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Agronomic and phenotypic plant traits as indicators for environmental risks of genetically modified plants

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Abstract

Background For market approval of genetically modified plants (GMPs), the evaluation of agronomic and phenotypic plant traits is a standard requirement and part of the comparative assessment of the GMP and its conventional counterpart. This comparative assessment is a starting point for environmental risk assessment (ERA) and should inform all areas of risk. We scrutinize frequently used approaches to characterize GMPs in EU market applications and discuss their usefulness for drawing conclusions on risks related to the plant's ability to survive, persist or become invasive.

Results Our analysis shows that the agronomic and phenotypic characterization of GMPs, although based on guidelines, is confined to plant traits and test designs that are relevant for the quality control and agronomic performance of genetically modified (GM) crops. We provide evidence of how methodological approaches frequently applied during the agronomic and phenotypic characterization of the GMP could be improved and complemented to better inform on potential phenotypic changes relevant to assessing environmental risks. These approaches refer to (i) the assessment of the survival of GM seeds and plants (e.g., volunteers); (ii) the consideration of environmental exposure and (iii) improved methodological approaches for the assessment of biotic and abiotic stress responses for GMPs.

Conclusions The comparative assessment of agronomic and phenotypic plant traits currently does not provide suitable data to draw conclusions on environmental risks relating to the persistence and invasiveness of the GMP. Ecologically more realistic assessments should be part of the phenotypic characterization of GMPs and need guidance and decision criteria to be implemented in ERA. This is of considerable importance, as new genomic techniques are expected to increase the diversity and complexity of GM plants and traits, particularly stress tolerance, which may affect the survival of GMPs in the environment.

Keywords GMO, Genetically modified plants, Risk assessment, Persistence, Invasiveness, Environmental risks, Agronomic and phenotypic characterization

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Background

When releasing a genetically modified plant (GMP) into the environment, adverse environmental effects may occur directly or indirectly, for example, through the spread, establishment and persistence of the GMP in the environment or the transfer of the inserted genetic material to the same organism or other sexually compatible organisms.

Since the beginning of GMP cultivation in the early 1990s, scientists have addressed environmental risks of

GMPs related to their potential persistence and invasiveness. These risks comprise the ability of GMP to survive, outcross, become persistent or invasive in agricultural or (semi) natural habitats, including the environmental consequences thereof, such as (i) genetic assimilation (replacement of wild genes by crop genes) and reduction of the genetic diversity of wild populations; (ii) contamination of seed pools/seed production/other varieties/land races affecting seed quality; (iii) demographic swamping if hybrids have lower fitness than their wild parents, resulting in population decline or extinction of vulnerable populations; (iv) replacement of wild populations and other plants by GM hybrids with higher fitness; (v) the evolution of new or more resistant weeds affecting control options, e.g., resulting in additional pesticide loads and (vi) disturbance of ecological interactions in off-field habitats [1–10].

To date, some GM crops have established free-ranging populations, either as volunteer, feral, weedy, or wild populations, partly creating environmental problems [2, 11]. The ability of a specific crop to establish a volunteer (within cultivated fields) or feral (outside cultivated fields) population is tightly linked with its phenotype. For example, crops that establish feral populations often exhibit certain ferality traits, such as seed dormancy and the ability to establish a seed bank. Additionally, other common characteristics have been linked to the potential to survive or persist in semi-natural or natural habitats [2, 12, 13]. Apart from inherent traits of the respective crop species, the feralization process itself may involve multiple genomic changes that affect morphology, growth and metabolic traits [14].

For crop genes in general, spontaneous hybridization and gene flow between crops and wild relatives facilitate the escape of transgenes into (semi)natural habitats [7, 15, 16]. Ellstrand, Prentice, Hancock [3] compiled one of the first syntheses on gene flow between domesticated plants and wild relatives and its evolutionary consequences. To understand this, the selective value of the GM trait plays an important role. Traits such as insect resistance can provide a selective advantage to crop–wild hybrids [e.g., 10, 17, 18. However, the fitness benefit or cost of a specific novel trait depends not only on the trait itself, but also on the genetic background and particularly the environment in which the plant thrives [2, 10, 19–21]. Such fitness benefits may only become evident in the long term [15].

Not only may the intended GM trait affect fitness, unintended changes of the genetic modification may also occur that manifest themselves in the phenotype of the GMP and can have an impact on the survivability of the GMP. Unintended changes are defined as "…consistent changes which go beyond the intended change(s) resulting from the genetic modification" (Commission Directive (EU) 2018/350). For example, fitness benefits related to vegetative and fecundity traits have been observed in GM and non-GM herbicide-tolerant plants due to the interference of the EPSPS gene (mediating glyphosate resistance) with key metabolic processes involved in plant growth and development [22, 23] and references therein]. Additionally, unintended effects on the stress response of GM plants are possible if fatty acid profiles are genetically modified [24]. Unintended changes in seed physiology and germination characteristics may change the ability of the GM plant to germinate under less than optimal environmental conditions and therefore increase the likelihood of survival, becoming more persistent or invasive in non-agricultural habitats. With the increased application of new genomic techniques (nGT), e.g., site-directed nucleases such as CRISPR-based approaches, applications targeting complex plant traits, e.g., food quality traits, can be expected to increase in the future [25]. Compared to classical genetic modification techniques, nGT allow the introduction of simultaneous modifications into multiple alleles, into all members of a gene family or into different functional genes. In addition, they enable knockouts and silencing of multiple genes with possible unintended effects, as observed, e.g., with plant susceptibility genes [26, 27]. Multiplexing several genes can result in a considerably altered phenotype, such as de novo domesticated crops [28]. With nGT applications, effects on the environment are likely to increase in scale, as nGT extend modifications beyond classical GM crops [26, 29].

As in most countries worldwide, in the EU, any authorization for a release of genetically modified organisms (GMO) in the environment (import and/or cultivation) is linked to an a priori environmental risk assessment (ERA). This includes an assessment of the potential 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 (Directive 2001/18/EC; Commission Directive (EU) 2018/350, 9). In the EU, GMO risk assessment is conducted by the European Food Safety Authority (EFSA). In its guidance document, EFSA [9] describes a problem formulation approach and staged approach with different stages of information requirements to test hypotheses concerning persistence and invasiveness of the GMP or any of its wild relatives if vertical gene flow is relevant. Information needed for the formulation of risk hypotheses during the staged approach may base on data generated by applicants, data from the scientific literature, or from any other relevant sources [9]. Event specific information from the agronomic and phenotypic characterization in this respect is needed to inform on growth, reproduction, and overwintering characteristics of the GMP to check for intended and unintended differences to the conventional counterpart. The agronomic and phenotypic characterization—together with the compositional and molecular characterization—is the basis for the comparative approach and serves as the starting point for problem formulation [9]. In addition, the outcome of the comparative assessment is meaningful in the overall weight of evidence for the ERA.

Table 1 GMP applications evaluated regarding studies relevant for persistence and invasiveness of the GMP in the context of the agronomic and phenotypic characterization

No.	GM trait	Regulatory status	Scope of application
	Oilseed rape/mustard (*B. napus*, *B. juncea*)		
1	Herbicide tolerance	A	FFIP
2	Herbicide tolerance	A	FFIP
3	Herbicide tolerance and male sterility	A	FFIP
4	Fatty acid content	P	FFIP
5	Herbicide tolerance and male sterility	P	FFIP
	Potato		
6	Amylopectin content	W	FFIP & C
7	Amylopectin content	W	FFIP & C
8	*Phytophthora* resistance	W	FFIP & C
	Soybean		
9	Fatty acid content	A	FFIP
10	Herbicide tolerance	A	FFIP
11	Fatty acid content, herbicide tolerance	W	FFIP
12	Herbicide tolerance, nematode resistance	A	FFIP
	Maize		
13	Lysine content	W	FFIP
14	Drought resistance	A	FFIP
15	Herbicide tolerance and RHS	P	FFIP
16	Insect resistance, herbicide tolerance	A	FFIP
17	Herbicide tolerance	A	FFIP

RHS RoundUp Hybridization System, *A* adopted scientific opinion by EFSA, *P* pending, *W* withdrawn, *FFIP* food and feed use, import and processing, *C* cultivation

In the EU, the comparative assessment of the agronomic and phenotypic plant characteristics is laid down in the respective EFSA guideline [30]. In general, it represents a standard agronomic evaluation of the crop. The guidance document lists specific and prescriptive plant characteristics of relevance that need to be assessed (e.g., Tables 1 and 2 in [30]). Additional assessments of plant traits may be provided by applicants on a case-by-case basis (depending on the scope of the application, the trait or the crop species). In this context, EFSA refers to certain plant traits with relevance for persistence and invasiveness, such as seed dormancy and seed survival traits [30]. These are considered relevant for crop species that are able to persist in agricultural fields (e.g., potato, oilseed rape) and/or species that are able to establish temporary or persistent feral populations (e.g., oilseed rape). Assessments carried out in the agronomic and phenotypic characterization are important to generate information on potential differences in plant characteristics, which are then used to inform problem formulation and risk hypotheses on the potential persistence and invasiveness of the GMP.

Against this background, we analysed the practice of agronomic and phenotypic characterization in market applications of GMPs and discussed their usefulness to provide information on potential changes in the GMP's

Table 2 Seed germination and dormancy assessments in GMP applications (number of studies in brackets)

	Oilseed rape (10)	Soybean (5)	Maize (5)
Germination tests	Standard/warm germination	Warm/optimal germination	Warm/optimal germination
	Cold germination (10 ℃)	Cold germination (10 ℃)	Cold germination (10 ℃)
	Seed cold tolerance (– 5 ℃) (1)	n.a	n.a
	Germination after induction of secondary dormancy (1)	n.a	n.a
Parameters assessed	Germinated seeds (normal/abnormal)	Germinated seeds (normal/abnormal)	Germinated seeds (normal/abnormal)
	Ungerminated seeds	Ungerminated seeds	Ungerminated seeds
	Seed viability (Tz-test) (3)	Seed viability (Tz-test) (4)	Seed viability (Tz-test) (5)
Dormancy test	Secondary dormancy (1)	n.a	n.a
Seed treatment	Fungicides, insecticides (2), sterilized (1), n. i. (5), untreated (2)	Fungicides (1), sterilized (1), n. i. (3)	Fungicides (3), n. i. (2)

℃ degree Celsius, *n.a.* not assessed, *n. i.* not indicated

ability to outcross, survive and persist in or outside agricultural fields.

Methods

We scrutinized the methodological approaches for agronomic and phenotypic characterization in GMP applications. As outlined above, the major part of the comparative assessment of agronomic and phenotypic plant traits is based on the evaluation of standard agronomic parameters in field trials, as outlined in [30]. As these are uniform across applications, the focus was on any additional assessments, which were submitted to further support the agronomic and phenotypic characterization beyond the standard agronomic set of parameters and which address plant traits relevant for survival, persistence or invasiveness. For the selection of additional experiments contained in GMP applications, we queried the GenTG database hosted by the German Federal Agency for Nature Conservation (BfN). The database lists meta-information on reports submitted with GMP applications since 2001. We queried the database using the search strings "persistence", "invasive", "ecol*", "ecol* and environ*", "volunteer", "germination", "pollen" and "fitness" in the title or in the keywords of studies. All retrieved reports were checked for their relevance. For each search term, the following number of studies were retrieved: "fitness" = 0, "persistence" = 0, "invasive" = 0, "ecol and environ" = 132, "ecol" = 54, "volunteer" = 5, "germination" = 48, and "pollen" = 21. The applications identified with this search were narrowed down by the following criteria: (i) applications that contain several (more than one) studies; (ii) applications of different crop types to include crops differing in their ability to become persistent and invasive due to their intrinsic biological characteristics; (iii) applications with different traits with particular relevance for persistence or invasiveness (e.g., drought tolerance); (iv) applications of different applicants (within a crop type) to cover different assessment approaches and (v) applications of GMPs with stacked traits.

Overall, we identified 82 studies of relevance in 17 selected applications for four different crop types (see Table 1). We use the term "report" for studies compiled by the applicant in a single document, which is clearly identifiable by a study or report number. In many cases, such reports covered several different studies or experiments, which differed in their purposes and methodological designs. These were counted as separate studies. Thus, the number of studies reviewed in our analysis sometimes exceeds the number of reports identified for the individual applications.

We categorized the studies retrieved according to their indicated purpose into the following categories: (i) assessment of germination and dormancy; (ii) assessment of pollen characteristics; (iii) volunteer assessment and (iv) assessment of ecological and environmental interactions of the GMP. The last category included two types of assessments, the assessment of interactions of the GMP with biotic stressors (i.e. pests, diseases) and with abiotic stressors (i.e. cold, drought conditions).

Results

Containment levels

In total, 82 studies presented in 17 applications contained assessments that we considered relevant for indicating risks with respect to survival, persistence or invasiveness of the respective GM crop type. Most of the studies evaluated environmental and ecological interactions of the GMP, followed by germination and dormancy assessments of GM seeds, assessment of pollen characteristics and volunteer assessments (Fig. 1). The studies were carried out at three different levels of containment: studies conducted in the laboratory or growth/climate chambers using different parts of the GMPs (e.g., seeds, pollen or tubers), studies conducted with whole plants grown in pots in the greenhouse, and studies conducted under field conditions (Fig. 2). Only a few assessments were conducted in the greenhouse, e.g., to evaluate ecological and environmental interactions for GM potato or GM maize. All studies evaluating germination and pollen characteristics took place in the laboratory or in climate chambers. Field trials were used to evaluate ecological and environmental interactions (all types of GM crops) and for volunteer assessments (GM oilseed rape and maize).

Assessment of germination and dormancy of GM seeds

For all types of plants, except potato, studies were submitted that investigated the germination and/or

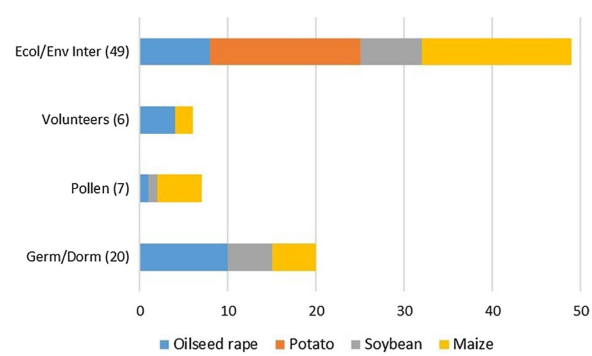

Fig. 1 Classification of 82 studies with relevance for the persistence and invasiveness of GMP contained in 17 GMP applications according to their assessment purpose (total numbers of studies for each category in brackets)

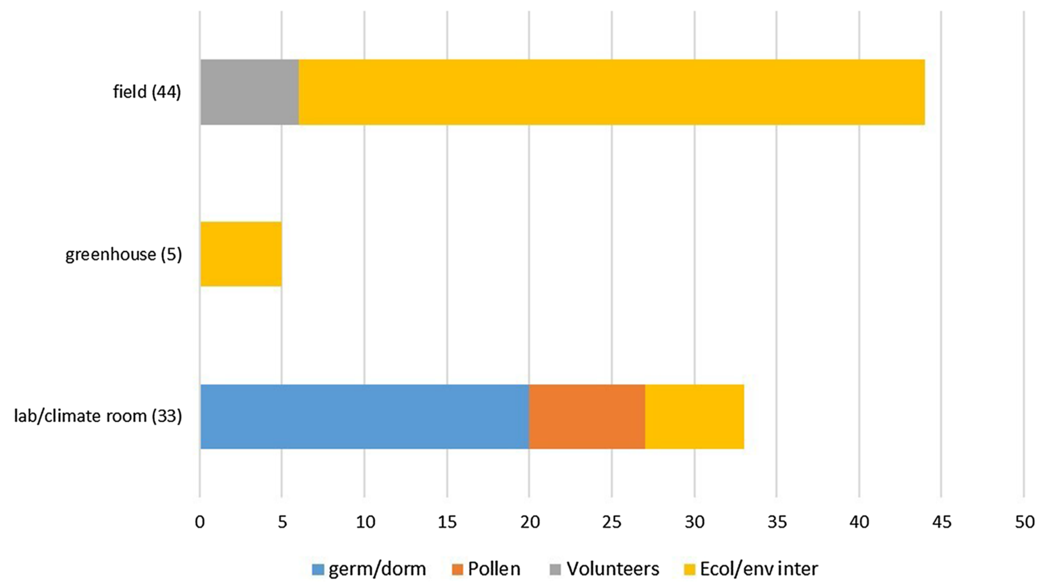

Fig. 2 Containment levels of 82 studies with relevance for the persistence and invasiveness of the GMP contained in 17 GMP applications (total number of studies for each containment level in brackets)

dormancy of seeds (20 studies, Fig. 1). The purpose of the studies, as indicated by the applicants, was the assessment of seed quality, such as germination ability, seed viability or seed health. For the tests, the applicants used standard protocols for seed testing according to AOSA (Association of Official Seed Analysts), ISTA (International Seed Testing Association) or SCST (Society of Commercial Seed Technologists). Nevertheless, the test designs differed among applications and studies, e.g., with respect to the germination tests applied, dormancy assessments or seed treatments (Table 2).

In general, applicants carried out standard warm germination tests in which the seeds were exposed to constantly optimal temperatures (e.g., 20 ℃ or 25 ℃). These tests were usually complemented by germination tests using alternating temperature regimes as well as cold germination tests that exposed the seeds to cooler temperatures (i.e. 10 ℃) for a few days followed by optimal temperatures. According to the applicants, cold germination tests were used to reflect less than ideal temperature conditions, e.g., as experienced in early spring when seeds are sown into agricultural fields. In one application (GM oilseed rape), seeds were also exposed to frost conditions (Table 2). Seed treatments differed between applications and studies. Some experiments used seeds with fungicide treatment (oilseed rape, soybean and maize); in the case of oilseed rape, seeds were also subjected to an insecticide treatment. Not all seeds were sterilized before the germination assessment (Table 2). Non-germinated seeds were usually tested for viability using a tetrazolium test to distinguish dead from viable seeds, thereby identifying dormant seeds. One study investigated GM oilseed rape seeds for secondary dormancy.

Assessment of GM pollen characteristics

We identified seven pollen studies in seven applications (Table 3). Pollen was collected from GMPs from field trials or grown in a growth chamber. Pollen viability and morphology were examined. All assessments comprised the determination of the percentage of viable pollen via a common staining technique, the measurement of grain diameter and the description of the general pollen morphology via microscopic examination. In the case of drought-tolerant maize, reduced soil moisture conditions were applied during the first reproductive growth stages of maize grown in the field. In all other cases, the environmental conditions during the production and dehiscence of pollen of plants grown in fields, which could affect pollen viability, were not reported.

Assessment of GM volunteer emergence

GM volunteer plants were assessed in six studies from two applications (maize and oilseed rape; Table 4). Generally, GM seeds were planted in agricultural plots, and the number of emerging plants (i.e. GM volunteers) was assessed. The purpose of the studies was to evaluate management options to control the occurrence of GM volunteers (i.e. herbicide-tolerant volunteers) in the subsequent crop. In studies with herbicide-tolerant oilseed rape, either chemicals (e.g., herbicides against

Table 3 Pollen viability assessments in GMP applications (number of studies in brackets)

	Oilseed rape (1)	Soybean (1)	Maize (5)
Plants derived from	Growth chamber	Field trial	Field trial
Environmental conditions	21°/18 ℃ (day/night), 16 h photoperiod	n.i	Well-watered and water-limited conditions
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, general morphology
Methodology	Alexander's stain, micrographs	Alexander's stain	Alexander's stain, Lugol staining, microscopic examination

n.i. not indicated, ℃ degree Celsius

dicotyledonous plants) and/or mechanical measures (e.g., ploughing) were applied to manage GM oilseed rape volunteers. No study has compared volunteer occurrences between GM and non-GM plants without management measures for volunteer control. Except for one study, the occurrence of volunteers in the year following GM crop cultivation was not assessed. The occurrence or survivability of GM oilseed rape outside fields, i.e. in field margins or ruderal sites, was not assessed in any of the studies. In one GM maize application (drought-tolerant maize), a specific number of maize seeds was deliberately scattered in either agricultural fields (including weed control) or unmanaged areas (e.g., natural grassland and pastures with varying degrees of ground cover). Drought stress conditions were not specifically applied or reported, which might have provided information on the ability of drought-resistant GM maize to survive.

Assessment of ecological and environmental interactions of the GMP

All applications contained information on ecological and environmental interactions of the GMP, which comprised an evaluation of the response of the GMP to biotic stressors (pests and diseases) as well as abiotic stress conditions.

Response to abiotic stress conditions

Field studies were conducted to assess the response of the GMP to abiotic stress in the case of GM oilseed rape, GM soybean and GM maize (Table 5), while assessments under contained conditions were carried out with GM potato and GM maize (Table 6).

Applicants recorded the incidence of different abiotic stress conditions for the respective GMP at the different locations where field trials took place. The observed stress conditions comprised heat, cold, drought, excess moisture or flooding, nutrient/nitrogen deficiency, soil compaction, wind damage, sunscald, mineral toxicity and hail injury. As the abiotic stress conditions varied between years, sites and observations, these conditions were only reported if they were causing injury to the plants by visual, qualitative assessment of the plant damage using an ordinal rating scale. In the application of drought-tolerant GM maize, a field study assessed the response of the GMP to drought stress, which was actively induced, by halting irrigation of the plants for a short period of time during the late vegetative, early reproductive growth stage (Table 5).

Studies conducted under contained conditions applied artificially induced abiotic stress to the GMPs (high amylopectin, *Phytophthora*-resistant potato and drought-tolerant maize, Table 6). For GM potatoes, applicants studied the freezing tolerance of potato tubers in climate chambers. The tubers were exposed to minimum temperatures around the freezing point for a short period, and then the ability of the GM tubers to resprout was assessed. All stress response assessments of maize were conducted for drought-tolerant GM maize, examining the response of the GMPs planted in pots to different abiotic stress conditions (e.g., cold, heat, drought and salt stress, Table 6). For the assessment of drought stress, either a single or several drought levels were imposed on the plants. In these studies, different endpoints were assessed, such as growth, development, yield or physiological plant parameters.

Response to biotic stressors

Studies assessing the response of the GMP to biotic stressors were generally carried out in field trials (Table 7). Only for GM potato, additional experiments were carried out under contained conditions evaluating the response of GM potatoes to different pathogens by use of manipulated experiments.

Table 4 Assessment of GM volunteers in field studies in GM oilseed rape and GM maize applications

	Oilseed rape	Oilseed rape	Oilseed rape	Maize	Maize
Assessment area	Agricultural plot	Agricultural plot	Agricultural plot	Agricultural plot (not used for maize cultivation)	Unmanaged area
Methodology	Sowing of GM and non-GM plants together with the cover crop (various densities)	Scattering of GM seeds after harvest (various depths) followed by shallow cultivation	Assessment of occurrence of GM volunteers in the course of a variety trial	200 maize seeds per plot scattered in fall by hand	Sowing of maize seeds (approx. 100 per plot), variable ground cover in unmanaged areas
No. of sites	1	1	2	3	4
Control measures applied	Herbicide treatments	Shallow cultivation followed by herbicide treatment	Shallow cultivations followed by herbicide treatment or ploughing in fall and tillage in spring	Herbicide treatments	None
Type of following crop/crop rotation	Winter barley, winter wheat, sugar beet and without crop	None	Wheat, linseed	None	Not applicable
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) plus additional phenotypic parameters
Duration of experiment	7–9 months	2 months	2–4 months	6–9 months	Not indicated (assessments made in the following year)

The information for oilseed rape in the first column is compiled from two studies, which were conducted in two years with slightly different design

Table 5 Assessment of the plant response to abiotic stress in field studies in GMP applications (number of studies in brackets)

Stressor selection	Oilseed rape (4)	Soybean (4)	Maize (11)	
	Incidence	Incidence	Incidence	Drought stress
Artificially induced abiotic stress	No	No	No (6)	Yes (5)
Parameters assessed	Plant damage, stressor symptoms	Plant damage, stressor symptoms	Plant damage, stressor symptoms	Plant damage, stressor symptoms (3); growth, physiol. parameters, yield (2)
Assessment method	Visual observation (ordinal scale)	Visual observation (ordinal scale)	Visual observation (ordinal scale)	
Observations	4 per season	4 per season	4 per season	4 per season
Specific stress conditions induced	No	No	No	Water limitation (5)

Incidence: selection if either actively causing plant injury in the plots or likely to occur in the crop during a given observation period

Table 6 Assessment of plant response to abiotic stress under containment in GMP applications (numbers of studies in brackets)

Plant	Potato (2)	Maize (4)	Maize (1)
Containment	Climate chamber	Growth chamber (2), greenhouse (2)	Greenhouse
Abiotic stress assessed	Cold stress	Cold, heat, drought, salt stress	Drought stress
Artificially induced stress	Yes	Yes	Yes
Experimental design	Tubers placed on soil surface and tubers covered with soil (10 cm and 20 cm depth)	Plants grown in pots (V3 or V4 growth stage)	Plants grown in pots (V4/V5 growth stage)
Test regimes	2 experiments with low starting temperatures, gradually lowering to minimum temperatures followed by gradual thawing	3 stress treatments (mild, moderate, severe)	1 drought cycle
Parameters assessed	Survival rate of tubers (developing sprouts were assessed after 2–3 weeks at 18 ℃)	Growth and development parameters: 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 conductance, leaf extension rate (LER), ion leakage and relative water content (RWC)
Duration of stress in days	8–12	Cold: 8, Heat: 5, Drought: 15, Salt: 12	6

In field studies, applicants generally reported damage to the GMP caused by insect pests and diseases if these occurred during the growing season and at a level that actually caused plant injury. Hence, reporting biotic stressors depended on the natural occurrence of these stressors, which varied across the individual sites. A systematic monitoring and reporting of the incidence of arthropod pests and diseases was not carried out in any of the applications (Table 7). In addition, maintenance pesticides (i.e. fungicides, herbicides, or insecticides) were generally applied during the field trials, often without indication of which stressors were controlled.

Biotic stressors in field trials were largely assessed by qualitatively rating the insect damage to the plant (or by recording disease symptoms) on an ordinal (categorical) scale. In one application, at a few sites, quantitative assessments were used to comparatively evaluate the abundance of the European corn borer (ECB) and corn earworm (CEW) on the GMP. Generally, few efforts were made to identify the sampled insect pests or pathogens taxonomically to species or strain level, instead general terms were frequently used (e.g. leafhopper, aphids, virus) or classification was restricted to the genus level (see Table 7). Similarly, life stages of insect pest were not differentiated, e.g. larval stages of ECB.

Discussion

We analysed the methodological approaches of studies submitted for the agronomic and phenotypic characterization of GMPs to determine whether these provide information on environmental risks of the respective GM crop. Since 2003, approximately 120 applications for market authorization of GMPs have been submitted in the EU. Therefore, the 17 dossiers analysed represent only a fraction of the available applications. A large

Table 7 Assessment of plant response to biotic stress in field trials or under contained conditions in GMP applications (number of studies in brackets)

	Potato (15)	Oilseed rape (6)	Soybean (7)	Maize (10)
Level of containment	Climate chamber or greenhouse (4), field trials (11)	Field trials	Field trials	Field trials
Biotic stressors selected	3 Pests: CPB, Aphids, *Globodera* sp. 7 Diseases: *Phytophthora, Erwinia*, *Alternaria,* PVX, PVY, unspecified virus, *Synchytrium endobioticum*	Incidence (varied among observations and between sites)	Incidence (varied among observations and between sites)	Incidence (varied among observations and between sites)
Artificial inoculation	Yes (*Phytophthora*, PVX, PVY, *Globodera* sp *S. endobioticum*) No (all other)	No	No	No
Application of plant protection products in field trials	Yes (4), no (3), n. i. (8)	Yes (5), n.i. (1)	Yes (3), n.i. (4)	Yes (9), n.i. (1)
Parameters assessed	Pests: relative susceptibility (*Globodera*), abundance (CPB), species diversity, abundance (aphids) Diseases: % infected leaf area or plants or crop canopy, qualitative rating (1–10 scale), % resistant plants	Pests and diseases: visual rating of symptoms or damage on a 0–3, 1–4 or 1–9 scale, yes/no classification, number of plants with aphids	Pest and diseases: Visual rating 1–9/1–4 scale, 0–5 scale (e.g. aphids, leafhoppers), Visual estimate 0–100% or % plant tissue or leaf area affected Abundance of 6 most abundant pest arthropods (beat sheet sampling) (1) Disease/damage type recorded if incidence greater than 30% (1)	Pests and diseases: Visual estimation on qualitative scale (6) Yellow sticky trap for 6 most abundant pest arthropods (1) Quantitative assessment (3): ECB: number of larvae, holes, feeding galleries,, length of feeding galleries in stalks) CEW: abundance, damage rating on 0–9 scale
Protocols used	Official tests for entrance on the Dutch Variety List, Dutch Plant Protection Service, EPPO Standards (EPPO 1999, EPPO 2005, EPPO PP1/213(2)	N.i	N.i	N.i. (for CEW: method adapted from Widstrom 1967)

n. i. = not indicated; incidence = those that were actively causing plant injury in the study area or were likely to occur during a given observation period

part of the agronomic and phenotypic characterization of GMPs is uniform due to the requirement to assess a standard set of agronomic plant traits, which is usually done in all GMP applications since the respective guidelines were published in 2015. In this respect, the usefulness of the EFSA guidance for agronomic and phenotypic characterization [30] must be acknowledged, as it provides detailed guidance on the assessment of standard agronomic GM crop traits under optimal agronomic conditions.

Apart from this standard data set, applicants can opt for additional studies on plant characteristics on a case-by-case basis, as outlined by EFSA [30]. As these are optional, the decision on what to assess and how is left to applicants and no specific guidance on traits, parameters or test designs for such additional experiments exists thus far. According to our analysis, studies that focus on the performance of the GM crop in the agronomic context show few methodological differences between individual studies and applications. We note that seed and plant survival is not addressed in these studies, specifically in non-agricultural habitats, which are environments most likely exposed if the GMP is considered for import purposes. In addition, our analyses show that the methodological designs of these studies only considered optimized environmental conditions in agricultural fields but ignored other than optimal conditions, e.g., if the GMP is spilled and spread into (semi)natural habitats. The emerging methodological and conceptual shortcomings will be discussed in the following.

Consideration of plant traits that inform about the survival of the GMP

Persistence is considered the ability of a GMP to establish sustained and permanent populations, which are no longer dependent on the supply of diaspores from GM crop cultivation [31], either in agricultural or in (semi) natural habitats. The meaning of the term "invasiveness" is more complex, as it is derived from invasion biology and refers to non-native species [32]. Additionally, in EU legislation, invasiveness refers to an organism outside its natural range, which threatens biodiversity or ecosystem services (Regulation (EU) No 1143/2014). In the context of GMPs, the term invasiveness has thus far not been defined but generally refers to increases in the abundance or population sizes of a GMP often with a competitive effect on other species [4, 33]. Generally, the persistence or invasiveness of plants are long-term ecological processes; in the context of ERA, an ex ante assessment is only possible by the use of appropriate indicators (for further discussion, see [31, 34]).

If certain plant traits can be defined that act as triggers for these processes, then these could be subject to testing in the context of an agronomic and phenotypic assessment of the GMP. A number of plant characteristics have been linked with invasiveness and persistence in crop plants (so-called ferality traits; see [12, 35]), weeds (e.g., discussion in [4]) or invasive species (e.g., [36, 37]), and some were also discussed in the context of GMO risk assessment [13]. However, the power to predict invasiveness in ERA is limited, as interactions with the receiving environment and other factors influence the success of an invasive plant [38].

Nevertheless, certain plant characteristics are known to be linked to the dispersal of plants in the environment and the survival of the GMP in general and can act as triggers or indicators for processes related to persistence and invasiveness. Specifically, two major pathways enable the spread of a GMP into the environment—seed and pollen dispersal [2, 39]—as well as lateral spread in plants that disperse vegetatively, e.g., potatoes and grasses [40]. In the context of the assessment of risks due to persistence and invasiveness, seed survival and soil seed bank dynamics therefore play an important role. Consequently, changes in seed and pollen characteristics, such as pollen viability, the number of seeds produced by the plant, seed germination, seed dormancy and seed survival, or spread and survival of vegetative plant parts such as tubers, can be informative to assess the potential of a GM crop to become persistent or invasive in and outside agricultural fields.

The importance of seed traits with respect to plant survival and related environmental risks is also acknowledged in the guidance document by EFSA, which recommends a set of additional, seed survival-related endpoints with relevance for persistence and invasiveness [30]. For species that are able to build up a soil seed bank, EFSA recommends additional assessments for seed survival under field conditions (one year viability testing of buried seeds and survey of volunteers in subsequent years). However, these are suggested for potentially persisting species such as oilseed rape or potato. For non-persisting species such as maize or soybean, such assessments are not considered necessary in the present guideline [30]. Although seed germination was frequently assessed in the studies analysed, its focus was on seed viability of the GMP under optimal germination conditions as encountered upon sowing in the field. The assessments were, in general, not designed to address suboptimal germination conditions that seeds may encounter if spilled outside agricultural habitats and the germination of harvested seeds usually imported into the EU. In addition, seed survival was not considered in any of the studies, with the exception of a few sporadic volunteer assessments; however, mostly with another purpose than to address seed survival in or outside fields. Therefore, the applicants did not follow the recommendations outlined by EFSA [30] with regard to the voluntary assessment of additional endpoints relevant for persistence and invasiveness.

In addition to ensuring the quality of seed for sowing, seed assessments should also aim at the detection of alterations in the crop`s ability to survive under suboptimal conditions. Changes in fitness-related plant characteristics, including seed germination, have been reported to occur in GM crops, specifically under stress conditions, due to unintended alterations of metabolic pathways in the GMP, which are also involved in plant growth, development or stress response [22, 41–44]. Such changes may likewise affect persisting or non-persisting crop species. We therefore consider an extended assessment of seed survival during the agronomic and phenotypic characterization of GMP necessary for all types of GM crops.

In addition, seed germination under laboratory conditions is a weak predictor of the ability of crop seeds to survive under field conditions. Laboratory germination rates can differ significantly from seedling emergence under field conditions, which is often much lower [45]. Seed germination and survival under field conditions is context-specific, depending on environmental conditions such as soil type, burial depth, and other factors [46, 47]. Seedling emergence, especially under non-optimal field conditions, is difficult to predict from seed germination rates assessed in laboratory test settings. Correlation studies showed that the standard seed germination test for maize seeds was not a good predictor of field emergence [48]. In a comparative assessment with Lucerne seeds, the standard germination test did not show a correlation with seedling emergence, either in the glasshouse or in the field [49]. We assume that such a weak correlation also applies to plant emergence from spilled seeds, which enter non-agricultural, seminatural habitats with other than optimal germination conditions.

Consequently, an assessment of seed germination and seed survival in situ under optimal, e.g., managed, as well as suboptimal, i.e. unmanaged conditions, would add ecological realism to predictions derived from laboratory seed germination tests. An assessment of seed survival under managed conditions in agricultural fields that are optimized for seed sowing and germination, e.g., by use of well-designed volunteer studies, should be a standard assessment for GM crops intended for cultivation purposes. This assessment would give a maximum estimate of volunteer survival [50]. In the case of GMP applications, including import in their scope, suboptimal germination conditions should be included in the experimental design of field assessments to account for unintended spread into non-agricultural habitats. Such suboptimal conditions could be established by artificial tilling of harvested seeds into fields without standard agricultural management measures (e.g., seed treatments, fertilization, ploughing, herbicide application and irrigation). Assessment of surviving plants in the following season can be performed similarly to volunteer assessments under standard field management. Specifically, for GM ERA purposes, Kjellson, Simonsen [51] proposed a general method for field assessment of seed germination and survival, either short-term (less than 3–5 years) or long-term (more than 3–5 years).

For vegetatively propagating plants, the growth and survival of vegetative plant parts (e.g., potato tubers or stolons of perennial grasses) would also need to undergo an assessment, as these plant structures are relevant for spread and persistence, either in or outside the agronomic context. For example, potato tubers are often left in fields after harvest, particularly if they are small [52], with estimations of up to 400,000–500,000 tubers per ha left in European fields (see [53] and references therein). Under field conditions, a range of factors can influence the winter survival of potato tubers, such as the soil depth, rate of acclimation in fall, variation in snow cover, lowest temperature, midwinter thaw periods, and rate of deacclimation in spring (see references in [54]). Potato volunteers are also considered important perennial weeds. Long-term assessments of the frost tolerance of potato under field conditions have shown that potato seeds are able to survive frost temperatures and produce seedlings and tubers in the following year [53, 55].

Specifically for plants that are able to spontaneously outcross and hybridize with wild relatives, the survival of GM hybrids may be affected by genomic interactions of the parental genetic backgrounds [1, 11, 56]. For these cases, assessing seed germination and survival not only of the GMP itself, but also of GM crop–wild hybrids would be justified, e.g. by testing of experimental crossings.

Assessing seed and plant survival in the agronomic and phenotypic characterization of GMPs will provide a first useful indication of potential differences between the GM crop and its non-GM counterpart with respect to survival and persistence as a preceding step for the ERA. The results of this assessment should then feed into the problem formulation in ERA and can provide a robust basis for the definition of risk hypotheses.

Consideration of the relevant environmental exposure of the GMP

Environmental risk results from the exposure of the environment to a specific hazard. While the potential hazard of a GMP becoming persistent or invasive is influenced by the crop, its biology, and the novel GM trait, the environmental exposure and therefore the specific receiving environment depends on the specific use of the GMP during the authorization period. Consequently, the scope of the GMP application may affect the potential of the GM plant to survive, persist or become invasive (Fig. 3). Under cultivation conditions, the exposure and therefore the potential environmental impact are expected to be higher than under mere import conditions [30]. Hence, assessments should be aligned to different exposure scenarios. However, in the GMP applications analysed, we could not identify any link between the scope of the respective application and the additional studies submitted for the agronomic and phenotypic characterization.

Particularly for applications with cultivation scope (see, e.g., the GM potato application), assessments with respect to the plant's ability to survive (e.g., volunteer tubers) under field conditions are necessary. Similarly, potato seeds are a relevant exposure pathway for the GMP, as they can produce volunteer potatoes in succeeding crops. For other crops (oilseed rape and maize), seed germination under optimal field conditions and volunteer assessments are of limited relevance for plant survival in off-field habitats if only import of seeds is intended in the EU. In combination with seed germination characteristics assessed in the laboratory (e.g., germination, dormancy), an assessment of the in situ survivability of the plant can provide useful information on the survival and persistence of GM crops depending on the scope of the application and thus environmental exposure.

Additionally, pollen viability studies should be linked to environmental exposure of the respective GMP. Altered pollen characteristics can affect pollen viability of the GMP and are therefore suggested as a case-specific endpoint specifically relevant in the cultivation context [30]. Changes in pollen viability may affect the plants' ability to produce seeds by selfing, which can then give rise to, e.g., potato volunteers in the year following cultivation. For import purposes of any type of GM crop, the assessment of pollen viability is of limited environmental relevance.

The response of the GMPs to biotic and abiotic stressors was evaluated in all analysed applications, independent of the scope of the application. This was because these assessments are needed as part of the standard data set in the agronomic and phenotypic characterization

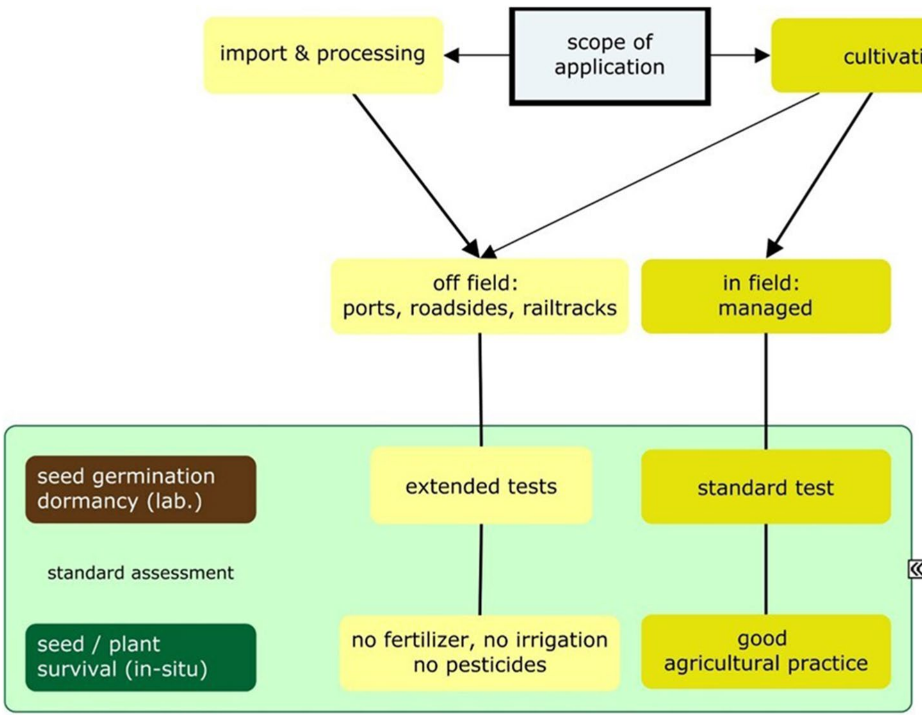

Fig. 3 Relationship between scope of application and test conditions in the agronomic and phenotypic characterization of GMPs with relevance for ERA

according to the current guidelines [30]. Therefore, the assessments were not linked to the GMP's scope or environmental exposure.

In general, all studies provided in the analysed GMP applications did not take into account that GMPs will not only be cultivated in agricultural fields but may be spilled and spread into non-agricultural habitats, e.g., along transport routes, during harvesting, handling and reloading activities or due to animal dispersal. In these (seminatural or natural) habitats, GM seeds or plants will encounter less than ideal (i.e. suboptimal) germination and growing conditions, different than those prevalent in agricultural fields. Hence, study designs that refer to the survivability of the GMP (e.g., germination, dormancy of seeds, seed or plant survival) need to account for such (suboptimal) environmental conditions, specifically for GMPs under import conditions. For both scopes of application (import and cultivation), spillage, spread and occurrence in non-agricultural habitats cannot be excluded and may lead to survival and persistence of the GMP outside the agronomic context [57]. Crops such as oilseed rape may be spilled at reloading points or along transportation routes when cultivated or imported but also occur in countries without cultivation or import activities, e.g., due to admixtures in other crop types [58, 59].

We therefore recommend a differentiation of test conditions for studies that assess the germination and survival of the GMP according to the scope of application and therefore environmental exposure. For import applications, environmental exposure due to spillage should be taken into account by considering suboptimal (i.e. non-managed field conditions). For cultivation applications, both optimal and suboptimal conditions must be considered to reflect cultivation and spillage conditions.

Consideration of methodological approaches that inform about environmental risks

The methodological approaches in the four categories of studies were specifically tailored to assess the agronomic performance of the respective GMP. The aim of the seed germination tests was to ensure seed quality for seeds used in field trials for comparative assessments, as needed in the respective guidance [30]. Pollen viability tests were conducted to ensure the yield of the crop. Control options of herbicide-tolerant volunteers were tested in volunteer assessments in the field. Studies to assess the response to biotic and abiotic stressors were carried out to fulfil the data requirements prescribed by [30]. In general, the methodological approaches used in the studies were not appropriate to derive conclusions on environmental risks, specifically any potential changes in the GMPs' ability to survive, persist or become invasive. The specific limitations and methodological flaws of the individual study categories are discussed below.

Seed testing

To assess seed germination, the applicants used tests and protocols according to international seed testing standards. These test methods are suitable for assessing different aspects of seed quality and have been validated, e.g., by the ISTA validation program [60]. The main purpose of seed germination tests is to assess seed quality in terms of seed viability and health and to ensure that seeds germinate uniformly and at a high percentage, a criterion relevant for international seed trade [60]. Standard germination tests (e.g., warm germination) are generally used for labelling purposes and provide an idea of field emergence under favourable conditions. Cold germination tests are used to assess germination under less than ideal conditions, simulating field conditions in early spring but not winter conditions to which seeds may be exposed when spilled. In laboratory tests, conditions can be controlled and give reproducible results, which is important from a seed quality perspective. Although applicants applied various temperature regimes according to the AOSA/ISTA rules, these temperature regimes (minimum of 10 ℃ for maize and soybean, for a few days only) are far from reflecting natural temperature conditions in Europe to which spilled seeds may be exposed. Crop seeds, which are spilled and dispersed outside agricultural habitats, e.g., due to harvest and transport losses or accidental spillage encounter suboptimal environmental conditions. Such conditions are context-specific and affect the survival and persistence of seeds [61]. Hence, the informative value of standard seed germination tests, as currently carried out in GMP applications, is limited with respect to the survival of GMPs under suboptimal conditions.

Oilseed rape seeds can persist in soil over prolonged periods due to their ability for secondary dormancy [62]. Although the applied tetrazolium test was used to indicate primary dormancy, secondary dormancy or non-dormancy can only be tested by specific test methods [62–66]. Seed dormancy can also be affected unintentionally by genetic modification, e.g., if key regulatory endogenous phytohormones such as abscisic acid and gibberellic acid are affected [67].

In addition, other seed tests are available that can better indicate the germination of seeds under suboptimal or unfavourable conditions, such as seed vigour testing, biochemical tests (conductivity) or seedling performance tests. The respective protocols are available even in a standardized form [68, 69]. The effects of specific stress conditions, e.g., drought stress, on seed germination can be assessed by using osmotic regulation substances (e.g., mannitol and polyethylene glycol), which create osmotic stress potentials, thereby simulating drought conditions. [70–73]. For example, Saffariha, Jahani, Potter [74] assessed the effect of a range of different abiotic stress conditions 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 specific abiotic stress conditions.

In addition, harvested seeds that are spilled during transport of imported GMPs are likely to lack any fungicidal seed treatment, as is usually applied in standard laboratory seed testing to ensure germination. Soil fungi are important mortality factors for seeds [75]. In addition, seed treatments and seed priming can have a range of effects on germination, dormancy and seedling emergence [76, 77]. Grains that may be spilled after harvest will have different genetic backgrounds differing in their germination characteristics. They may also have different proportions of transgenic DNA. In general, grains imported into the EU differ from seed tested by applicants, as they correspond to the F2 generation, while seed used for cultivation is F1 seed. Additionally, non-harvested seeds are of relevance for the survival and spread of a GMP. For GM potatoes, seedlings can also form from true potato seeds (berries), which can then produce tubers and give rise to potato volunteers in the year following cultivation. Such true potato seeds can survive in soil for at least 6 years [78].

We recommend considering these aspects to reflect unintended spillage and spread of GM seeds into non-agricultural habitats, either during cultivation, harvest or import. Extended seed testing should therefore include (i) germination tests without fungicide or insecticide treatment of seeds; (ii) testing seeds used for sowing (F1, cultivation scope) and harvested seeds (F2, for cultivation and import purposes); (iii) laboratory seed tests considering suboptimal and/or stress conditions; (iv) testing of secondary dormancy in addition to primary dormancy and (v) testing germination of non-harvested seeds (e.g., TPS for potato) in case of cultivation scope.

Pollen viability assessment

Pollen viability is important for seed set of the crop but also affects vertical gene transfer and therefore hybridization between crop and wild plants. The current practice of pollen viability assessment during the agronomic and phenotypic characterization of GMPs serves the purpose of ensuring pollination of the respective crop and therefore yield. It usually uses pollen from plants either reared in the laboratory or field and relies on common staining techniques such as Alexander's stain [79] to assess the viability of pollen. This methodology has major shortcomings, as it frequently stains old or dead pollen and shows no correlation with germination. It is known to have a high false positive rate, thereby overestimating pollen viability and consequently seed set in the field [80].

To improve the usefulness and predictability when assessing pollen viability in the context of the agronomic and phenotypic characterization of GMP, we recommend adapting the currently applied test systems and complementing them with seed set assessments [81, 82]. In vitro germination tests provide a more reliable assessment of pollen viability if an optimized growth medium is used for the respective species [80, 83, 84]. These tests require the use of growth media optimized specifically for the pollen of the respective plant species. Protocols to assess the effect of different types of environmental stressors on pollen viability (e.g., temperature, humidity, UV-B radiation) are also available [82].

Assessing seed set in the field in addition to laboratory tests accounts for the fact that environmental conditions during pollen production, in particular physical factors such as water balance and humidity, temperature stress and UV radiation, considerably affect pollen viability. The (de)hydration state of the pollen after dehiscence is an important factor for viability as well as drought and heat stress during pollen development. The longevity of pollen can be drastically reduced under ambient atmospheric conditions. It is known that the pollen of some plant species, e.g., potato, is more resistant to temperature stress, while pollen from other species is not (see review in [80, 82, 85]).

Such an improved assessment would not only benefit the interpretability of the assessments with respect to the yield of the GM crop but would also provide an estimate of the ability of the GMP to cross-pollinate with any wild relative, particularly if seed set with hybrid progeny are also taken into account (if relevant for the respective crop). This could support the assessment of the outcrossing and hybridization ability of the GMP that needs to be conducted in detail during ERA for GM crops that are able to hybridize with wild relatives and that are intended for cultivation.

Volunteer assessment

Crop plants can form volunteers in subsequent crops. These volunteers are generally controlled by the use of specific management measures, mostly the application of one or several herbicides [86]. The selective advantage of novel GM traits, e.g., an herbicide-tolerance trait, can lead to increased survivability and consequently changes in the occurrence of herbicide-tolerant volunteers in agricultural fields, often associated with an increase in the number and use of herbicides involved. This has been demonstrated by the aggravation of management problems of herbicide-tolerant volunteers in non-GM and GM crops [86]. In recent years, problems with glyphosate-resistant volunteer maize, particularly in soybean crops, have arisen in addition to the longer-known volunteer problems of herbicide-resistant oilseed rape [86–88].

If a plant is able to evade commonly used management measures and herbicide applications, this may be an indication of the plant's ability to establish a persistent volunteer population. Herbicide tolerance traits from GM creeping bentgrass (*Agrostis stolonifera*) have been shown to persist in the environment even in the absence of herbicide application [89, 90]. Studies with the model plant GM *Arabidopsis thaliana* have demonstrated increased fitness effects (e.g., increased fecundity) resulting from the overproduction of EPSPS, also in the absence of glyphosate application [22, 91]. Any information on potential changes in a GM plant's ability to establish a volunteer population is therefore important for the ERA of GM crops, particularly if the genetic modification involves a trait that confers a selective advantage to a stressor (e.g., herbicide tolerance, drought tolerance).

As shown in the analysed GMP applications, volunteer assessments focused on the evaluation of management options for volunteers rather than evaluating potential changes in volunteer occurrences as an indicator for increased survival or persistence of the GMP. Information on short-term changes in volunteer populations can provide an indication of whether the GMP has a selective advantage and increased survivability under optimized environmental conditions. This is important to complement the informative value of seed germination tests (see also above), specifically for GMPs intended for cultivation purposes. To our knowledge, there are no standardized protocols that are suitable to assess differences in volunteer occurrence between GM and non-GM crops. Such protocols are still to be developed, taking into account common practices, such as crop rotation and tillage. These protocols could consider active tilling of a determined seed number into a fallow plot at harvest time.

Volunteer dynamics are highly context-dependent and vary according to local conditions. Such long-term changes in GM plant populations and related effects on biodiversity therefore have to be considered during the commercial use of the product and the whole authorization period of the GMP, in particular in the context of post-market environmental monitoring.

Biotic and abiotic stress response assessment

If a GMP has an improved tolerance of biotic or abiotic stress conditions compared to its non-GM counterpart, it may also survive better in or outside agricultural habitats [17, 92]. Abiotic stress tolerance is based on the expression of multiple stress-responsive genes that are involved in a range of metabolic pathways [93]. In many cases, not only single but also multiple abiotic stress tolerances are modulated by regulatory proteins such as transcription factors [93, 94]. If plant hormones (e.g., abscisic acid, jasmonic acid, ethylene or salicylic acid) are modified or affected by genetic modification, interactions between signalling pathways may lead not only to changes in the response to abiotic but also biotic stressors [94].

In the analysed GMP applications, the response of the GMPs to biotic and abiotic stress was evaluated in the context of field trials that were carried out for the comparative assessment (e.g., compositional analysis) of the GMP and its non-GM counterpart. The assessments were aligned to fulfil the data requirements outlined in [30]. Prevalent pests and diseases or abiotic stress conditions (e.g., frost, cold, etc.) were recorded based on their occurrence at the individual sites without systematic measurement, taxonomic identification, reporting or statistical evaluation of results. In the field experiments with drought-tolerant maize, relevant drought conditions were either not achieved or not reported. This pure observational assessment of erratically occurring biotic or abiotic stress in combination with pesticide application and an inadequate assessment method represents an insufficient basis to draw conclusions on potential changes in the response of the GMP to stress conditions in the field. Hence, the conclusions made by applicants that no differences between the GMP and the conventional plants with respect to biotic or abiotic stressors were detected cannot be supported by the methods applied and data provided. Most importantly, scientifically sound assessment methodologies are needed to increase the informative value when evaluating the response of the GMP to biotic or abiotic stressors in the context of agronomic and phenotypic assessments.

Potential changes in the response of a GMP due to exposure to stress only become evident at the phenotypic level if stress is actually imposed (for overview see [1]). To elicit a response of the GMP in field trials during the agronomic and phenotypic characterization, the relevant stress condition must be ensured, ideally in a separate experimental setup of field trials. Appropriate abiotic stress conditions can be ensured by choosing trial sites for which the respective stress conditions can be expected (e.g., water-scarce locations). Alternatively, artificially inducing stress conditions, e.g., by controlling water supply in time and intensity by irrigation, is possible [95]. Importantly, the relevant stress conditions must be defined together with selection of the relevant stressor (e.g., pest species, specific abiotic stress condition) and the appropriate assessment methodology before field trials are started. Additionally, continuous monitoring of actual growing conditions during the season is needed to form the basis for interpretation of results.

Experience and guidance for methodological approaches to assess plant responses to biotic and abiotic stress conditions are available from international organizations and the scientific literature. For the evaluation of biotic stressors (e.g., arthropod pests and pathogens), a range of protocols for manual field assessments are available, e.g., from variety testing of new crop varieties but also from the European and Mediterranean Plant Protection Organization [96]. It is important that the exact methodology chosen is aligned to the respective biotic stressor (e.g., type of pest species) that is subject to assessment, also considering the respective life stages of the assessed insect pest as these may have different feeding preferences or susceptibilities to plant toxins [97, 98]. For many pest species, preseason or parallel monitoring is needed, while for those with infrequent occurrences, artificial infestation experiments will be needed. Such artificial infestation experiments using (non-target) pest species have been carried out for a pyramided *Bt* maize event under field conditions [99]. Methods to select, prioritize and test important non-target herbivores and pests, e.g., for maize, are also available from the scientific literature [100]. In addition, it must be ensured that the standard agricultural practice, as usually applied in field trials, does not interfere with the assessment of a specific pest or pathogen.

To date, no standardized protocols are available for phenotyping the response of the GMP under abiotic stress conditions. A range of experimental approaches are reported in the scientific literature, depending on crop type and type of abiotic stress, including high-throughput phenotyping methods [101–104]. In crop breeding, the use of targeted drought stress conditions is common practice when assessing drought stress responses. For this purpose, guidance on how to achieve targeted drought stress conditions and the recording of relevant phenotypic traits is available in field manuals issued by the International Maize and Wheat Improvement Centre [105]. In combination with an assessment of plant survival (see above), gene expression data as well as proteomic and metabolomic profiling [106–108], such assessments could provide useful information on the GMP's ability to survive and perform under drought stress conditions.

Further guidance is therefore needed to implement a focused and scientifically sound assessment including criteria for crop-specific stress conditions, appropriate assessment approaches, and monitoring and reporting of stress conditions and results. This is particularly important, as, under climate change scenarios, due to erratic weather conditions different stress conditions are likely to occur over a range of combinations regarding stress types, affected growth stages, intensities and durations. Not all conditions can be taken into consideration during agronomic and phenotypic characterization; hence, a focus is needed depending on the individual GM crop and GM trait. For different GM crop types, different abiotic stresses will be of relevance. In general, drought conditions are likely to increase in agricultural crops in Europe under climate change conditions. For summer crops such as maize or soybean, heat and drought conditions are already of relevance [109], while for winter crops such as potato, cold tolerance and the overwintering performance of the crop are important. We therefore recommend an improved assessment of these two stress conditions, which would benefit the agronomic and phenotypic characterization of GMPs.

Conclusions

The persistence and invasiveness of a GMP in its receiving environment can entail risks for biodiversity and ecosystem services; therefore, its assessment is an important cornerstone in ERA. As a starting point for ERA, the agronomic or phenotypic assessment can be used to inform risk assessors about potential differences between the GMP and the non-GM counterpart with respect to the GMP's ability to survive or persist in or outside agricultural fields. As our analysis shows, the agronomic and phenotypic characterization is currently not fit to inform about these aspects and a weak starting point to identify unintended environmental effects of the GMP. We notice that applicants generate standard data that characterize product quality aspects and agronomic risks, while environmental risks are poorly addressed. We criticize that the scope of the application (import and/or cultivation) and the resulting different exposure routes of the environment to the GMP are not taken into consideration when defining study designs and test parameters. Consequently, the usefulness of the submitted agronomic and phenotypic data is of limited use for risk assessors to draw conclusions on environmental risks.

We propose implementing a range of conceptual and methodological aspects in the agronomic and phenotypic characterization of GMPs, which would improve the assessment of environmental risks, specifically in terms of the ability of the GMP to survive and persist in the receiving environment. These proposals refer to an extended assessment of GM seed and plant survival, both in the laboratory and under field conditions, also taking other than optimal environmental conditions into account. These would not only be of relevance for GM crops but also for any type of GM plant. The consideration of the receiving environment when characterizing GMPs accounts for the possibility of accidental spillage and unintended exposure of the environment, e.g., during import, transport, or processing activities. Specifically, for GMP applications intended for cultivation in agricultural fields (GM crops), the assessments of potential changes in volunteer occurrences in the field provide first insights into the potentially increased survivability of GMP with consequences for environmental and agronomic protection goals. The current practice of assessing a standard set of agronomic plant traits together with a few selected additional assessments, without link to environmental exposure, should not be used to justify the absence of environmental risks with respect to the persistence and invasiveness of the GMP. At the same time, improved methodological approaches and standardized protocols are needed when assessing the response of GMP to biotic and abiotic stress conditions. This is a necessary cornerstone to improve the informative value of such assessments, not only for the evaluation of the agricultural performance of the respective GM crop but also for the indication of environmental risks.

In order to be useful for ERA, an adaptation of the EFSA guidelines for the assessment of agronomic and phenotypic traits of GMPs would be useful. Guidance would improve when referring to methodological and conceptual approaches that are suited to better integrate ecological realism in laboratory assessments (e.g., seed germination), as well as to further phenotypic assessments that will enable risk assessors to conclude on potential effects on different protection goals in field and off-field habitats. Together with further methodological guidance for the selection and assessment of biotic and abiotic stressors, the currently performed unspecific profiling of GM crops would be avoided and better inform the ERA.

Ecological processes such as survival, persistence or invasiveness can only be approximated during risk assessment, as these are complex, context-specific and long-term. Any observed unintended agronomic and/or phenotypic changes in a GMP are useful as indicators. However, this is only possible if appropriate methodological approaches and relevant environments are considered. The results can then be linked to the problem formulation step in ERA, complemented by a comprehensive, case-by-case and hypothesis-driven testing strategy and validated by post-market environmental monitoring once the GMP is placed on the market or released into the environment. Only then can the comparative safety approach fulfil its original intention to act as the starting point for problem formulation in ERA.

Abbreviations

GMO	Genetically modified organism
GMP	Genetically modified plant
GM	Genetically modified
EFSA	European Food Safety Authority
ERA	Environmental risk assessment
EPPO	European and Mediterranean Plant Protection Organization
EU	European Union
ISTA	International Seed Testing Association
AOSA	Association of Official Seed Analyst
nGT	New genomic techniques

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Author contributions
MO and MD conceptualised the study; MD and MM analysed the data presented in the GMP applications. MD and MM drafted the manuscript. All authors reviewed, edited and approved of the final manuscript.

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Availability of data and materials
Data sharing is not applicable to this article as no datasets were generated during the study. Restrictions to the public availability apply to the contents of the unpublished regulatory industry studies which are in parts confidential. The analysis was restricted to the methodology used in these studies. According to European law, access on information on environmental risk assessment may not be kept confidential and may be requested from the respective authorities.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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