg)
	flower biology/time to flowering or maturity/flowering period	Date of ear emergence (date)
	fertility/vernalisation requirement	No fitting methods in investigated VCU protocols
	attractiveness to pollinators	No fitting methods in investigated VCU protocols
	pollen shed/viability/compatibility/morphology	No fitting methods in investigated VCU protocols
	Seed dispersal ability/seed shatter ability	No fitting methods in investigated VCU protocols
	Tuber survival and characteristics (number/size/bud number	Not applicable
	Vegetative (horizontal) growth (plant/colony diameter)	Density of grass cover, density of grass cover at the end of vegetation, tillering-density after each cut except for the last one, sparsity of the stand at the end of vegetation (visually, 1 to 9 scale)
Seed	seed size/morphology/moisture	No fitting methods in investigated VCU protocols
	seed number/weight	No fitting methods in investigated VCU protocols
	seed longevity/survival	No fitting methods in investigated VCU protocols
	seed germination characteristics	No fitting methods in investigated VCU protocols
	primary/secondary dormancy	No fitting methods in investigated VCU protocols
	volunteers in subsequent crops	No fitting methods in investigated VCU protocols
All stages	response to naturally occurring insects and pathogens (biotic)	No fitting methods to investigate response to naturally occurring insects in investigated VCU protocols Pathogen occurrence is assessed visually on a scale from 1 to 9. Pathogens include rust (yellow rust, black rust), fusarium (including *Microdochium nivale*), Sclerotinia (dollar patch disease), etc.
	response to abiotic stress (heat, drought, excess of water)	Deficiencies in crop stand before winter, deficiencies in crop stand after winter, winter damage, damage after last late

Life Cycle Stage	Characteristics	Suitable methods and parameters in the VCU protocols
		frost (visually, 1 to 9 scale); No fitting methods in investigated VCU protocols to assess for heat and drought stress

D Annex: Standardized Methods for Seed Testing (ISTA, AOSA)

Both, ISTA (International Seed Testing Association) and AOSA (Association of Official Seed Analysts), have published standardized methods for seed testing. For example, ISTA issues International Rules for Seed Testing (ISTA rules) in order to "develop, adapt and publish standard procedures for sampling and testing seeds, and to promote uniform application of these procedures for evaluation of seeds moving in international trade" (ISTA 2022, www.seedtest.org). Methods for principal crop species worldwide are available but not only for agricultural but also for e.g. spices, herbs or medicinal species. The methods to different aspects of seed quality and have been validated by the ISTA validation program. However, not all ISTA rules and chapters are free of charge but must be purchased online.

Similarly, AOSA and SCST (Society of Commercial Seed Technologists) publish handbooks and rules for seed testing which are not free of charge for non-members (https://analyzeseeds.com/publications/?v=dfd44cc06c1b).

The seed laboratory of the Iowa State University is a public seed-testing lab in the US and offers seed testing services for the industry for more than 300 crop and other plant species. It also publishes a range of seed testing methods (https://seedlab.iastate.edu/portfolio-items/).

D.1 Testing seed germination potential

The germination potential is a central quality criterion of seed, which is assessed under standardized laboratory conditions that are optimized for a respective crop species according to method specifications of ISTA and AOSA (AOSA Rules for Testing Seeds 2021, ISTA-Rules 2022). The conditions addressed therein refer to a standardized substrate/medium such as paper or sand and to a temperature range that ideally corresponds to the respective crop species. The temperature is usually above 10°C in any case, mostly at 20 or 25°C and rudimentarily designed with an alternating temperature to mimic a day/night cycle. An overview of the different options on the validated methods are shown in Table 24.

Tab. 24: Methods for standard germination tests (AOSA Rules for Testing Seeds, 2021 and ISTA-Rules 2022)

Species	Rules	Temperature (°C)	First count (days)	Final count (days)	Recommendations for breaking dormancy
Agrostis stolonifera	AOSA	15-30, 10-30, 15-25	7	28	Light, KNO_3, prechill
	ISTA	20<=>30, 15<=>25, 10<=>30	7	28	KNO_3, prechill
Brassica napus	AOSA	20, 15-25	3	7	-
	ISTA	15<=>25, 20	5	7	KNO_3, prechill
Glycine max	AOSA	20-30, 25	-	7	Hard seeds
	ISTA	20<=>30, 25	5	8	-

Species	Rules	Temperature (°C)	First count (days)	Final count (days)	Recommendations for breaking dormancy
Zea mays	AOSA	20-30, 25	4	7	-
	ISTA	20<=>30, 25, 20	4	7	Use CCP for TP method
Solanum spp.	AOSA	20-30	3	14	Light and KNO_3
	ISTA	20<=>30	3	14	Imbibe in 1,5 % GA_3 for 24 h

Also the recommendations by the Iowa State University refer to a warm germination test as a standard germination test. This is used for seed labeling purposes and informs on field emergence under favorable conditions, using temperature ranges of 15°-25°C or 20°-30°C for up to 28 days. The viability of ungerminated seed at the end of the germination period is usually tested by a tetrazolium test. A modified version is the sand germination test, which assesses field emergence under favorable conditions. This test suppresses fungi and guarantees uniform water uptake, especially in low moisture seeds (e.g. soybean). Using sand for germination reduces the variability in results better than if soil is used as a substrate.

D.2 Possibilities to improve assessments based on state-of-the art methods

Temperatures below 10°C are not supported by validated methods. However, methods are available that refer to breaking a possible dormancy or try to mimic natural stress conditions, like a time-period with cold weather conditions in the field for the germination process. Standardized substrates but also non-standardized substrates like natural soil can be used.

The results describe the total germination ability, which is composed of normal seedlings and any hard seeds. In addition, abnormal seedlings, freshly ungerminated and dead seeds are to be reported. In the case of reproducibility beyond the regular test period resulting by the final count as specified for the requested cultivar in the table, the hard and freshly ungerminated seeds are the main focus of interest. For this purpose, standardized test procedures like the tetrazolium test that show the viability of seeds are used. Assessing the viability serves as indication of the potential for later germination.

In terms of seed vigor, a few species can also be tested by means of the accelerated aging (AA) test (according to ISTA). Starting from a certain moisture content of the seeds at high temperature (41°C+/-0.3°C) and a defined period, accelerated ageing is provoked and followed by a germination test with reduced number of grains. Similarly, an approach with the controlled deterioration (CD) test for Brassica species at 45°C±0.5°C is available. Furthermore, for a number of species, the Radicle Emergence Test (RE) can also be used. Narrowly defined temperature range and time duration specifications as well as the minimum length of the radicle are relevant here.

Seed vigor testing

Seed vigor is defined as “the sum of properties that determine the activity and performance of seedlots of acceptable germination in a wide range of environments” (ISTA 2015, cited in Finch-Savage & Bassel 2016). Finch-Savage & Bassel (2016) extensively describe the environmental influences and mechanisms affecting seed performance in the field as well as methods to measure seed vigor.

The object of seed vigor testing is to provide information about the planting value in a wide range of environments and/or the storage potential of seed lots. There are several possibilities to determine the activity and performance of seed lots of acceptable germination associated with aspects like the rate and uniformity of seed germination and seedling growth, or the emergence ability of seeds under unfavorable environmental conditions and further on the performance after storage, particularly the retention of the ability to germinate.

The AOSA has published specific rules for seed vigor testing, including different seed vigor test methodologies, e.g. aging tests, cold stress tests, conductivity tests, seedling performance tests (e.g. seedling growth rate test), etc. (see www.analyzeseeds.org)

Testing of seed germination at cold temperature regimes (cold germination test) is also proposed in order to assess seed germination under "less than ideal, but not severe conditions" (https://seedlab.iastate.edu). For maize and soybean the recommended temperature regimes is 10°C for 7 days, followed by optimum germination temperatures at 25°C for another 7 days. The quick cold test uses 10°C for 3 days only and 25°C for 4 days. Cold tests are usually simulating field conditions in early spring. Adapted cold tests (e.g. saturated or sand cold test) estimate seed emergence under mildly stressful conditions (cold and wet). The seeds need stronger root and shoot development under these conditions. For the sand cold test, seeds are covered by sand saturated to 70 % of their water holding capacity. Temperature regimes are 10°C for 7 days without light, followed by 25°C for another 7 days (also 3 days and then 4 days is possible as a quick method).

The Accelerated aging test estimates germination under less than ideal conditions and the storage potential of seeds. It can be used with many types of seed. The seeds are exposed to high temperature and humidity in a special chamber for 48-96 hours, depending on the seed type. Seeds are then grown for a time period similar as in the warm germination test. This test also indicates the relationship between emergence in the laboratory and in the field (Hay et al. 2018).

In the following, the objective of such tests is outlined.

Controlled deterioration (CD) test for *Brassica* spp.

For Brassica species, a controlled deterioration test is specified at raised moisture content (20%) and raised temperature (45±0,5°C) for 24 hours as well as a radicle emergence test at 20±1°C for 30 hours (ISTA 2022, Chapter 15) and a tetrazolium test (ISTA 2022, Chapter 6).

The test exposes seeds to a high temperature while at a specified and constantly raised seed moisture content. These conditions cause seeds to deteriorate, or age rapidly. The moisture content of a seed sample is raised before the seed are placed at the raised temperature, thus ensuring that all samples tested are exposed to a predetermined degree of deterioration during the test. High vigor seeds retain a high germination after deterioration, while the germination of low vigour seeds is reduced (ISTA 2022).

Accelerated ageing (AA) test for *Glycine max*

An accelerated ageing test is outlined for soybean, exposing seeds to 95% relative humidity and 41±0,3°C for 72 hours in an ageing chamber (ISTA 2022, Chapter 15). This stress test exposes seeds for short periods to high temperature and high relative humidity (about 95%). During the test, the seeds absorb moisture from the humid environment and the raised seed moisture content, along with the high temperature, causes rapid seed ageing. High vigour seed lost will withstand these extreme stress conditions and age more slowly than low vigour seed lots. Thus, after AA high vigour lots retain a high germination, whilst that of low vigour lots is reduced (ISTA 2022).

Radicle emergence (RE) test

A slower rate of germination is an early physiological expression of seed ageing, the major cause of reduced vigour. The rate of germination of all validated species are accurately reflected in a single count of radicle emergence early in germination and this single count relates closely to other expressions of the rate of germination. High counts of radicle emergence early in germination are indicative of high seed vigour; low counts indicate low seed vigour.

Such a test is validated for species like Brassica napus and Zea mays (ISTA 2022). For maize a radicle emergence test is specified at 20±1°C for 66 hours or at 13±1°C for 144 hours (ISTA 2022, Chapter 15).

Dormancy in germination testing

Dormancy is the reason why some seed lots cannot reach the optimum germination capacity. The seed is not yet physiologically mature or its germination capacity is negatively influenced by substances such as treatment agents (secondary dormancy). The test results therefore show a higher proportion of hard, freshly ungerminated or dead seeds. There are a number of methodical approaches to break dormancy. Preferably, a phase of temperatures between 5 to 10°C with a duration of up to seven days or even longer is used. Potassium nitrate (also calcium nitrate), gibberellic acid (GA3), sulfuric acid, heating up to 40°C or mechanical approaches such as scoring or piercing are also used. There are the different recommendations for breaking dormancy for the different species (see Table 24).

The International Rules on Seed Testing provide a recommendation for dormancy breaking methods in Brassica napus with application of a KNO3 solution and a prechilling temperature at 5-10°C (ISTA 2022). Also for *Agrostis stolonifera* recommendations are provided with the application of a KNO3 solution and a prechilling temperature of 5-10°C as well as a tetrazolium test (ISTA 2022).

The International Rules on Seed Testing do not provide a recommendation for dormancy breaking methods in maize or soybean (ISTA 2022).

Literature

AOSA (2021). AOSA Rules for Testing Seeds. Volume 1. Principles and Procedures. Association of Official Seed Analysts, Wichita, KS 67205 USA

ISTA (2022). International Rules for Seed Testing. International Seed Testing Association. Bassersdorf, Switzerland

E Annex: Selected studies on plant fitness, persistence, survival or weediness of GMP

Tab. 25: Studies of assessment of crop-wild hybrid fitness under experimental field or greenhouse conditions.

Crop-wild hybrid	Study	Assessment	Parameters assessed	GM trait
Maize x teosinte	Guadagnolo et al. 2006	Relative fitness of maize x teosinte hybrids (GM, non-GM)	germination rate, plant survival, plant height, seed set, dry mass, days to flowering, pollen viability	HR (glyphosate)
B. napus x *B. rapa*	Hauser et al. 1998a, b	Fitness of F1, F2 hybrids, BC1 generation	seed development, survival in the field to harvest, no of pods, no of seeds per pod, pollen viability	Non-GM
B. napus x *B. rapa*	Moon et al. 2007	Comparison of fitness of *B. rapa* and GM crop-wild hybrid (with insect infestation) (greenhouse experiment)	No of seeds per plant, dry weight per plant and per m^2	IR (Bt)
B. napus x *B. rapa*	Allainguillaume et al. 2006	Fitness of hybrids in natural habitats (survey for spontaneous F1 hybrids in semi- natural habitats	Seed return (mean seed no per pod, flower number, pod number, total seed number), seed viability, pollen viability	Non-GM
B. napus x *B. rapa*	Vacher et al. 2004	Fitness of crop-wild hybrids in natural habitats (herbivore pressure, plant density)**	Plant final biomass (stem height x width^2), no of flowers, seed mass, germination rate	IR (Bt)
B. napus x *B. rapa*	Hooftman et al. 2014	Demographic models of F1 hybrid seed bank dynamics in agricultural and semi-natural habitats	Germination, survival rates among life stages, fecundity (pods, seeds per pod), seed viability, seed bank survival (overwinter and annual survival)	Non-GM
B. juncea x *S. arvensis*	Warwick & Martin 2013	Characterisation of hybrid generations	Pollen viability, self-compatibility, no of flowers, ploidy level, plant height, pod set, seed weight/plant	HR (ALS)
G. max x *G. soja* (wild soybean)	Guan et al. 2015	Performance of hybrid progeny (F1, F2 generation)	Vegetative growth period, pod number, seed number, above-ground biomass, 100-seed weight	HT

Crop-wild hybrid	Study	Assessment	Parameters assessed	GM trait
G. max x *G. soja* (wild soybean)	Liu et al. 2021	Hybrid fitness with and without weed competition (greenhouse experiment)	Emergence rate: emergence number / number of seeds sown) Performance without competition: length/width of cotyledons, length/width of leaves, plant height, above-ground biomass, pollen viability, pod number/plant, filled seeds no per plant, 100-seed-weight (composite fitness) Performance with competition: plant height, above-ground plant biomass, pod number per plant, filled seed number/plant, 100-seed weight (composite fitness)	HT
Soybean (*G. max*) x wild soybean (*G. soja*)	Kuroda et al. 2012	Hybrid fitness	Seed dormancy: % winter seed survival, % seed hardness, seed coat colour Seed production: total seed number, total seed weight, 100-seed weight, total pod number, stem dry weight, stem length Flowering phenology: days to first flower Survival: total number of seeds expected to germinate in the following year	Non-GM
H. annuus x *H. annuus* (wild sunflower)	Mercer et al. 2007	Relative fitness of sunflower crop-wild hybrids compared to wild sunflowers under competition and herbicide application	Seedling height, early growth, flowering phenology (first day of flowering), survival to seed production, head size, head number, number of seeds per head	HT (ALS)
C. pepo x *C. pepo* (wild squash)	Laughlin et al. 2009	Assessment of fitness of BC2 and BC3 hybrids with and without virus pressure	Female/male flower number, fruit number, seed number per plant, pollen production, biomass	Virus-resistance
O. sativa x *O. rufipogon*	Song et al. 2004	Relative fitness of rice hybrids	Seed germination, seedling survival rate, plant height, flag leaf area, days to flowering, no tillers, panicles, spikelets, pollen viability, pollen longevity, seed set of self-pollination	Non-GM
O. sativa x *O. sativa* (weedy rice)	Xia et al. 2016 (abstract only)	Crop-weed hybrid progeny (F1, F2) fitness under insect pressure	Fecundity (?)	IR (Bt)

Crop-wild hybrid	Study	Assessment	Parameters assessed	GM trait
O. sativa x *O. rufipo-gon*	Yang et al. 2017	Crop-wild hybrid offspring (F1-F3) fitness without herbicide application	Plant height, no tillers/plant, flowering time (days for 1, 30, 50% plants to flower), no panicles/plant, no seeds/plant, 1000-seed weight, seed set ratio, ration of tiller regeneration Seed burial experiments (seed germination ratio at 0, 20, 40, 60 days)	HT (gly)

Tab. 26: Studies of assessment of GM plant fitness, persistence, survival or weediness under experimental field conditions or in natural habitats.

Crop	Study	Assessment	Parameters assessed	GM trait
Maize	Crawley et al. 2001	Survival in natural habitats	survival over 10 years	HR (glu)
Maize	Raybould et al. 2012	Establishment of feral maize in uncultivated plots and buffer strips	Replacement capacity, establishment, emergence of ferals	IR, IR+HR
Maize	Sammons et al. 2014 (Monsanto)	Persistence within and outside cultivation	No of plants in fall/spring (in plots) Early/final stand count, no of ears per lot/per plant, seed produced per plot , replacement value (no seeds produced/no of seeds sown) (in noncultivated land areas)	Drought-tolerant MON87460
Soybean	Ko et al. (2016) only Abstract in English available	Weediness assessment	Burial experiments: viability and survival (after 1, 2, 6, and 10 months), competition experiments with weeds	?
Oilseed rape	Hails et al. 1997	Fitness of OSR (GM/non-GM) in comparison with S. arvensis	seed survival/seed persistence: no of viable seeds after 1 and 2 years in soil	kanamycin-tolerant, HR (glu)
Oilseed rape	Londo et al. 2011	Changes in fitness-associated traits under herbivores and glyphosate pressure	Vegetative biomass, seed production (seed number and biomass)	Stack HT (gly), IR (Bt)
Oilseed rape	Walker et al. 2004	Volunteer potential, Seed loss estimation in comparison with two weed species (S. arvensis, B. nigra) at cultivated/uncultivated sites	Seed burial experiment (6, 12, 18 months), no of ungerminated seeds; no of germinated seedlings per species	modified fatty acid content: high stearate, laurate line)
Oilseed rape	Stewart et al. 1997	Fitness under natural conditions with insecticide treatment and insect infestation	Plant establishment, no of surviving plants, damage rating, defoliation, seed production	IR (Bt)
Oilseed rape	Crawley 1993, 2001	Survival of GM plants in natural habitats: Field experiments over 12 habitats and 3 and 10 years, respect.	Seed survival (seedling/adult plant density), mean seed production per plant, finite rate of increase, seedling establishment	Kanamycin-tol., HR (glu)

Crop	Study	Assessment	Parameters assessed	GM trait
Potato	Conner et al. 1994	Field performance	(phenotypic) shoot appearance, plant survival, yield (no tubers/plant, weight of each tuber)	Several GM lines (nptII)
Potato	Mustonen et al. 2009	Volunteer plants from tubers	% survival of tubers	Non-GM
Potato	Kim et al. 2010	Volunteer plants from tubers	No of volunteers, No of tubers per harvested plant, tuber volume and vertical distribution in soil (of volunteer plants)	drought- tolerant
Potato	Crawley et al. 2001	Survival of tubers in different natural habitats	Fraction of tubers that produce mature plants	(Bt, pea lectin)
Potato	Lawson 1983	Volunteers from potato seeds	No/weight of berries, no of seeds/berry, no of seeds/ha, no of potato seedlings/m2, no of tubers/seedling, tuber weight and size/plant, tuber yield per seedling	Non-GM
Arabidopsis thaliana	Fang et al. 2018	fitness related traits (under stress conditions)	Survival, seed germination (normal, heat and drought stress conditions), leaf area, plant height and branching, no of siliques per plant, no of seeds per silique and per plant, biomass, auxin content	HR (gly)
GM crops	Kos et al. 2011	weediness	Seed size, flowering period, seed bank, vegetative period (for pre-screening), also other traits	unknown
Creeping bentgrass	Gardner et al. 2003	Fitness in bluegrass (*Poa pratensis*) or mixture of *P. pratensis* and *Lolium perenne*	Lateral spread (mean plant diameter)	HT (gly)

F Annex: Methods for the evaluation of biotic stressors of GM crops (interactions with pests and diseases)

F.1 Introduction – selection of pest species

A large number of organisms could potentially interact directly or indirectly with genetically modified plants (GMP). In the frame of environmental risk assessment of GMP it is not reasonable to consider all the pests, beneficial organisms and destructive organisms concerned. For this reason, the literature research for suitable methods from horticulture and arable farming was focused on insect pests, since they can, in most cases, be observed and determined in the field without additional necessary analysis. Furthermore, there is a lot of information available on their rearing. This is required to carry out experiments with artificial infections as well as experiments in the laboratory and in the greenhouse. Insect pests are directly interacting with the host plant and their environment. In some cases, they will also carry part of the host plant material and distribute it in the environment, or they become part of the food network.

Insect pests with economic relevance, which are frequently found in Europe and for which breeding protocols are available, were condensed into a table with 66 pest (group) - host plant combinations. Quarantine pests were not taken into account. Thirteen of these accounted for potatoes, eleven for corn, 21 for oilseed rape, and 14 for soybeans. Additionally, seven were taken into account for the creeping bentgrass. Eleven of the combinations were especially relevant for seedlings, another 24 were only relevant for maturing plants. In 31 cases, both seedling and maturing plants were targets of pests. Fifteen combinations aimed at subterranean plant organs like roots or tubers. In 21 cases, the focus was on leaf damage in multiple ways. In nine pest – host combinations, the pest is mining inside the plant organs or inside of galls or the fruit, which makes it hard to fight them with common pesticides. In 16 cases, the pests are sucking plant sap and some of them are vectors for plant diseases.

In total seven species of aphids, 16 species of beetles, six species of gnats and flies, four bug species, two sawfly species, eleven butterfly and moth species, one mealy bug species, one spider mite species and two thrips species were considered, partially as representative for groups or species complexes.

In the next step, the most important pests were defined based on the rating above and summarized in Table 27. The citations of the respective papers describing the methods for conducting experiments under laboratory conditions are listed in Table 28.

F.2 Selection criteria for pest assessment methodology

The pests relevant for Europe that damage the selected crops were collected in a table based on the EPPO Global Database and the "Beratungsschriften der Bundesanstalt für Pflanzenschutz". Besides the affected crop, the relevant growth stage (seedling, adult) and the type of damage (e.g. mines in stems and leaves) were noted. The estimation of relevance was divided into multiple sub-points, each with a 5-level scale.

Economic relevancy

The ranking of economic relevance is based on a practice-oriented assessment. For further selection of relevant pests, we only considered them economically relevant, if there were reports of damages every few years (3).

1. In general damage is rare and only occurs locally in years of extreme climate
2. Damages of economic relevance occur only in years of extreme climate
3. Damages of economic relevance occur every few years
4. Strong economic damages occur every year
5. Tremendous economic damages occur every year

Distribution in Europe, geographic relevance

The regions of Europe, which are used here, are based on the definition of regions of Europe using country borders. They have been suggested by the „Ständiger Ausschuss für geographische Namen“ (StAGN) in 2005. Except for Eastern Europe, which is increased in size because of Russia, the remaining regions are similar in size. For the relevancy estimation of the distribution of pests, mostly data of the Fauna Europaea Database was used. For further selection of relevant pests, we considered them geographically relevant if they occurred in at least three regions or about half of all European countries (3). We also made an exception for pests that were still actively spreading across the continent, knowing that some data on them was outdated.

1. The organism has a limited distribution, only occurs in one region of Europe (<5 Countries)
2. The organism can be found in at least two regions (5-14 Countries)
3. It has been observed in at least three regions (15-24 Countries)
4. It is missing in only one region or a few countries (25-34 Countries)
5. It can be found in every region and in most countries. (>34 Countries)

Estimate of practicability

The estimation of practicability is based on three combined criteria:

The duration of a single generation, the challenges of rearing, and the practicability of the necessary experiments.

Duration of a generation

Since a duration of more than a year per generation (1) would require a lot of experience in rearing to avoid potential failures and since field experiments would need a screening for suitable plots of land, replicability of experiments is rather low.

1. Over a year
2. Between nine and twelve months
3. Between six and nine months
4. Between three and six months
5. Less than 3 months

Challenges of rearing

Rating the rearing process was necessary, because it guarantees that small scale experiments in labs and greenhouses can be executed and, if necessary, repeated. In addition, it enables field trials independent from local pest populations. In most cases, the challenge level of rearing is rather low. In some cases, there are sensitive stages (e.g. larvae, 3), which need a constantly humid substrate. Many species need live plants (4), which makes especially rearing leaf-mining organisms difficult. It is based on the pest's biology and rearing protocols.

1. Other mandatory requirements (e.g. live animal feed)
2. High fatality of larvae and other sensitive stages
3. Sensitive stages in development, other challenging aspects
4. Climate controlled room with day/night cycle, humidity and temperature control and additionally live plant feed/habitat
5. Climate controlled room with day/night cycle, humidity and temperature control, can live off dead feed or artificial feeding medium

Practicability of experiments

The practicality of standard methods (EPPO, VCU, Lindner (2006) etc.) for trials under field conditions was assessed. Experiment practicability estimates how much effort has to be put into phenotypically quantifying or estimating the damage caused by a pest. A lot of effort is for example necessary to investigate underground damage (roots, rhizome, tubers etc.), combined with an uneven distribution of the pests in the substrate (2). The evaluation of experiment practicability is mostly based on screening protocols and gardening experiments. Once the selection of example organisms was made, additional research was conducted on methods for small-scale experiments in labs and greenhouses. These methods are mainly experimental approaches from applied research on plant pests.

Practicability is determined based on the following considerations:

1. Observation requires a large sample size: Highly impracticable: a lot of manual labor, low significance in output data
2. Observation depends on multiple external factors: Very challenging/difficult: e.g. a lot of factors influence the outcome, uneven distribution of pests in the field/soil
3. Observation requires screening of a big portion of the plant in detail: Work intensive: e.g. every leaf has to be screened
4. Observation is necessary over a longer time: Somewhat work intensive: not necessarily challenging, but the experiment has a long duration
5. Observation is possible in a time efficient or even automatic way: Simple: can be analyzed automatically or easily quantified, e.g. image analysis, leaf area measurement, weighing, counting

F.3 Selected pest species for individual crops

F.3.1 Potato (*Solanum tuberosum*)

Wireworms (*Agriotes* spp.) are relevant for all of Europe. There are differences in wireworm species damaging potatoes depending on the field trial location chosen. They were chosen to represent soil-dwelling insects that feed on the potato tubers. Their influence on the crop is strongly climate and weather dependent and depends on other vegetation in the field. High economic damage is possible in years when a long dry spell precedes the harvest of potato. The diversity of wireworm species present on trial sites has to be taken into account when planning rearing and lab experiments. For field trials, a monitoring in the preceding year is necessary to find a suitable plot. Lab and greenhouse experiments are less practical because of the long lifecycle.

The potato beetle (*Leptinotarsa decemlineata*) was chosen to represent insect pests feeding on leaves and other plant organs above the ground. Under optimal circumstances, it can occur in masses and cause high economic damages, because the larvae can skeletonize the plants. The species is relevant for most of Europe and both its occurrence and damage are well quantifiable. Rearing them is not challenging and both field trials and lab/greenhouse experiments are easily conductible.

In potatoes, aphids (Aphidoidea) can potentially cause economic damage by transmitting diseases (e.g. potato leaf roll virus, Y-virus). However, in most cases, the symptoms cause no economic losses.

F.3.2 Maize (*Zea mays*)

Similar to potatoes, relevant wireworm species (Agriotes spp.) are taken into account. They are representing soil-dwelling insects feeding on the roots, especially during seedling stage. The remaining factors are the same as above.

The corn rootworm (*Diabrotica virgifera virgifera*) was chosen to represent both insects feeding on roots and insects feeding on leaves, as well as the plant’s stigmata. They are very specialized and require corn to finish their life cycle. Without crop rotation, their population will increase manifold and they will cause a lot of damage. The larvae will damage the seedlings, in many cases causing lodging at a later stage of development (“goose-necking”). The adults can cause reduced fertilization and therefore reduced yield. The corn rootworm does not (yet) occur in some corn cultivation areas of Germany, France, Romania, Italy and Belgium. Both rearing and field trials, as well as lab/greenhouse experiments are possible. Evaluation in cell based field trials are work intensive (evaluating the damage to roots, setting up eclectors), but well executable. Lab and greenhouse experiments do not pose a challenge.

The European corn borer (*Ostrinia nubilalis*) represents insects that feed on leaves and stem; and protect themselves by mining inside the plant. Under optimal circumstances (organic corn, weather/climate, crop parts remain above soil after harvest) large economic damage is possible. The corn borer is relevant for most of Europe. Rearing them is challenging, but executable, if no winter dormancy is simulated. Quantifying damages is practicable, but needs a large sample size, depending on the circumstances. If combined with rearing, experiments in lab/greenhouse are possible.

While aphids (Aphidoidea) cause damages primarily by feeding or secondarily by transmitting viral diseases, they are irrelevant for the most common diseases in corn.

F.3.3 Oilseed rape (*Brassica napus*)

The rape stem weevil (*Ceutorhynchus napi*, Gyllenhaal, 1837) and /or the cabbage stem weevil (*Ceutorhynchus pallidactylus*, Marsham, 1802) are representing the insects that feed on and mine in stems of oilseed rape. Depending on the date of sowing, the temperatures/development of the plant, the temporal and spatial proximity to other Brassicaceae, there is a high damage potential. Some Brassicaceae dependent weevils occur all over Europe and have a big range of hosts. Rearing them can be pretty challenging, since they require live plants to feed on. Field trials are possible, but should be preceded and combined with a monitoring via pan traps. Lab/greenhouse experiments will need to be combined with rearing of the weevils or live traps (kairomones). A visual evaluation of the damage is possible, but impractical because of oilseed rape growing very dense towards the end of the season.

The turnip sawfly (*Athalia rosae rosae*) was chosen to represent insects feeding on oilseed rape leaves. It thrives in high temperature and dry environment and has multiple peaks of flight activity over the year. Economic damages occur in regions with a high portion of summer oil crops (e.g. high amount of mustard). The pest occurs all over Europe. Rearing them is not challenging. For field trials, it is necessary to monitor the location in the preceding year via pan traps. Lab/greenhouse experiments need to be combined with rearing.

The rape beetle (*Brassicogethes aeneus*) represents insects feeding on oilseed rape flower buds. It is almost omnipresent in Europe and always occurs in areas of oilseed rape cultivation. The economic damage is highly dependent on the oilseed rape development and the temperatures (flowering time and duration). Field trials should always be combined with pan trap monitoring. Damage can be quantified optically during flowering or by using yield loss when comparing treatments.

The cabbage stem flea beetle (*Psylliodes chrysocephalus*) and the turnip flea beetles (*Phyllotreta atra*, *P. cruciferae*) represent insects feeding on leaves. They occur in areas of Brassicaceae cultivation all over Europe and damage especially young seedlings short after germination (reduced development speed). The larvae need live plants in which they mine (leaves, stems, roots), which increases the difficulty of rearing them. Field trials should be combined with a preceding pan trap monitoring. Lab/greenhouse experiments are best combined with rearing of the pest.

Cutworms (Noctuidae) were chosen to represent soil-dwelling insects feeding on roots and leaves. They can be found all over Europe. Rare mass occurrences can lead to economic loss. Rearing is simple, because they accept an artificial diet. Lab and greenhouse experiments are easy to execute in combination with rearing. Field trials are more challenging, because the pest might be unevenly distributed.

In oilseed rape, aphids (Aphidoidea) can potentially cause economic damage by transmitting diseases (e.g. bean common mosaic virus). However, in most cases, the symptoms cause no economic losses.

F.3.4 Soybean (*Glycine max*)

The bean seed fly (*Delia platura* and *Delia florilega*) represents insect pests, which damage especially the seeds or young seedlings. They occur almost all over Europe and cause damage depending on the weather around germination. Rearing is potentially possible, but there might be a difference in locally relevant species. It might be best to focus on pan trap monitoring and field trials.

The southern green stinkbug (*Nezara viridula*) was chosen to represent bigger sucking pests, which can cause a lot of damage to the crop. It already occurs in about half of the countries of Europe. Rearing is easy, but resource intensive (feeding). Both field trials and lab/greenhouse experiments are easily executable. Optical evaluation of crop quality can be used for damage estimation.

The European red mite (*Panonychus ulmi*) represents acari on soybeans. It occurs in about half of the countries in Europe and can lead to economic damages under the right circumstances (weather). Because of their small size and limited mobility, it might be beneficial to combine both field trials and lab/greenhouse experiments with rearing to cause an artificial infestation.

The painted lady (*Vanessa cardui*) was chosen to represent insect pests feeding on leaves. It occurs almost all over Europe and is one of the main pests on soybean. It is easy to rear, since it accepts artificial diet. Both field trials and lab/greenhouse experiments could be executed. For field trials, it might be beneficial to cause an artificial infestation.

In soybean, aphids (Aphidoidea) can transmit for example the bean common mosaic virus, but it is currently not relevant for Europe.

F.3.5 Creeping bentgrass (*Agrostis stolonifera*)

So far, the most dangerous pest for creeping bentgrass found in literature was the larvae of the Japanese beetle (*Popillia japonica*). Since it is a quarantine pest, it was regarded as not relevant for Europe. Other insects polyphagous on grasses are the larvae of the small heath (*Coenonympha pamphilus*) and speckled wood (*Pararge aegeria*), that have been mentioned as users of bentgrass. The larvae of sod webworms (*Crambus* spp.) are similarly polyphagous and feed on both roots and aboveground tissue of different grasses. Cutworms (*Agrotis ipsilon*, *Peridroma saucia*) are also feeding on grasses, but they only rarely occur in masses. Of the Blissidae, two species, which can cause economic damage in masses, occur in almost all of Europe on grasses (*Dimorphopterus spinolae*, *Ischnodemus sabuleti*). However, they are not as dangerous as the true chinch bug (*Blissus leucopterus*).

There are more pests mentioned in literature for creeping bentgrass, especially in context of golf court lawn maintenance, which will cause optical damage and only rarely lead to decay of parts of the lawn. Of these, only a small portion of species is also relevant for Europe. Other taxa were not listed because of their low distribution or low damage potential. For example, multiple owlet moths (*Spodoptera* spp.), which are either quarantine pests or have a smaller range of distribution than the sod webworms, or have a different host spectrum. In addition, weevils (*Sphenophorus* spp.) were disregarded, because the genus prefers warm and wet meadows. Mole crickets were mentioned in literature, but the most dangerous species (*Scapteriscus* spp.) are quarantine pests in Europe. Related species are not as dangerous and rarer.

Aphids can be found on grasses (e.g. *Rhopalosiphum padi*), but would only be relevant, if they also transmitted a specific disease.

F.4 Rearing and application protocols

To gauge the level of knowledge on rearing and application of the chosen insect pests, a separate search for published literature was performed. The data gained that way was partially also used to inform the “Challenges of rearing” rating. The main keywords used were the species name and “rearing”. For application protocols, we focused on different damage types, e.g. feeding on leaves or roots. The data is summarized in Table 28.

In most cases, the host plants used in the protocols can be exchanged with other plants the insect feeds on for practicability. In some cases the protocol of a closely related insect species can be used, e.g. with noctuids.

Application protocols are meant as examples and can be adapted more freely, as long a few points are considered. All experimental units have to be treated the same way (e.g. same number, age and sex ratio of pests applied to each individual plant or experimental plot). External pest influence should be excluded (e.g. with net tents). The data gained should be quantifiable, or at least have a clearly defined grading system. The sample size and number of replicates needs to be big enough to allow for statistical analysis, especially, if only a small difference is expected.

F.5 Methods for the Assessment of insect pests and protocols

To be able to plan experiments concerning plant-pest interactions, it is important to know what challenges to expect in the field, like low pest population, high effort manual monitoring and so on. Based on this information some experiments are better suited for greenhouse/laboratory approaches. In the following, suitable methods are collected that might be used directly or need further refinement to meet the exact demands for GMO risk assessment. It is important to consider that methods and equipment will continue to improve, and both their precision and practicability need to be evaluated in every specific experimental setup. The EPPO protocols used as a base for the evaluation only give a general direction of what should be done to quantify the pest impact, but don’t always list specific methods on how to do so. If precise measurement of e.g. leaf area affected or damaged is not practical, for example with seedlings or mite infestation, it is advisable to use ordinal scales. Ordinal scales need an adequate number of classes (e.g. ≥ 5) to allow for good distinction between treatments, using the median and non-parametric tests (e.g. Mann-Whitney U test, Kruskal Wallis H test, and Spearman’s rho or rank correlation coefficient). Ordinal scales do not allow for further calculations based on the data. Some of the available non-EPPO protocols use ordinal scales for damage assessment. These are meant for time efficient unaided damage estimation (e.g. naked eye observation of mites and their damage on soybean). Digital image analysis and other technical aids, enable the development of new quantification protocols to measure the pest impact more accurately, making ordinal scale approaches obsolete. The precision achievable this way is especially a concern when attempting to compare plants with very similar genotype, like in context of this project.

There are methods that are better suited under different conditions and with different research questions. There also are ways to minimize the risk of an experiment failing because of a lack of pest occurrence, which differ based on the pest species in question. Experiments in greenhouse/lab drastically increase the amount of work to maintain the individual test units. Therefore, the sample size for such experiments should be adequate, to allow for statistical analysis, but might not reach the same size as in field experiments.

In this chapter, we list pests relevant for the investigated crop species, appropriate rearing protocols, diet options and what to keep in mind when designing experiments. For easier navigation of the document, we included the most important information in a short summary for every host-pest pair. The relevant experimental aspects are marked as "technically possible" if they are executable on small scale and anything that would also be executable with a justifiable expense of time and work is listed as "practical". Not everything that is technically possible is economically executable on a big scale, e.g. in the field.

Some insect rearing procedures or experimental setups can be quite challenging and will require additional work (especially field experiments). In some cases, in particular for soil dwelling pests and pests with a multi-year lifecycle, they will require monitoring of the pests in the previous season to make sure there is an established pest population. Other field experiments might require artificial infestation ("artificial infestation suggested"), to ensure that an adequate amount of pests is present, especially for pests with infrequent mass occurrences. In any case, the field experiments should be replicated in multiple locations where the crop would be cultivated commercially and in multiple successive years to cover a range of different climatic conditions. This is especially important for pests that have climatic preferences or thrive under drought conditions. Most pests require active monitoring, as described in the respective protocols (EPPO 1997c, a, b, d, 2002, 2004b, c, a, 2005, 2008, 2019, 2020a, b), during the experiment ("parallel monitoring") to determine population density, development speed or to be able to distinguish between multiple species that cause visually similar damage. In other cases observing and quantifying the damage is sufficient (EPPO 2004d, 2006).

The EPPO protocols listed are always based on field experiments, but the damage quantification methods described in these protocols can also be used for greenhouse/lab experiments.

In case of wireworms, corn-rootworms and red mites, it is highly advisable to use greenhouse or laboratory experiments to ensure an evenly distributed infestation. With potato beetles, aphids, European corn borer, turnip sawfly, flea beetles, cutworms, bean seed fly, southern green stink bug and painted lady, a greenhouse/lab approach would allow for more control over the experimental setup. Only with oilseed rape weevils and rape beetles, the field experiments might have more advantages because both need adult host plants and occur frequently in the field.

However, in most cases this approach only works in combination with rearing of the target pests, since they should have grown up under similar conditions and should be of about the same age. In case of infestation with adult, sexually reproducing individuals, the same number of male and female individuals should be introduced in every experimental unit.

While many methods for remote sensing and other computer supported quantification methods are in development, there currently are no accepted standardized methods to assess and quantify insect occurrence or damage. Within a timeframe of a few years, this might change. Here, we list examples of technologies that have the potential to become standard for these applications in the future.

There already are “autonomous” insect traps, based on an attraction mechanism and a camera, which, in combination with a machine-learning algorithm, count and identify relevant insects (e.g. Bjerge et al., 2021). As the algorithms become better, this might be a good way to reduce the workload on pest monitoring for certain species (moths, small bugs, small cicadas, fruit flies), while this approach (mostly because of sticky sheets or low frequency of image recording) is not suitable for bigger bugs/beetles (e.g. *Halyomorpha halys*, *Nezara viridula* etc.). There also still are issues with the algorithms lacking data and the determination of the pest species needing human confirmation.

To investigate changes in leaf area in some cases destructive methods, like clipping and scanning are used (Fleck et al., 2012). With a sticky piece of millimeter-paper and a high contrast background, photos, in combination with image analysis software (e.g. FIJI) would be a non-destructive way to measure leaf area, similar to the approach by Nasution et al. (2021). This and similar approaches utilizing digital image analysis could further be used to count e.g. mites on the leaf (using high-resolution photos) and calculate the mite population density per cm^2 leaf area.

Remote sensing using drones or satellite images can give a general idea of plant health by e.g. estimating chlorophyll content (Elarab et al., 2015), but it is currently not developed well enough to be able to infer information on e.g. pest abundance, which, similar to plant health itself, depends on multiple factors. At least for now, the most reliable results are still produced by manually assessing a representative sample of plants in the field.

Overall, an adequate sample size (e.g. 80-100 datapoints over all replicates in a single treatment) is important to detect whether the plants under investigation have an altered influence on the insect pest (e.g. feeding deterrent, repellent, higher attractiveness) compared to their sister variety. Similarly, we suggest to utilize a grid-based systematic random sampling procedure like described by Wulfsohn (2010) to reduce location based sampling bias within field plots. By sampling the plants this way, the potential impact of differences in lighting, soil factors and other external influences (location bias) on the outcome is statistically reduced. In a greenhouse/lab setup where all the experimental units will be sampled, physically distributing the plants similar to a Latin-square (Freeman, 1979) is advised for the same effect.

F.5.1 Potato

Wireworm

Wireworms (*Agriotes* spp.) are relevant for all of Europe. There are differences in wireworm species damaging potatoes depending on the field trial location chosen. They were chosen to represent soil-dwelling insects that feed on the potato tubers. Their influence on the crop is strongly climate and weather dependent and depends on other vegetation in the field. High economic damage is possible in years when a long dry spell precedes the harvest of potato. The diversity of wireworm species present on trial sites has to be taken into account when planning rearing and lab experiments. For field trials, a monitoring in the preceding year is necessary to find a suitable plot. Lab and greenhouse experiments focusing on new infestation or adult emergence are less practical because of their long lifecycle.

Rearing: very challenging, Cuthbert (1962); Kölliker et al. (2009)

Artificial diet: no

Field experiments: requires high effort monitoring in the previous season, they are technically possible, and work intensive, but finding a fitting location is challenging; artificial infestation via adult beetles technically possible, but not practicable

Greenhouse/lab experiments: very challenging, overall technically possible and practicable

For determining the damage of wireworms in potatoes, the tubers are analyzed based on the EPPO standard PP1/046(3) (2004d). In a field experiment, the sample size would be 100 tubers per plot with each up to 10 tubers per plant (minimum 10 plants/plot) and equal distribution of tubers between replicates. The damage would be classified based on the number of holes with classes for “no holes”, “1-2 holes”, “3-5 holes” and “>5 holes”.

Possibilities to improve assessments based on state-of-the art methods:

In a greenhouse/laboratory setting, the potatoes might be either grown in pots or presented directly to the wireworms, for feeding, for an adequate amount of time. This allows for an even distribution of wireworms between the experimental units, but also requires documentation of the larvae’s developmental stage (measurement of head capsule width) and feeding behaviour.

In the field, the minimum 10 investigated potato plants per plot should be chosen based on a predetermined grid to reduce sample location bias. The holes in the tubers could also be counted without grouping them in classes to use the hole number as a scalar parameter.

Potato Beetle

The potato beetle (*Leptinotarsa decemlineata*) was chosen to represent insect pests feeding on leaves and other plant organs above the ground. Under optimal circumstances, it can occur in masses and cause high economic damages, because the larvae can skeletonize the plants. The species is relevant for most of Europe and both its occurrence and damage are well quantifiable. Rearing them is not challenging and both field trials and lab/greenhouse experiments are easily conductible.

Rearing: yes, Gelman et al. (2001)

Artificial diet: yes, Gelman et al. (2001)

Field experiments: requires low effort monitoring in the previous and in the experiment season, overall low difficulty, technically possible and practicable; artificial infestation via adult beetles technically possible

Greenhouse/lab experiments: low difficulty, overall technically possible and practicable

For the potato beetle the EPPO standard PP1/012(4) (2008) is used to estimate the damage. In the field, at least 10 marked plants should be observed for infestation by potato beetles and their larvae. The change in leaf area should also be evaluated on a plant-by-plant basis and the larvae development should be recorded over time.

Possibilities to improve assessments based on state-of-the art methods:

This method can also be used in greenhouse or pot experiments in the laboratory with no or only minor changes.

In the field, the 10 investigated potato plants per plot should be chosen based on a predetermined grid to reduce sample location bias. Depending on the plot size, an increase in sample size is also advisable to cover more ground (e.g. 1 plant/2m²). The protocol could be expanded by methods to quantify leaf area (e.g. as described in Fleck et al., 2012).

Aphids

Aphids are only rarely relevant based on their plant damage alone. In most cases, the damage to the plant is caused by transmitted diseases, and not the aphids themselves, which are also covered in VCU protocols (e.g. potato leaf roll virus, potato virus Y). Artificial infestation with virus-infected aphids can be done in a fashion based on Hossain et al. (2021), who infested 10% of sugar beet plants with virus-infected aphids at an early stage in plant development.

Rearing: yes, Nilsen et al. (2013); Li and Akimoto (2018)

Artificial diet: semi artificial, Li and Akimoto (2018)

Field experiments: requires monitoring in the experimental season, challenging and work intensive, but overall technically possible; artificial infestation via adult aphids technically possible

Greenhouse/lab experiments: challenging and work intensive, but overall technically possible

For aphids on potato, the EPPO standard PP1/230(1) (2004a)) can be applied especially for Myzus persicae and Macrosiphum euphorbiae. Twenty-five plants per plot are monitored for aphid occurrence and their development, and for virus infection (optical and via molecular biological testing).

Possibilities to improve assessments based on state-of-the art methods:

When artificially infesting field plots or in greenhouse/laboratory experiments, the same amount of asexually reproducing wingless or winged females should be used per experimental unit for infestation.

In the field, the plants should be chosen based on a predetermined grid, to reduce sample location bias. Depending on the plot size, an increase in sample size is also advisable to cover more ground (e.g. 1 plant/1m²). The monitoring would be focusing on the flight period of the relevant aphid species, which will require parallel monitoring of the pest during the experimental period.

Late blight (*Phytophtora infestans*)

Late blight occurs in all potato-growing regions of the world. Warm, humid weather favours its occurrence. Yield losses are often 20 to 40%, and total yield loss can occur under extreme infestation pressure (Radtke et al., 2000). In Ireland, Dowley et al. (2008) determined mean yield reduction of 10.1 t/ha in the untreated control compared to the variants with fungicide application over a 25-year period. Rakotonindraina et al. (2012) found yield reductions in untreated controls of susceptible cultivars Bintje and Desirée of 76.9 and 72.1%, respectively, compared to the variant with fungicide application, as an average of a four-year series of trials at INRA's Pluodaniel Experimental Station, Brittany, France.

The French guideline (GEVES, 2021b) for assessing the susceptibility of candidate varieties to late blight provides for the inclusion of reference varieties of different susceptibility from different maturity groups and the Bintje variety as a susceptible variety. According to the cultivation plan in the form of a block trial with two replicates, each test and control variety is adjacent to plots of the Bintje variety. There are five plants per plot. With variety Bintje, infestation can start early in the season and relevant infestation pressure can build up as the season progresses.

With the appearance of the first spots, weekly observations and records are made until the foliage is completely infested.

The extent of infestation is assessed visually based on the percentage of diseased foliage, following the scoring scheme developed by the British Mycological Society: Infestation values 0 (no symptoms), 0.1 (1 to 4 leaf spots), 1 (up to 10 spots), 5 (up to 50 spots), 10 (=10% foliage destruction), 25, 50, 75, 90, 100 (=100 % loss of foliage) will be assigned to the plot (Large, 1952).

The use of a natural source of infection in this test method based on a highly susceptible cultivar is instrumental in achieving sufficient infestation pressure to reveal differences in varietal responses.

Possibilities to improve assessments based on state-of-the art methods

Regarding new phenotyping methods, it is referred to the Plant Village Dataset for developing mobile disease diagnostics (Hughes et al., 2015) as an image dataset. In the case of late blight, CNN (Convolutional Neural Networks) models were tested by Chakraborty et al. (2022) using this database for automation-assisted infestation assessment. In this work, the VGG16 (Visual Geometry Group, 16 weight layers) model was able to achieve over 97% accuracy in classifying between healthy potato leaves and symptoms of early blight and late blight.

F.5.2 Maize

Wireworm

Similar to potatoes, relevant wireworm species (*Agriotes* spp.) are taken into account. They are representing soil-dwelling insects feeding on the roots, especially during seedling stage. The remaining factors are the same as above.

Rearing: very challenging, Cuthbert (1962); Kölliker et al. (2009)

Artificial diet: no

Field experiments: requires high effort monitoring in the previous season, they are technically possible, and practicable, but finding a fitting location is challenging; artificial infestation via adult beetles technically possible, but not practicable

Greenhouse/lab experiments: practicable and technically possible, but challenging

For determining the damage of wireworms in maize, emergence is observed based on the EPPO standard PP1/046(3) (2004d). In a field experiment, the number of healthy and damaged or missing plants is checked at 75% emergence and another time when most plants passed the critical seedling stage (5-6 leaf stage). At that point, 25 random plants per plot are checked for holes at the base of the plant.

Possibilities to improve assessments based on state-of-the art methods:

In the field, the first evaluation should take into account multiple rows or row parts (e.g. 4-5 lengths of 5 m) per plot to reduce sample location bias. Similarly, the 25 randomly chosen plants of the second evaluation should be chosen based on a predetermined grid to reduce sample location bias.

Because of the high damage potential of wireworms, a lab/greenhouse setup would need a high maize-to-wireworm ratio, enough substrate for wireworm survival and a satisfactory number of replicates to work in a greenhouse/laboratory setting. It allows for an even distribution of wireworms between the experimental units, but also requires documentation of the larvae's developmental stage (measurement of head capsule width) and feeding behaviour.

Corn-rootworm

The corn-rootworm (*Diabrotica virgifera virgifera*) was chosen to represent both insects feeding on roots and insects feeding on leaves, as well as the plant's stigmata. They are very specialized and require corn to finish their life cycle. Without crop rotation, their population will increase manifold and they will cause a lot of damage. The larvae will damage the seedlings, in many cases causing lodging at a later stage of development ("goose-necking"). The adults can cause reduced fertilization and therefore reduced yield. The corn-rootworm does not (yet) occur in some corn cultivation areas of Germany, France, Romania, Italy and Belgium. Both rearing and field trials, as well as lab/greenhouse experiments are possible using adult beetles or eggs under natural conditions or with artificial infestation. Evaluation of root damage in cell based field trials are work intensive, but well executable. They require extraction and cleaning of the roots, as well as setup of ground covering cages for bug catching (eclectors). Lab and greenhouse experiments do not pose a challenge.

Rearing: challenging, Jackson (1986)

Artificial diet: partially as addition to regular food

Field experiments: requires low effort monitoring in the previous season, damage evaluation is work intensive, but of low difficulty and technically possible; artificial infestation via adult beetles or eggs (with slightly wet substrate) is challenging, but technically possible

Greenhouse/lab experiments: challenging and work intensive but technically possible

The EPPO standard PP1/212(2) (2011), which is used to quantify damage caused by Diabrotica larvae is based on the Node injury scale by Oleson et al. (2005). A fully damaged crown root ring has a value of 1.00, the 2 decimals allowing the evaluation of partially damaged rings. At least 10 plants per plot are dug up; the roots are carefully washed and then investigated for damage. The distance between individual plants should be at least 1 m.

Possibilities to improve assessments based on state-of-the art methods:

In the field, the plants should be chosen based on a predetermined grid, to reduce sample location bias. Depending on the plot size, an increase in sample size is also advisable to cover more ground (e.g. 1 plant/2m²). Similar to wireworms, a greenhouse/laboratory setup needs a high maize-to-larvae ratio, enough substrate for larvae survival and a satisfactory number of replicates. Using a predetermined amount of eggs (based on determining the number of eggs in the substrate statistically), allows for an even distribution of larvae between the experimental units.

European corn borer

The European corn borer (*Ostrinia nubilalis*) represents insects that feed on leaves and stem, and protect themselves by mining inside the plant. Under optimal circumstances (organic corn, weather/climate, crop parts remain above soil after harvest) large economic damage is possible. The corn borer is relevant for most of Europe. Rearing them is challenging, but technically possible. Quantifying damages is practicable, but needs a large sample size, depending on the circumstances. If combined with rearing, experiments in lab/greenhouse are possible.

Rearing: challenging, but technically possible, Shorey and Hale (1965)

Artificial diet: yes, Shorey and Hale (1965)

Field experiments: requires monitoring in the previous season damage evaluation is work intensive, but of low difficulty and technically possible; artificial infestation via adult moths technically possible, but challenging and work intensive

Greenhouse/lab experiments: challenging and work intensive but technically possible

For Ostrinia nubilalis, the EPPO standard PP1/013(3) (1997c) lists a sample size of 20 plants with 5 plants each taken from 4 different rows per plot. The plants would be cut close to the ground and split open. Both the number of holes and the number of larvae as well as their location in the stem would be recorded for each plant. Further damage to the plant (broken stem, feeding traces on cob) would also be recorded. This monitoring would be repeated multiple times during the season, starting with milky ripeness (BBCH 75) and ending shortly before harvest.

Possibilities to improve assessments based on state-of-the art methods:

In the field, the plants should be chosen based on a predetermined grid, to reduce sample location bias. The grid would shift for every observation date. For lab experiments, it is recommended to e.g. record development speed of and other effects on the larvae fed with either a natural diet or an artificial diet based on chopped corn stalks (e.g. based on Shorey and Hale (1965)). In a greenhouse, it might be possible to work under field-like conditions if live adults are introduced to separate enclosures (aeraria) with maize plants. The sample size should still amount to at least 10 plants per variant and observation date.

In a greenhouse setting, the maize plants need to be grown to whorl stage (BBCH 25-30) to be attractive for egg deposition by adult moths (Udayagiri and Mason, 1995) and allow for hatching larvae to feed under realistic, field-like conditions.

There currently are no satisfying methods to non-destructively investigate *in-situ* how many larvae inhabit a corn stalk (Keszthelyi et al., 2020).

Aphids

Aphids are only rarely relevant based on their damage alone. In most cases, the damage to the plant is caused by diseases transmitted by aphids, which are less relevant for corn. Artificial infestation with infected aphids can be done in a fashion similar to Hossain et al. (2021), who infested 10% of sugar beet plants with pathogen-infected aphids at an early stage in plant development.

Rearing: yes, Nilsen et al. (2013); Li and Akimoto (2018)

Artificial diet: semi artificial, Li and Akimoto (2018)

Field experiments: requires monitoring in the experimental season, challenging and work intensive, but overall technically possible; artificial infestation via adult aphids technically possible

Greenhouse/lab experiments: challenging and work intensive, but overall technically possible

For aphids on maize the EPPO standard PP1/245(1) (2005) can be applied for Rhopalosiphum padi, Rhopalosiphum maidis, Metopolophium dirhodum, Sitobion avenae and Schizaphis graminum. Ten plants per plot are monitored for aphid occurrence, splitting the plant in a bottom, middle and top section. In case of sweet corn, the focus should lie on the leaves around the cob.

Possibilities to improve assessments based on state-of-the art methods:

In the field, the plants should be chosen based on a predetermined grid, to reduce sample location bias. Depending on the plot size, an increase in sample size is also advisable to cover more ground (e.g. 1 plant/2m²). The monitoring would be focusing on the flight period of the relevant aphid species, which will require parallel monitoring.

When artificially infesting field plots or in greenhouse/laboratory experiments, the same amount of asexually reproducing wingless or winged females should be used per experimental unit for infestation.

F.5.3 Oilseed rape

Rape stem weevil and cabbage stem weevil

The rape stem weevil (*Ceutorhynchus napi*, Gyllenhaal, 1837) and /or the cabbage stem weevil (*Ceutorhynchus pallidactylus*, Marsham, 1802) represent the insects that feed on and mine in stems of oilseed rape. Depending on the date of sowing, the temperatures/development of the plant, the temporal and spatial proximity to other Brassicaceae, there is a high damage potential. Some weevils feed exclusively on Brassicaceae and occur all over Europe. Rearing them is pretty challenging, since they require live plants to feed on. Field trials are possible, but should be preceded and combined with a monitoring via pan traps. Lab/greenhouse experiments will need to be combined with rearing of the weevils or live traps (kairomones). A visual evaluation of the damage is possible, but impractical because of oilseed rape growing very dense towards the end of the season.

Rearing: very challenging, Ganga Visalakshy and Krishnan (2001); Smith et al. (2009)

Artificial diet: not specifically, Earle et al. (1966); Sue et al. (1980)

Field experiments: requires monitoring in the previous and experimental season damage evaluation is work intensive, but of low difficulty and technically possible; artificial infestation via caught adult beetles is technically possible

Greenhouse/lab experiments: challenging and work intensive, but technically possible

For the two weevils mining in oilseed rape stems, the EPPO standard PP1/219(1) (2002) can be applied. It advises to both assess the occurrence and identity of adult weevils via yellow water pan traps, as well as assessing the number of larvae between flowering and end of flowering (BBCH 65-70). Twenty plants per plot should be checked for larvae by cutting them and splitting them open. The number of larvae and the area they inhabit should be recorded for each plant. Additionally visible symptoms of feeding damage in the plot (stunted growth, loss of leaves, increased number of lateral shoots) should be recorded.

Possibilities to improve assessments based on state-of-the art methods:

Depending on the number of adults caught in the water traps (or actively introduced to infest the plants), the number of plants to investigate should be increased to ensure a satisfactory sample size. In the field, the plants should be chosen based on a predetermined grid, to reduce sample location bias. The grid would shift for every observation date, since the sampling is destructive. There currently are no satisfying methods to non-destructively investigate *in-situ* how many larvae inhabit an oilseed rape stalk (Keszthelyi et al., 2020).

The lack of published artificial diets for Ceutorhynchus spp. larvae makes it difficult to conduct small-scale experiments in the lab. Field-like greenhouse experiments similar to the one listed for the European corn borer are possible. An artificial infestation of the experimental units, using caught adults, should be timed based on plant phenology.

Turnip sawfly

The turnip sawfly (*Athalia rosae rosae*) was chosen to represent insects feeding on oilseed rape leaves. It thrives in high temperature and under dry conditions and has multiple peaks of flight activity over the year. Economic damages occur in regions with a high portion of summer oil crops (e.g. high amount of mustard). The pest occurs all over Europe. Rearing them is not challenging. For field trials, it is necessary to monitor the location in the preceding year via pan traps. Lab/greenhouse experiments need to be combined with rearing.

Rearing: yes, Sawa et al. (1989)

Artificial diet: yes, Macedo et al. (2005)

Field experiments: requires monitoring in the previous season; overall, of low difficulty, practicable and technically possible; artificial infestation via adult sawflies is technically possible

Greenhouse/lab experiments: of low difficulty and technically possible, but work intensive

For the turnip sawfly the EPPO standard PP1/233(1) (2004b) can be applied. In the field, 25 plants per plot are marked and the larvae counted. The leaf area loss should also be estimated on a plant-by-plant basis on the marked plants in a defined timeframe.

Possibilities to improve assessments based on state-of-the art methods:

In the field, the plants should be chosen based on a predetermined grid, to reduce sample location bias. Depending on the plot size, an increase in sample size is also advisable to cover more ground (e.g. 1 plant/2m²). In a greenhouse or lab setting, the experiment can be executed in a similar fashion with artificial infestation with a predetermined number of adults or larvae of the same development stage per plant. All plants would need to be enclosed separately.

In case of greenhouse/laboratory experiments, the plants should provide enough leaf mass for one or ideally multiple generations of sawflies.

Rape beetle

The rape beetle (*Brassicogethes aeneus*) represents insects feeding on oilseed rape flower buds. It is almost omnipresent in Europe and always occurs in areas of oilseed rape cultivation. The economic damage is highly dependent on the oilseed rape development and the temperatures (flowering time and duration). Field trials should always be combined with pan trap monitoring. Damage can be quantified optically during flowering or by using yield loss when comparing treatments.

Rearing: challenging, Bromand (2009); Seimandi (2018)

Artificial diet: no

Field experiments: the beetle is likely to be found (almost) everywhere suitable, monitoring during experiment season suggested, damage determination is challenging and work intensive; artificial infestation with caught or reared adult beetles is technically possible.

Greenhouse/lab experiments: very challenging, since they require blooming plants for damage determination, overall technically possible, but not practicable.

The EPPO standard PP1/178(3) (2004c) is used for determining the effect on rape beetles. In a field setting, 50 main shoots would be selected in the center area of a plot to count the beetles, either visually on the shoot or via beating net/tray. For repetition it would be advisable to mark the observed plant and observe the plants under similar conditions (daytime, weather, wind speed, temperature) again after a few days (BBCH 55-62).

Possibilities to improve assessments based on state-of-the art methods:

In the field, the plants to observe should be chosen based on a predetermined grid, to reduce sample location bias. Depending on the plot size, an increase in sample size is also advisable to cover more ground (e.g. 1 plant/2m²). For a final evaluation, the yield of the different plots is compared.

Since the actual damage to flower buds and subsequently yield loss strongly depend on the timing of flowering and beetle phenology, lab or greenhouse experiments that provide valuable data are challenging. Artificial infestation with caught or reared rape beetles would need to be precisely timed to cover the late development of flower buds before blooming, where most damage occurs.

Cabbage stem flea beetle & Turnip flea beetle

The cabbage stem flea beetle (*Psylliodes chrysocephalus*) and the turnip flea beetles (*Phyllotreta atra*, *P. cruciferae*) represent insects feeding on leaves. They occur in areas of Brassicaceae cultivation all over Europe and damage especially young seedlings short after germination (reduced development speed). The larvae need live plants in which they mine (leaves, stems, roots), which increases the difficulty of rearing them. Field trials should be combined with a preceding pan trap monitoring. Lab/greenhouse experiments are best combined with rearing of the pest.

Rearing: yes, Nagalingam and Costamagna (2019)

Artificial diet: no

Field experiments: requires monitoring in the previous and experiment season challenging, but both practicable and technically possible; artificial infestation with caught or reared adult beetles technically possible

Greenhouse/lab experiments: practicable and technically possible, but challenging

For *Phyllotreta* species on oilseed rape, the EPPO standards PP1/218(2) (2020a) and PP1/073(4) (2020b) are used. In the field, yellow water pan traps are used to determine which species are present. At low infestation rates (e.g. with only 2-5% loss of leaf area), a selection of plants (e.g. 50/plot) is rated as “damaged” or “not damaged” and the number of holes is recorded. At high infestation rates (80% or more damaged plants) 25 plants per plot are classified based on the damage (0-2%, >2-5%, >5-15%, >15-30%, >30-50%).

Possibilities to improve assessments based on state-of-the art methods:

To reduce location bias in the field, the observed plants should be selected based on five clusters of 10 plants along a row. The clusters should be evenly distributed in the field. If the infestation rates are high, the cluster size can be reduced to five, observing every second plant in the row. If the plants or the sections are marked, the same plants can be observed multiple times until BBCH 14.

The experiments can be conducted in a similar fashion in a greenhouse or lab setting. Individual trays with multiple seedlings can be used per aerarium with multiple individual aeraria acting as replicates. A predetermined number of adult insects is then used for artificial infestation (Block based choice experiment).

Cutworms

Cutworms (Noctuidae) were chosen to represent soil-dwelling insects feeding on roots and leaves. They can be found all over Europe. Rare mass occurrences can lead to economic loss. Rearing is simple, because they accept an artificial diet. Lab and greenhouse experiments are easy to execute in combination with rearing. Field trials are more challenging, because the pest might be unevenly distributed.

Rearing: challenging, Shorey and Hale (1965)

Artificial diet: yes, Shorey and Hale (1965)

Field experiments: requires monitoring in the previous season, monitoring in the experiment season suggested, finding a fitting location is challenging, overall both practical and technically possible; artificial infestation with adult moths is technically possible

Greenhouse/lab experiments: challenging, but overall both practical and technically possible

To determine the damage of cutworms, the EPPO standard PP1/249(1) (2006) is employed. Maize and other plants would be treated the same way. In the field, four lengths of 10 m would be investigated for healthy, damaged and cut/missing plants at emergence, BBCH 12-14 and 14-20 days later.

Possibilities to improve assessments based on state-of-the art methods:

In the field, the four lengths should be distributed evenly over the plot to avoid location bias. If marked, the investigated plants can be observed over time.

The experiments can be conducted in a similar fashion in a greenhouse or lab setting. Individual trays with connected substrate-space with multiple seedlings can be used per aerarium with multiple individual aeraria acting as replicates. A predetermined number of adult moths is then used for artificial infestation for example in a block based choice experiment.

Aphids

Aphids are only rarely relevant based on their damage alone. In most cases, the damage is caused by transmitted diseases. Artificial infestation with infected aphids can be done in a fashion similar to Hossain et al. (2021), who infested 10% of sugar beet plants with infected aphids at an early stage in plant development.

Rearing: yes, Nilsen et al. (2013); Li and Akimoto (2018)

Artificial diet: semi artificial, Li and Akimoto (2018)

Field experiments: requires monitoring in the experimental season, challenging and work intensive, but overall technically possible; artificial infestation via adult aphids technically possible

Greenhouse/lab experiments: challenging and work intensive, but overall technically possible

In this case, the EPPO standard PP1/324(1) (2019) can be applied for *Brevicoryne brassicae*, *Myzus persicae*, *Lipaphis erysimi* and *Macrosiphum euphorbiae*. Twenty-five plants per plot are monitored for aphid occurrence and their development, and for virus infection (optical and via molecular biological testing).

Possibilities to improve assessments based on state-of-the art methods:

In the field, the plants should be chosen based on a predetermined grid, to reduce sample location bias. Depending on the plot size, an increase in sample size is also advisable to cover more ground (e.g. 1 plant/1m²). The monitoring would be focusing on the flight period of the relevant aphid species, which will require parallel monitoring.

When artificially infesting field plots or in greenhouse/laboratory experiments, the same amount of asexually reproducing wingless or winged females should be used per experimental unit for infestation.

Cylindrosporiose wilt (*Cylindrosporium concentricum*)

Cylindrosporiosis wilt is the anamorph of leaf spot disease (*Pyrenospeziza brassica*) and one of the most important biotic stressors to oilseed rape in France and the United Kingdom (Paul, 2012). In general, mild leaf spot disease occurs in temperate climates in Europe, Australasia, and Southeast Asia. Among the brassicas, canola is considered susceptible. McCarntney et al. (2007) mention yield losses of up to 22% in the United Kingdom, and Bradburne (1999) even of up to 50%.

The French VCU protocol (GEVES, 2021b) provides for a series of measures to establish relevant disease infestation: Small plot trials with at least 100 plants per plot and 30 to 35 plants/m² are sown in four replications towards the end of the local sowing time interval. To increase infection pressure, four contaminated canola stalks from the previous year are laid out per square meter. In addition, sprinkling or misting is to be applied twice a day for 20 minutes in the fall. In the event of spring drought, this should be continued from the beginning of flowering. Disease observations record intensity and infested area according to the 1-9 scale 1 =healthy, 3 <25%, 5 <50%, 7 <75%, and 9 >75% infested plants and are conducted on leaves and later on stems and pods.

Sclerotinia Stem Rot (*Sclerotinia sclerotiorum*)

In winter oilseed rape, yield reduction caused by this disease can reach 30% or more, mainly due to lower numbers of kernels per area, lower seed weight and higher preharvest seed losses (Paul, 2012). Kirkegaard et al., (2006) reported 1.3% yield loss for each percentage point of infected plants. A smaller, but still relevant, yield impact of 0.5% for each percentage point of infested plants was found by Del Rio et al. (2007).

Natural infection

In VCU protocols (AT, CZ), natural infection is the source of infection with Sclerotinia disease. However, the extent of infestation strongly depends on the number of overwintering sclerotia in the soil and on weather conditions, especially at flowering time. In order to increase the infection pressure, a kind of provocation area can be created by repeated cultivation of winter oilseed rape on the same area, which is in contrast to the usual recommendation in good agricultural practice. By applying such methodology it is possible to observe the susceptibility behaviour of varieties more reliably (see soybean).

Approaches with targeted artificial inoculation

Bradley et al. (2006) conducted spray infection with ascospores suspensions in field trials followed by misting over several weeks. Significant differences in the response of the varieties could be found in the field trials, though the disease incidence levels varied depending on year and location. Concerning the use of ascospores as inoculum agent Derbyshire and Denton-Giles (2016) indicated that, it is delicate and tedious to reliably provide sufficient ascospores as inoculum in the laboratory.

Various pathogen bioassays with different artificial infection techniques under defined environmental conditions have been developed:

For the petiole inoculation technique (PIT, according to Del Rio et al., 2001), the leaf blade of the third fully developed leaf is removed and the petiole shortened to 2.5 cm from the stem. A pipette tip with mycelium of Sclerotinia sclerotiorum on potato dextrose agar as inoculum is inverted over the petiole. Data recording over a period of 6 or up to 12 days included the number of days until irreversible wilting and also the scores 0 to 4 of the plant lesions on the last day of wilt observation (Zhao et al., 2004) or plant mortality on each day as basis for an area- under-disease-progress-curve (Bradley et al., 2006). In both cases, the results showed significant differences in the response of the genotypes. The validity of these results under field conditions was not investigated in the first study and could not be confirmed in the second study.

A detached leaf assay (DLA) was applied also by Bradley et al. (2006, see above) as a method in controlled environments. A plug (5 mm) of a Sclerotinia culture on potato dextrose agar is placed in the centre of the leaf. The infected leaves were incubated for 24 h at 21°C. However, the genetic differences in susceptibility towards stem rot observed in the field trials could not be confirmed in the DLA. Rather, all genotypes showed similar infestation behaviour.

Infected toothpicks pierced into canola stems were used successfully by Zhao and Meng (2003) for revealing phenotypic variation in a rapeseed population. Resistance recording (size of stem lesions) was carried out on mature plants.

However, injuring sound plants is to be seen critical as infection barriers such as the epidermis are bypassed by these approaches: “Resistance to Sclerotinia sclerotiorum in Brassica napus is a result of retardation of pathogen development, both on the plant surface and within host tissues.” (Garg et al., 2010).

Possibilities to improve assessments based on state-of-the art methods

Gupta et al. (2020) developed an inoculation technique in the field without injury to the plants. Fresh mycelium on potato dextrose agar wrapped with a water-soaked cotton swab to the internode region in the lower stem area served as the source of infection. The length and width of the stem lesions were taken as criteria for disease development of Sclerotinia stem rot. The method, which more or less simulates the way of natural infection via fallen petals in the leaf axils, also showed good correlations to infestation occurrence under natural conditions.

Garg et al. (2008) applied a cotyledon-inoculation-assay, primarily developed for legumes, in B. napus. Cotyledons of ten-day-old seedlings from 32 genotypes were inoculated with four droplets of a Sclerotinia sclerotiorum mycelial suspension under controlled conditions. Genotypes showing significant differences in the response to the Sclerotinia infestation in the first run were chosen for two further repetitions of the treatment. The correlations of the genotype reactions between the different runs and to available field data were significantly positive. Thus, the cotyledon inoculation method can be seen as a rapid and reliable screening test of B. napus genotypes for their resistance against stem rot.

Furthermore, with regard to a very natural and reliable infection process, the methods with agar plugs wrapped in cotton swabs seems to be the most suitable. It does not require any injuries to plant parts, which facilitate the penetration of the Sclerotinia fungus. The water-saturated swab maintains the necessary moisture in the infection area sufficiently well, even under field conditions.

An overview of artificial infection methods applied can be found in Derbyshire and Denton-Giles (2016), according to which the non-injuring agar plug method is used very frequently. The quantification of the infestation is mostly achieved by detection of the lesion size.

F.5.4 Soybean

Bean seed fly

The bean seed fly (*Delia platura* and *Delia florilega*) represents insect pests, which damage especially the seeds or young seedlings. They occur almost all over Europe and cause damage depending on the weather around germination. Rearing is potentially possible, but there might be a difference in locally relevant species. It might be best to focus on pan trap monitoring and field trials.

Rearing: challenging, Ishikawa et al. (1983); Guerra et al. (2017)

Artificial diet: yes, Ishikawa et al. (1983)

Field experiments: requires monitoring in the previous season, monitoring in the experimental season is suggested, overall challenging, but both practical and technically possible; artificial infestation with adult flies is technically possible

Greenhouse/lab experiments: challenging, but both practical and technically possible

To quantify the damage of bean seed flies, the EPPO standard P1/034(2) (1997a) is utilized. In the field, the plants are investigated for damages at 50% plant emergence until no more additional emergence is visible.

Possibilities to improve assessments based on state-of-the art methods:

In the field, damages, missing plants and deformities are recorded for seedlings along 4-5 lengths of 1m, evenly distributed over the plot to reduce location bias.

The experiments can be conducted in a similar fashion in a greenhouse or lab setting. Individual trays with connected substrate-space with multiple seedlings can be used per aerarium with multiple individual aeraria acting as replicates. A predetermined number of adults is then used for artificial infestation for example in a block based choice experiment.

Southern green stinkbug

The southern green stinkbug (*Nezara viridula*) was chosen to represent bigger sucking pests, which can cause a lot of damage to the crop. It already occurs in about half of the countries of Europe. Rearing is easy, but resource intensive (feeding). Both field trials and lab/greenhouse experiments are easily executable. Alternatively, the damage can be estimated based on visual cues of the harvested crop.

Rearing: yes, Medal et al. (2012)

Artificial diet: no

Field experiments: requires monitoring in the previous season, overall challenging, but practicable and technically possible; artificial infestation with adult bugs is suggested

Greenhouse/lab experiments: overall challenging, but practicable and technically possible

In lack of an EPPO standard to quantify the damages caused by the southern green stinkbug, a combination of the EPPO standard for potato beetles (2008) and a harvest damage screening are suggested. In the field, at least 10 marked plants per plot should be observed for infestation by the Southern green stinkbug and their nymphs. The nymphs should be counted and their development recorded over time. This method can also be used in greenhouse or pot experiments in the laboratory. Depending on the number of replicates, the total amount of observed plants should be equal to or exceed 40 observed soybean plants per variant in field trials.

Possibilities to improve assessments based on state-of-the art methods:

In the field, the 10 investigated soybean plants per plot should be chosen based on a predetermined grid to reduce sample location bias. Depending on the plot size, an increase in sample size is also advisable to cover more ground (e.g. 1 plant/2m²). To quantify the damage on the harvested crop, soybeans should be harvested plot by plot and a sample of 100 beans per plot should be investigated for visual deformations and entrance wounds caused by the bugs. For this purpose the beans can be dyed with acid-fuchsin solution and checked for UV autofluorescence of lignin (Giacometti et al., 2020).

On a smaller scale, similar experiments can be conducted also in a greenhouse/laboratory setting using whole plants. Alternatively, fresh soybeans (like the vegetable soybeans edamame) can be presented to N. viridula in a choice experiment, before being analyzed using the methods mentioned above.

European red mite (and other mites)

The European red mite (*Panonychus ulmi*) represents acari on soybeans. It occurs in about half of the countries in Europe and can lead to economic damages under drought conditions. Because of their small size and limited mobility, it might be beneficial to combine both field trials and lab/greenhouse experiments with artificial infestation.

Rearing: challenging, Bustos et al. (2009)

Artificial diet: no

Field experiments: requires monitoring in the previous and experimental season, very challenging and work intensive, but overall technically possible; artificial infestation is required

Greenhouse/lab experiments: challenging and work intensive, but technically possible

In lack of an appropriate EPPO standard, it is suggested to quantify both the damages and the mites per leaflet in representative samples. Similar to EPPO standards P1/192(2) (1997b), PP1/112(2) (1997d) and (1997e), 10 plants per plot are marked for mite observation. The mites on the plants are quantified on at least one leaflet per plant. The different life stages (egg, nymph, adult) should be recorded separately.

Possibilities to improve assessments based on state-of-the art methods:

In the field, the 10 investigated soybean plants per plot should be chosen based on a predetermined grid to reduce sample location bias. Depending on the plot size, an increase in sample size is also advisable to cover more ground (e.g. 1 plant/2m²). Of each plant 12 leaflets (based on Storck et al. (2012)) in the lower to middle plant height are chosen and the number of live mites is determined by beating them onto a sheet of paper (other leaflets on the same leaf are temporarily sleeved with small paper envelopes). If the whole leaf is covered with webs and live mites, the whole leaf is sampled and counted as three leaflets.

In the field, a high infestation is only possible during a drought period. Semi field setups with artificial infestation and a rain shield or field-like greenhouse experiments might be beneficial. There is also an increased fringe and neighboring effect that needs to be taken into account.

Additionally, the damages can be estimated based on the state of the leaves (Jardine, 2020). The classification is as follows:

0 No spider mites or injury observed.

1 Minor stippling on lower leaves, no premature yellowing observed.

2 Stippling common on lower leaves, small areas or scattered plants with yellowing.

3 Heavy stippling on lower leaves with some stippling progressing into middle canopy. Mites present in middle canopy with scattered colonies in upper canopy. Lower leaf yellowing common. Small areas with lower leaf loss. (Spray Threshold)

4 Lower leaf yellowing readily apparent. Leaf drop common. Stippling, webbing and mites common in middle canopy. Mites and minor stippling present in upper canopy. (Economic Loss)

5 Lower leaf loss common, yellowing or browning moving up plant into middle canopy, stippling and distortion of upper leaves common. Mites present in high levels in middle and lower canopy.

Because of the size of mites, it is very important to use very fine meshes when containing the individual experimental units in a greenhouse or laboratory setting.

Painted lady

The painted lady (*Vanessa cardui*) was chosen to represent insect pests feeding on leaves. It occurs almost all over Europe and is one of the main pests on soybean. It is easy to rear, since it accepts artificial diet. Both field trials and lab/greenhouse experiments could be executed. For field trials, it might be beneficial to use artificial infestation.

Rearing: yes, Shorey and Hale (1965)

Artificial diet: yes, Shorey and Hale (1965)

Field experiments: requires monitoring in the previous season, overall of low difficulty, practicable and technically possible; artificial infestation with adult butterflies is suggested

Greenhouse/lab experiments: overall of low difficulty, practicable and technically possible

In lack of an appropriate EPPO standard to quantify the damage caused by painted lady larvae, the EPPO standard for potato beetles (2008) is adapted. In the field, at least 10 marked plants per plot should be observed for infestation by the painted lady larvae. They should be counted and their development recorded over time. This method can also be used in greenhouse or pot experiments in the laboratory. Depending on the number of replicates, the total amount of observed plants should be equal to or exceed 40 observed soybean plants per variant in field trials.

Possibilities to improve assessments based on state-of-the art methods:

In the field, the 10 investigated soybean plants per plot should be chosen based on a predetermined grid to reduce sample location bias. Depending on the plot size, an increase in sample size is also advisable to cover more ground (e.g. 1 plant/2m²).

In case of greenhouse/laboratory experiments, the plants should provide enough leaf mass for one or ideally multiple generations of Vanessa cardui.

Aphids

Aphids are only rarely relevant based on their damage alone. In most cases, the damage is caused by transmitted diseases, which are also covered in VCU protocols (e.g. bean common mosaic virus). Artificial infestation with infected aphids can be done in a fashion similar to Hossain et al. (2021), who infested 10% of sugar beet plants with infected aphids at an early stage in plant development.

Rearing: yes, Nilsen et al. (2013); Li and Akimoto (2018)

Artificial diet: semi artificial, Li and Akimoto (2018)

Field experiments: requires monitoring in the experimental season, challenging and work intensive, but overall technically possible; artificial infestation via adult aphids technically possible

Greenhouse/lab experiments: challenging and work intensive, but overall technically possible

In lack of a dedicated EPPO standard, the standard for oilseed rape (PP1/324(1) (2019)) can be applied especially for Aphis fabae, Myzus persicae and Acyrtosiphon pisum. Twenty-five plants per plot are monitored for aphid occurrence and their development, and for virus infection (optical and via molecular biological testing). When artificially infesting field plots or in greenhouse/laboratory experiments, the same amount of asexually reproducing wingless or winged females should be used per experimental unit for infestation.

Possibilities to improve assessments based on state-of-the art methods:

In the field, the plants should be chosen based on a predetermined grid, to reduce sample location bias. Depending on the plot size, an increase in sample size is also advisable to cover more ground (e.g. 1 plant/1m²). The monitoring would be focusing on the flight period of the relevant aphid species, which will require parallel monitoring.

When artificially infesting field plots or in greenhouse/laboratory experiments, the same amount of asexually reproducing wingless or winged females should be used per experimental unit for infestation.

Sclerotinia stemrot (*Sclerotinia sclerotiorum*)

The selection of Sclerotinia stem rot in soybean is primarily based on economic pest potential, widespread occurrence and the availability of methodological approaches for assessment. Thus, Sclerotinia stem rot in soybean is one of the most important diseases (Willbur et al. 2018) and occurs worldwide (Purdy 1979) with an extensive host plant range of over 400 plant species (Boland 1994). Another aspect contributing to the relevance of assessment as a risk for the crop stand the long survival of the sclerotia in the soil, which can last from two to five years (Adams et al. 1975, Cook et al. 1975, Schwartz et al. 1978) or up to even eleven years (Leite, 2005). In the US, stem rot was among the 10 most important soybean diseases in 6 out of 12 years (Warther et al. 2009). For every 10 % of infected plants, a 0.25 t/ha reduction in yield can be expected (Hartman et al. 2015).

Natural infection

Stem rot is a crop rotation disease. Repeated cultivation of Sclerotinia hosts can build up provocation plots due to the enrichment of sclerotia in the soil and thus increase the infestation pressure on the experimental plots. A short-term increase in sclerotia in the soil can also be achieved with the spreading and shallow incorporation (max. 5 cm) of infected plant residues or sclerotia from other infested areas, as was done, for example, by Wegulo et al. (1998) for their field experiments. But even then, weather conditions still strongly influence occurrence of the disease symptoms Stem rot incidence is favored on sites with high humidity and in rather denser crop stands.

Assessment scale (BAES, 2015) for infestation of soybean with stem rot under field conditions:

1 No infestation

3 Slightly infested (approx. 5% of plants with low or fewer plants with medium symptom expression).

5 Moderately infested (approx. 15% of plants with medium or fewer plants with strong symptom expression)

7 Heavily infested (approx. 25% of plants with medium or fewer plants with strong symptom expression)

9 Very severely infested (more than 50% of plants with strong symptom expression or fewer plants with such strong disease expression that plants die)

Methods with artificial infections:

Chen and Wang (2005) compared three inoculation methods under greenhouse conditions for the evaluation of soybean resistance towards Sclerotinia sclerotiorum:

Spray-mycelium method: Mycelia were grown in a culture medium of potato dextrose broth mycelia, homogenized and then sprayed out on soybean leaves

Drop-mycelium method: A drop of this homogonised culture medium is being dropped on the top of the main stem.

Both mycelium-based infection methods turned out to be less costly compared to the third approach, the cut-petiole method (Del Rio et al., 2001). Mycelial plugs were placed on the shortened petioles (2.5 cm) of the first trifoliate leaf using drinking straws. Observations started on the 3rd day and were continued until the 14th day.

Parameters targeted:

- plant mortality (%) and
- increase of the area-under-wilt-progress-curve (AUWPC).

Results of both, the spray and drop mycelium method, showed sufficient correlation with those of the cut petiole method. The high humidity in the growth chamber may have helped the good consistency of the data.

Possibilities to improve assessments based on state-of-the art methods

Botha et al. (2009) reported an even more comprehensive comparison of methods with six screening techniques for stem rot resistance on four soybean varieties under greenhouse conditions.

- Spray mycelium method (Chen and Wang, 2005)
- Drop mycelium method (Chen and Wang, 2005)
- Cut stem method (Vuong et al., 2004)
- Cotyledon method (Kull et al., 2003, modified by Botha et al. as not the cotyledons but the unifoliated leaves were infested)
- Petiole inoculation method (Del Rio et al., 2001) and
- Straw inoculation method (Auclair et al., 2004)

Parameters recorded aimed at the number of wilted plants (wilting incidence, %), the development of leaf lesions and the extent of plant wilting, both on a 0-to-5 scale.

The spray mycelium method always turned out as the most effective way of inoculation inducing the most severe disease symptoms. Cut stem, drop mycelium and straw inoculation resulted in a comparable disease incidence at a significantly lower level. Inoculation of petioles or cotyledons inoculation provided the mildest disease infection. In the discussion, the advantage of non-infringing methods, such as the spray mycelium-, the drop mycelium- and the modified cotyledon method, is pointed out due to their similarity to the natural infestation situation, as well as the advantage of being able to use resistant genotypes directly for further breeding.

Bastien et al. (2012) reported on a simple and highly reproducible artificial infection method using cotton pads as carrier medium for a mycelium suspension. The pads were wrapped around the flower bud of the first flower-bearing node. The method delivered plausible results both on the field and under greenhouse conditions.

With regard to a very natural and reliable infection process, the cotton swab method seems to be the most suitable. It does not require any injuries to plant parts, which facilitate the penetration of the Sclerotinia fungus. The water-saturated swab maintains the necessary moisture in the infection area sufficiently well, even under field conditions.

F.5.5 Creeping bentgrass

Response to insects/pathogens (biotic stressors)

Overall, not much is known about economically relevant pests on creeping bentgrass in Europe. Our only sources are from US-American literature, which was used to infer information for the European context.

So far, the most dangerous pest for creeping bentgrass found in literature was the larvae of the Japanese beetle (*Popillia japonica*). Since it is a quarantine pest, experiments with it would underlie strict regulations. Many alternative species damage creeping bentgrass to some extent, but not all occur in Europe. Other insects polyphagous on grasses are the larvae of the small heath (*Coenonympha pamphilus*) and speckled wood (*Pararge aegeria*), that have been mentioned as consumers of bentgrass (Settele et al., 2015). The larvae of sod webworms (*Crambus* spp.) are similarly polyphagous and feed on both roots and aboveground tissue of different grasses. Cutworms (*Agrotis ipsilon*, *Peridroma saucia*) also feed on grasses, but they only rarely occur in masses. Of the Blissidae, two species, which can cause economic damage if occurring in masses, occur in almost all of Europe on grasses (*Dimorphopterus spinolae*, *Ischnodemus sabuleti*). However, they are not as damaging as the true chinch bug (*Blissus leucopterus*).

There are more pests mentioned in literature for creeping bentgrass, especially in context of golf court lawn maintenance, which will cause optical damage and only rarely lead to decay of parts of the lawn (Harriman et al., 2015). Of these, only a small portion of species is also relevant for Europe. Other taxa were not listed because of their low distribution or low damage potential. For example, multiple owlet moths (*Spodoptera* spp.), which are either quarantine pests or have a smaller range of distribution than the sod webworms, or have a different host spectrum. In addition, weevils (*Sphenophorus* spp.) were disregarded, because the genus prefers warm and wet meadows. Mole crickets were mentioned in the literature, but the most damaging species (*Scapteriscus* spp.) are quarantine pests in Europe. Related species are not as damaging and rarer. Aphids can be found on grasses (e.g. *Rhopalosiphum padi*), but would only be relevant, if they also transmitted a specific disease. To our knowledge, there are currently no relevant diseases of creeping bentgrass associated with aphids.

Lepidoptera

Rearing: yes, Shorey and Hale (1965)

Artificial diet: yes, Shorey and Hale (1965)

Field experiments: requires monitoring, challenging and work intensive, but technically possible; artificial infestation with adult butterflies/moths is required

Greenhouse/lab experiments: challenging and work intensive, but technically possible

There currently is no standardized way of quantifying the damages caused to creeping bentgrass by lepidopteran larvae. Non-standard approaches determine the number of pests per area or the proportion of damaged and undamaged turf.

In general, the crop/pest combination is suitable for field and greenhouse/lab experiments. Semi field setups with artificial infestation and a rain shield or field-like greenhouse experiments would be beneficial.

Bugs

Rearing: yes, Bustos et al. (2009)

Artificial diet: no

Field experiments: requires monitoring, challenging and work intensive, but technically possible; artificial infestation with adult bugs is required

Greenhouse/lab experiments: challenging and work intensive, but technically possible

There currently is no standardized way of quantifying the damages caused to creeping bentgrass by bugs. Non-standard approaches determine the number of pests per area or the proportion of damaged and undamaged turf.

In general, the crop/pest combination is suitable for field and greenhouse/lab experiments. Semi field setups with artificial infestation and a rain shield or field-like greenhouse experiments would be beneficial.

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Tab. 27: List of investigated insect pests and their relevancy evaluation. PS: plant stage; ER: Economic relevancy; DEU: Distribution in Europe; DG: Duration of a generation; RC: Rearing challenges; EP: Experiment practicability; 1: S: seedling, A: adult; 2: L: larvae; A: adult; *: no rating of economic relevancy possible in European context

Crop	**PS (S, A)**	**Taxon**	**Insect**	**Damage**	**ER**	**DEU**	**DG**	**RC**	**EP**
Potato	S, A	Coleoptera	*Agriotes lineatus* etc. L	Feeds on tubers and plant base	4	5	1	3	2
Potato	S, A	Coleoptera	*L. decemlineata* L, A	feeds on leaves	4	4	5	4	4
Potato	S, A	Aphidoidae	*Myzus persicae* L, A	Vector of diseases	3	5	5	4	3
Corn	S, A	Coleoptera	*Agriotes lineatus* etc. L	Feeds primarily on roots and seedlings	4	5	1	3	4
Corn	S, A	Coleoptera	*Diabrotica v.v.* L, A	Feeds on roots, leaves and crop	3	2	2	3	4
Corn	A	Lepidoptera	*Ostrinia nubilalis* L	Mines inside the stem	4	5	2	3	4
Oilseed Rape	S, A	Coleoptera	*Agriotes lineatus* etc. L	Feeds on roots and seedlings	3	5	1	3	2
Oilseed Rape	S	Lepidoptera	*Agrotis segetum* L	Feeds on plant base	2	5	4	5	3
Oilseed Rape	S	Hymenoptera	*Athalia rosae* L	feeds on leaves	3	5	4	4	4
Oilseed Rape	A	Coleoptera	*Brassicogethes aeneus* A	Damages flower buds	4	5	4	3	3
Oilseed Rape	S, A	Aphidoidae	*Brevicoryne brassicae* L, A	feeds on plant phloem on the tip of the stalks	3	5	5	4	3
Oilseed Rape	S, A	Coleoptera	*Ceutorhynchus napi* L, A	Larvae mine inside the stem	4	3	2	3	3
Oilseed Rape	S, A	Coleoptera	*C. pallidactylus* L, A	Larvae mine inside the stem	3	5	2	3	3
Oilseed Rape	S, A	Coleoptera	*Phyllotreta atra* L, A	Larvae mine in roots/stem, adult feeds on leaves	3	5	4	4	4

Crop	PS (S, A)	Taxon	Insect	Damage	ER	DEU	DG	RC	EP
Oilseed Rape	S, A	Coleoptera	*P. cruciferae* L, A	Larvae mine in roots/stem, adult feeds on leaves	3	5	4	4	4
Oilseed Rape	S	Coleoptera	*Psylliodes chrysocephala* L, A	Larvae mine in roots/stem, adult feeds on leaves	4	5	3	4	4
Soybean	S	Diptera	*Delia florilega* L	Larvae feeds on seedlings	3	5	4	4	3
Soybean	S	Diptera	*D. platura* L	Larvae feeds on seedlings	3	5	4	4	3
Soybean	A	Heteroptera	*Nezara viridula* L, A	Damages the crop by feeding	3	3	5	2	3
Soybean	A	Tetranychidae	*Panonycus ulmi*L, A	can cause wilting	3	3	5	4	3
Soybean	A	Lepidoptera	*Vanessa cardui* L	feeds on leaves	3	5	5	4	4
Bentgrass	A	Lepidoptera	*Coenonympha pamphilus* L	feeds on leaves	*	5	3	4	4
Bentgrass	A	Lepidoptera	*Pararge aegeria* L	feeds on leaves	*	5	4	4	4
Bentgrass	A	Lepidoptera	*Crambus* spp. L	feeds on leaves and roots	*	4	3	4	4
Bentgrass	A	Lepidoptera	*Agrotis ipsilon* L	feeds on leaves	*	5	4	4	4
Bentgrass	A	Lepidoptera	*Peridroma saucia* L	feeds on leaves	*	5	4	4	4
Bentgrass	A	Heteroptera	*Dimorphopterus spinolae* L, A	feeds on plant sap	*	3	5	2	3
Bentgrass	A	Heteroptera	*Ischnodemus sabuleti* L, A	feeds on plant sap	*	5	5	2	3

Tab. 28: Overview of final list of relevant insect pests and example rearing and application protocols. The rearing notes point out, which rearing protocols will need adaptation. * Protocol potentially needs modification; ** Protocol needs modification

Insect species	**Damage**	**Rearing protocol**	**Application example**
Agriotes lineatus	Feeds on roots, tubers, plantbase, seedlings	Cuthbert 1962; Kölliker et al. 2009	Mankin et al. 2008; Sonnemann & Wurst 2012
Leptinotarsa decemlineata	Feeds on leaves	Gelman et al. 2001	Hitchner et al., 2008; Münzbergová & Skuhro-vec 2020
Myzus persicae	Disease vector	Li & Akimoto 2018; Nilsen 2013*	Srinivasan & Alvarez 2011
Diabrotica v.v.	Feeds on roots, leaves and crop	Jackson 1986	Mankin et al. 2008; Robert et al. 2012
Ostrinia nubilalis	Mines inside the stem, damages crop	Shorey & Hale 1965	Münzbergová & Skuhrovec 2020
Agrotis segetum	Feeds on plantbase, roots, tubers	Shorey & Hale 1965	Mankin et al. 2008; Sonnemann & Wurst 2012
Athalia rosae	feeds on leaves	Sawa et al. 1989	Münzbergová & Skuhrovec 2020
Brassicogethes aeneus	Damages flower buds	Bromand 2009; Seimandi et al. 2018	Münzbergová & Skuhrovec 2020
Brevicoryne brassicae	Feeds on plant phloem on the tip of brassica stalks	Li & Akimoto 2018; Nilsen 2013*	Srinivasan & Alvarez 2011
Ceutorhynchus napi	Larvae mine inside the stem	Ganga Visalakshy & Krishnan 2001; Smith et al. 2009**	Mankin 2008; Münzbergová & Skuhrovec 2020
C. pallidactylus	Larvae mine inside the stem	Ganga Visalakshy & Krishnan 2001; Smith et al. 2009**	Mankin 2008; Münzbergová & Skuhrovec 2020
Phyllotreta atra	Larvae mine in roots/stem, adult feeds on leaves	Nagalingam & Costamagna 2019*	Mankin 2008; Münzbergová & Skuhrovec 2020

Insect species	Damage	Rearing protocol	Application example
P. cruciferae	Larvae mine in roots/stem, adult feeds on leaves	Nagalingam & Costamagna 2019*	Mankin 2008; Münzbergová & Skuhrovec 2020
Psylliodes chrysocephala	Larvae mine in roots/stem, adult feeds on leaves	Nagalingam & Costamagna 2019*	Mankin 2008; Münzbergová & Skuhrovec 2020
Delia florilega	Larvae feeds on seedlings	Ishikawa et al. 1983; Guerra et al. 2017	Guerra et al. 2017
D. platura	Larvae feeds on seedlings	Ishikawa et al. 1983; Guerra et al. 2017	Guerra et al. 2017
Nezara viridula	Damages the crop by feeding	Medal et al. 2012	Münzbergová & Skuhrovec 2020
Panonycus ulmi	Can cause wilting	Bustos et al. 2009	Casey & Parella 2005; Opit et al. 2005; Shaw & Wallis 2007
Vanessa cardui	Feeds on leaves	Shorey & Hale 1965	Münzbergová & Skuhrovec 2020
Coenonympha pamphilus	Feeds on leaves	Shorey & Hale 1965*	Münzbergová & Skuhrovec 2020
Pararge aegeria	Feeds on leaves	Shorey & Hale 1965*	Münzbergová & Skuhrovec 2020
Crambus spp.	feeds on leaves and roots	Shorey & Hale 1965*	Mankin 2008; Münzbergová & Skuhrovec 2020
Agrotis ipsilon	Feeds on leaves	Shorey & Hale 1965*	Münzbergová & Skuhrovec 2020
Peridroma saucia	Feeds on leaves	Shorey & Hale 1965*	Münzbergová & Skuhrovec 2020
Dimorphopterus spinolae	Feeds on plant sap	Medal et al. 2012*	Münzbergová & Skuhrovec 2020
Ischnodemus sabuleti	feeds on plant sap	Medal et al. 2012*	Münzbergová & Skuhrovec 2020

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