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EPPO STANDARD ON SAFE USE OF BIOLOGICAL CONTROL

PM 6/5 (1) Host specificity testing of non-indigenous (classical) biological control agents used against invasive alien plants

Specific scope: This Standard describes the procedure for evaluating the host specificity of non-indigenous (classical) invertebrate and fungal biological control agents (BCAs) for use against invasive alien plants. The Standard covers guidance and best practice on the essential elements of this procedure, including taxonomic confirmation, life cycle studies, the optimal conditions to maintain BCAs, selection and maintenance of test plants, and host specificity tests.

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1 | INTRODUCTION

In the EPPO region, there are a number of invasive alien plant species that have a wide distribution. Due to the low success rates and high costs of implementing more traditional control methods, such as chemical control and mechanical removal, and the reduced number of active substances available to control invasive plant species, classical biological control (CBC) may be the only feasible method to control (widespread) invasive alien plant species.

As a management tool for controlling invasive alien plant species, CBC has been practised worldwide for over 100 years, with over 550 biological control agents (BCAs) used against approximately 220 plant species (Winston et al., 2014). Overall, CBC of invasive alien plants has proved effective, with over a quarter of biocontrol programmes resulting in complete control (where no other control methods are needed to suppress the target species) and 50%–70% achieving partial control (resulting in a substantial reduction of other control methods) (Hinz et al., 2020).

In recent years, CBC programmes have been implemented against a small number of invasive alien plant species in the EPPO region. Such programmes have included the control of *Reynoutria japonica* (formerly *Fallopia japonica*; Japanese knotweed) using the psyllid *Aphalara itadori* (Shinji) (Hemiptera: Aphalaridae) (Djeddour & Shaw, 2010); the control of *Acacia longifolia* (Sydney golden wattle) using the gall forming wasp *Trichilogaster acaciaelongifoliae* (Froggatt) (Hymenoptera: Pteromalidae) (López-Núñez et al., 2021); the control of *Crassula helmsii* (Australian stonecrop) using the mite *Aculus crassulae* Knihinicki & Petanović & Cvrković & Varia (Acarida: Eriophyidae) (Varia et al., 2022); and the control of *Hydrocotyle ranunculoides* (floating penny-wort) using the weevil *Listronotus elongatus* (Hustache) (Coleoptera: Curculionidae) (Walsh & Maestro, 2017). Additionally, a fungal BCA, *Puccinia komarovii* var. *glanduliferae* (Pucciniales: Pucciniaceae) has been released against *Impatiens glandulifera* (Himalayan balsam) (Pollard et al., 2021).

Demonstrating the specificity of the CBC agent to the target plant, prior to its release, is an essential component in the development of any biocontrol programme. Host specificity testing aims to predict any impacts that the BCA may have on non-target species in the proposed area of release (Blossey, 1995; Louda & Arnett, 2000; Schaffner, 2001). Regulators will evaluate this research when making their decision whether a BCA is safe to release into the environment or not. When evaluating previous CBC programmes, pre-release testing predictions for non-target impact have been shown to have an accuracy of more than 99% (Hinz et al., 2020).

This Standard describes the procedure for evaluating the host specificity of BCAs for use against invasive alien plants. The Standard can be used to produce supporting information required to complete sections and answer questions in EPPO Standard PM 6/2 (3) *Import and release of non-indigenous biological control agents* (EPPO, 2014) and PM 6/4 (1) *Decision-support scheme for import and release of biological control agents of plant pests* (EPPO, 2018).

For the first import of a BCA into the EPPO region for research, the guidance in the EPPO Standard PM 6/1 (2) *First import of non-indigenous biological control agents for research under confined conditions* (EPPO, 2023) should be followed. This includes conducting the research on non-indigenous BCAs under confined conditions.

ISPM 3 *Guidelines* for *the export*, *shipment*, *import and release of biological control agents and other beneficial*

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organisms (FAO, 2017) provides guidelines for risk management related to the export, shipment, import and release of BCAs and other beneficial organisms.

2 | DEFINITIONS

This section provides definitions and explanations of terms that are used in this Standard.

- *Biological control agent* (BCA) is defined as a natural enemy, antagonist or competitor, or other organism, used for pest control (ISPM 5, FAO, 2021).
- *Centrifugal phylogenetic method* is a protocol for evaluating the host range of a biological control agent (Wapshere, 1974).
- *Classical biological control* (CBC) is defined as the utilization of a non-indigenous natural enemy that shares a co-evolutionary history with the target pest or with its closely - related relative and introduced for permanent establishment and long-term control of a pest which has invaded an area.
- *Invasive alien species* is an alien species whose introduction and/or spread threatens biological diversity. For further explanation of the definition see the Appendix of ISPM 5 (FAO, 2021) for the terminology of the Convention on Biological Diversity in relation to the glossary of phytosanitary terms.
- *Physiological host range* is the range of species (hosts) a BCA is able to survive, reproduce and complete its life cycle on under optimal conditions (e.g., confined conditions).
- *Realized host range* is the range of species (hosts) that a BCA is able to survive, reproduce and complete its life cycle under natural conditions in the field.
- *Target and non-target effects* can be defined as follows: *target effects* – the impacts that a BCA has on the target pest; *non-target effects* – the impacts a BCA has on species other than the target pest.

3 | DETERMINE/VERIFY TAXONOMIC IDENTITY OF THE PROPOSED BCA

It is important to identify the proposed BCA (hereafter referred to as the organism) to allow its unambiguous recognition. The information on identity should include: order, family, genus, species and author (and date of description), and, where appropriate, subspecies, strain, or biotype, as well as common names and synonyms. An appropriate method should be used for identification of the organism [e.g., by morphology, a bioassay (e.g. pathotypes), and/or molecular methods]. However, molecular methods (such as the use of species-specific primers or sequencing) should be used in all cases where species delimitation cannot be achieved based on morphological criteria.

The origin and distribution of the source population should be well documented and, if field-collected, information should be provided on collection sites and dates. Site information may include GPS information (approximate latitude, longitude and altitude) and a description of the site (e.g. general characteristics) and habitat (e.g. grassland, riparian habitat) where the collections were made.

Reference (voucher) specimens of the source population of the organism should be deposited in a recognized collection facility (these depositions should be made before the organism is released). The name and location of institution(s) where reference (voucher) specimens are deposited, and any associated information (DNA barcoding or other), should be freely available.

If the organism is new to science, the identity of the organism should be confirmed by the appropriate experts and supported by a publication in a peer-reviewed scientific journal prior to release.

4 | LIFE CYCLE STUDIES AND OPTIMAL CONDITIONS TO MAINTAIN A PROPOSED BCA

The life cycle and feeding behaviour/infection parameters of the organism should be fully elucidated before host range testing can be completed.

This section is intended to provide an overview of concepts for lifecycle studies and conditions to maintain the organism. For culturing techniques for specific organisms or groups of organisms, the specialized literature (including recent publications) should be consulted.

4.1 | Invertebrate life cycle

To support culturing of invertebrates, a thorough understanding of feeding behaviour, mating behaviour, and oviposition preference is required. This information can be gathered from field observations in the native range (and non-native range if relevant), through scientific studies or consultation of the scientific literature. Optimum temperatures and light regimes for the development from immature stages to adults along with the suitable plant material for specific life stages should be determined. It is important to understand the preference for certain plant parts for feeding and oviposition, as well as the possible need of specific plant parts for the invertebrate organism to complete its life cycle.

4.2 | Invertebrate culturing and maintenance

The culturing of invertebrates should aim to produce and/or maintain sufficient high-quality individuals (of

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a particular life stage that are e.g., free from contaminants) to conduct replicated host specificity tests.

4.3 | Fungal life cycle

Optimum infection parameters (spore age, spore type and concentration, inoculum dosage, use of adjuvant/spore carrier (e.g., Tween, talc), temperature, humidity, dew period, etc.) and maintenance conditions (temperature, light intensity, adding of preservatives, etc.) need to be studied before host range testing can be conducted.

An understanding of the lifecycle of the fungal organism on its host, in particular the stage of the life cycle (spore type, mycelial-fragment suspension) that infects different ages or parts of the target species is required. It should be confirmed, if the fungal organism completes its full life cycle on the one host (the target species).

4.4 | Fungal culturing and maintenance

Depending on the biology of the fungal organism, it may be possible to maintain it in the laboratory on artificial media, e.g., agar, liquid broth. In the case of obligate biotrophs such as rust and smut fungi, it will be necessary to maintain the fungal organism on living plants. For these fungi it will be necessary to maintain fully susceptible biotypes of the target plant species (preferably co-evolved plant biotypes), to ensure pathogenicity and efficacy is preserved. These plants would also serve as positive control plants in any host range testing. Susceptibility testing of the invasive plant biotypes will also be required before host range testing can commence.

FIGURE 1 Fungal culture on agar slope for storage (Image courtesy of S. Thomas CABI-UK).

Bulk inoculum can be prepared for a series of host range tests and may be stored long term, for example on agar culture (Figure 1) or in liquid nitrogen.

5 | TEST PLANT SELECTION AND MAINTENANCE

5.1 | Compiling a test plant list

The compilation of a test plant list is a fundamental component of the development of any CBC programme. The plant species included in the list are used to evaluate the host specificity of the organism and therefore determine its safety.

Once an organism has been found feeding or infecting the target plant in field surveys (native range), the first step is usually to survey the surrounding plants, particularly those in the same family as the target plant. This type of survey can give a preliminary indication of whether the organism is a generalist or a specialist.

Before a test plant list is created, it is important to identify the hosts of the organism listed in the scientific literature.

The Centrifugal Phylogenetic Method (Wapshere, 1974) is the standard method for selecting plants for host range testing. Following this method, closely related plant species are selected from the same genus, tribe and/or family of the target species. The list is then expanded to more distantly related species in other families within the same order to the target species until the host range of the organism is circumscribed (Table 1). In addition, plant species with a similar morphology (relevant for organs or tissues that the organism utilizes) and/or biochemical composition to that of the target species are included in the test plant list. Finally, any plant species which the organism (or species similar to the organism) has previously been recorded on should be included in the list.

When compiling the test plant list, relevant information on plant phylogenetics should be consulted (Briese, 2002, 2005; Kelch & McClay, 2004).

When selecting test plant species for the above groups, particular emphasis should be given to (1) rare and endangered plant species that are congeners to the target species and (2) closely-related cultivated crops/species that are economically important where little information on entomological or mycological association is available, or where the plant host species has evolved in isolation to the proposed organism. Additionally, in consultation with stakeholders, other crops/species that are important economically or important for conservation and that share a similar

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TABLE 1 Selection procedure for test plant list.

Group	Phylogenetic relatedness to the target species	Notes
1	Other forms of the same species	Genetic and/or diagnostic-morphological types of the target species, e.g., varieties, cultivars, etc.
2	Other species within the same genus	Relevant to the region
3	Other members of the same tribe	Relevant to the region
4	Other members of the same subfamily	Relevant to the region
5	Other members of the same family	Species that have some morphological, or biochemical similarities to the target species.
6	Other members of the same order	Species that have some morphological, or biochemical similarities to the target species
7	Any other plant species	Species which the organism (or species similar to the organism) has previously been recorded on, and in consultation with stakeholders, other economic or conservation important species that share a similar habitat to the target species.

habitat with the target species may be included in the test plant list.

The species in the test plant list should be relevant to the area where the organism will be released. This may be an area within the EPPO region where the invasive alien plant is established (for example biogeographical regions) or a wider area. The composition of the flora of neighbouring countries should be considered when compiling the test plant list.

The proposed test plant list should be discussed and agreed with national or regional regulators at an early stage of the development of the biological control programme (Shaw et al., 2011).

See Appendix 1 for test plant list case studies.

5.2 | Filtering the test plant list

The final test plant list may include a large number of plant species that could be reduced to a more manageable number in consultation with relevant (i.e., national, regional) regulators. Care should be taken to ensure the most closely related species and other safeguard species (species which by definition occur in similar ecological habitats to the target species) remain on the list, but consideration may be given to omitting some of the more distantly related species only present in horticulture, for example.

Whilst selection of plants for testing should include those potential non-target plant species at highest risk, the availability of species for testing should also be considered. This may only become apparent once beginning to source material for the testing stage, and therefore this information may feed-back into a second filtering of the test list (Section 5.3).

A more condensed test plant list may be considered (in consultation with national or regional regulators) when testing a new strain or population of a species which has already been evaluated.

5.3 | Sourcing and maintenance of test plants

The test plant list should be developed with an understanding of the availability of the species listed. It is vital to ensure that the proposed test plants can be obtained and propagated for testing. The collection and maintenance of rare and endangered species should be carefully considered before including such species in the test plant list. Legal restrictions may prevent the collection of CITES[1] listed species from the wild, access and benefit sharing regulations may complicate import, and plant health restrictions may prevent the import of certain plant material.

All test plants (e.g., whole plants, rhizomes, seed) should be sourced from reputable suppliers and in case there is any doubt about the identity of a test species, its identity should be confirmed by a botanical expert or by molecular analysis. When sourcing test plants, consideration should be given to the variation of individuals within and between collections/populations, especially if differences in susceptibility of cultivars or subspecies have been reported.

Plants should be grown and maintained under protected conditions and all replicates should be subject to the same conditions. All plants should be kept healthy and pest free. When cut parts of the plants are used for the tests, field collected plants may also be used (e.g., branches).

A constant source of the target plant species is required and should be grown and maintained (in the same conditions as detailed above) to (a) maintain the population of the BCA (Section 4) and (b) for utilization in the host specificity testing experiments (Section 6).

[1]Convention on International Trade in Endangered Species of Wild Fauna and Flora.

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6 | HOST SPECIFICITY TESTING

Host specificity testing of non-indigenous organisms should be carried out under confined conditions.

Determining the host range of an organism is a critical step in the development of a CBC programme, as the safety of non-target species is paramount. Host specificity testing of organisms used against invasive plants is focused on evaluating the potential risk to non-target plant species within the proposed area of release (Ghosheh, 2005).

Species in the test plant list are assessed for their susceptibility to an organism in a series of replicated tests. The number of individual plants, and the number of plant species to use in each test, will depend on the type of test being conducted (e.g., no-choice test or choice test, see Sections 6.1.2 and 6.1.3 respectively), and other factors such as availability of space and equipment. The total number of replicates for each plant species tested should be scientifically sound for the experiment conducted. From experience, an absolute minimum number of 3 replicates should be used and where possible 6–10 replicates can capture variation within species and populations. Individual plants should only be used for one experiment and not reused. There are certain constraints, which may influence the total number of replicates (e.g., availability), and these need to be taken into account during the experimental procedure and analysis.

When initiating host range testing experiments, start to test the most closely related species in the test plant list first, and gradually expand the testing to include more distantly related species. More emphasis should be given to closely related species as these may be more likely to be attacked by the organism. For closely related plant species, if warranted, a higher number of individuals may be tested compared to distantly related species.

In all tests, representative healthy plant material of the appropriate tissue type and growth stage should be used to ensure that test conditions are as optimal as possible.

The conditions for host specificity testing may vary depending on the type or group of the organism, namely being an invertebrate or a fungus, and as such the following sections address these two groups separately.

6.1 | Specific aspects for invertebrate organisms

No set protocol or standard procedure exists for host specificity testing and each test should be developed and implemented specifically for each proposed invertebrate organism. This largely depends on feeding behaviour of the organism and/or the part of the plant on which it feeds, reproduces and/or completes its life cycle. Therefore, host range testing for different organisms may require a different experimental design. However, each experimental design should be recorded to ensure repeatability and should consider the following aspects:

- Number and quality (see Section 4.2) of the invertebrate organism to use in each test,
- Stage of the invertebrate organism (adult, immature stages or both),
- Number and quality of test plants,
- Specific plant parts used,
- Number of replicates per test,
- The duration of the test.

The data collected from host range testing may include assessments on:

- Feeding damage from adults and/or immature stages (e.g. expressed as percentage of plant material consumed, number of feeding marks, number of oviposition scars, or scale of damage),
- Oviposition of females (e.g. number of eggs and oviposition preference),
- Immature stage development (e.g. ability to reach adulthood, development time to each developmental stage such as instar, pupa and adult),
- Survival rate (e.g. number of generations that can be maintained on the non-target plant).

Results of host range testing should be analysed carefully with a good understanding and consideration of the biology and ecology of the invertebrate organism (Schaffner, 2001).

6.1.1 | Testing sequence – in general

The series of tests that is detailed in the following sections follows a tiered approach that allows the assessment of the physiological and the realized host range of the invertebrate organism. In most CBC programmes, the minimum sequence performed will be no-choice (Section 6.1.2) followed by choice tests (Section 6.1.3), although there are exceptions (see Varia et al., 2022). The testing sequence progressively reduces the number of plants exposed based on results of preceding tests, essentially removing unaccepted plant species at each stage, until only a few species remain to be tested under conditions which are as natural as feasible. Therefore, if a non-target plant species is not fed on by the invertebrate organism in no-choice tests, this species can be excluded from subsequent tests. Confined outdoor tests (Section 6.1.4) and open field tests (Section 6.1.5) may be used to assess the realized host range of the BCA.

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This method has proven reliable in determining the realized host range of BCAs (McFadyen, 1998), as shown historically by the low incidence of adverse effects from released BCAs to non-target plants (Hinz et al., 2020).

6.1.2 | No-choice tests

The initial testing of an invertebrate organism will include all test plant species selected in the test plant list (see Section 5). No-choice tests are usually simple with the aim of determining the initial response of the invertebrate organism to each of the test plant species individually.

The tests may involve placing individual invertebrate organisms (adults or immature stages) in a container (petri dish or other) with a cut leaf and/or other relevant plant parts or confining the invertebrate organism with a potted plant (Figures 2 and 3). Depending on the life cycle of the invertebrate organism, these tests are then run for a set period of time. For the duration of the test, the plant material offered should be maintained in an appropriate condition, i.e., watering of the potted plant and replacing cut leaves if mouldy or dry as required.

FIGURE 2 No-choice test set-up for *Aculus crassulae* (Image courtesy of S. Varia CABI, GB).

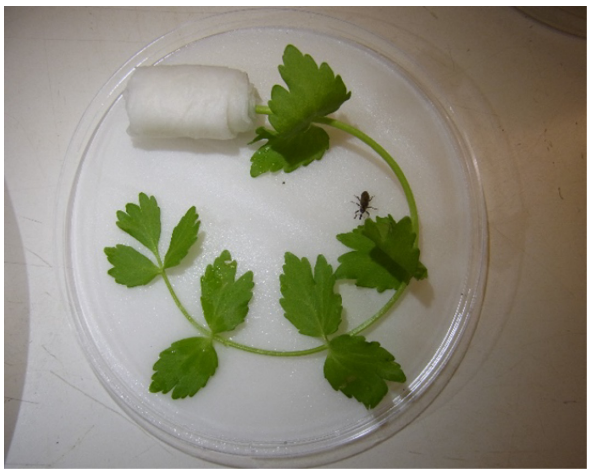

FIGURE 3 No-choice test in petri-dish for *Listronotus elongatus* (Image courtesy of D. Djeddour CABI, GB).

In all cases, positive controls (with the invertebrate organism and the target species) should be set up in parallel to confirm the validity of the experimental setup.

The tests are usually conducted using the stages of the invertebrate organism that are mobile and have the ability to actively select the plant species to feed or oviposit on. This may include both adults and immature stages depending on its life cycle. In cases where the immature stages are sedentary, and the female selects the plant species for oviposition it may not be necessary to test immature individuals separately.

It should be noted that no-choice tests will show the overall acceptability and give insights into the physiological host range. Not all species accepted may act as hosts under natural conditions (EFSA, 2015). Therefore, these tests should be followed by choice tests and, if required, open field tests to determine the realized host range of the invertebrate organism to predict which species are potentially at risk if it is released (Section 6.1.3).

6.1.3 | Choice tests

The next stage in host specificity testing is choice tests and includes all plant species that were selected by the invertebrate organism in the previously realized no-choice tests, i.e., either selected by the invertebrate organism for oviposition or for the immature stages which were able to complete their life cycle on the non-target plant.

Choice tests allow the invertebrate organism to freely select between the target plant and the non-target plant species under controlled conditions, usually in large cages or testing arenas (see Figure 4).

The number of individual invertebrates used in each test and the size of the testing arena will depend on several factors, such as mobility of the invertebrate (crawling, jumping, flying), or typical host selection behaviour of the species or group that allow the invertebrate to freely select between the target and non-target plants.

The number of non-target species to use in each test may vary depending on the invertebrate organism and the resources such as available space, cage size, plant size, etc. A good indication is to use two to four non-target species with the target plant and consideration should be given to the placement of individual plants in the cage to avoid grouping individuals of one species together. Where possible, all individual plants in choice tests should be at a similar growth stage. The stage of each plant for each test should take into consideration

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FIGURE 4 Choice test set-up for *Listronotus elongatus* (Image courtesy of D. Djeddour CABI GB).

the preference of the invertebrate organism for certain plant parts for feeding and oviposition, as well as the possible need of specific plant parts to complete its life cycle.

As with no-choice tests, choice tests should use the invertebrate stage that would make the choice under natural conditions, which may include adults and/or immature stages. The duration of choice tests is dependent on the feeding behaviour and the life cycle of the invertebrate organism. Feeding damage and/or oviposition and development on the target species will act as a comparison for the non-target species.

The no-choice tests determine the physiological host range of the invertebrate organism, while choice tests conducted under confined conditions begin to explore the realized host range. However, there may still be some utilization of the host by the invertebrate organism that requires further investigation. This is best done under conditions as close to natural conditions as possible (see Sections 6.1.4 and 6.1.5).

6.1.4 | Confined outdoor testing

The realized host range can be further studied under natural conditions once the physiological host range is determined. Authorization and appropriate safeguards may be required from the competent authority before the invertebrate organism is transferred from confined conditions to outdoor confined conditions.

Choice tests, under confined conditions can be difficult to replicate in natural conditions. Therefore, if there is the appropriate approved facility to conduct choice tests in large cages outdoors to approximate natural conditions while giving the researcher the ability to manipulate the exposure time and density of the invertebrate organism, this option should be explored.

The methods would be the same as those detailed in Section 6.1.3. However, cages may be required to be larger to limit cage effects, such as increased humidity and temperature. Typical field cage sizes start in the range of 1 m^3 or larger. Under certain circumstances, it may be possible to utilize smaller cages, but there should be good justification for that.

6.1.5 | Open-field testing

The most accurate information on realized host range of an invertebrate organism can be obtained from open-field tests conducted under natural conditions, in areas where the BCA is established, either after an authorized release or in the native range. In open-field tests, the test plants are cultivated alone or in intermixed plots (depending on the experimental design) with the target plant (see review by Schaffner et al., 2018). BCAs from the surrounding population are then allowed to freely select the test plants or target plant for feeding, oviposition and subsequent immature stage development. In some cases, the BCA population in the area may be too low to allow for a realistic assessment. It may therefore be necessary to carry out augmentative releases into the plot using individuals collected from natural populations or reared in the laboratory. Several different experimental designs exist for testing the realized host range of invertebrates and each invertebrate organism will pose its own challenges, with the design needing to be adapted accordingly (Schaffner et al., 2018).

6.2 | Specific aspects for potential fungal BCAs

As with invertebrate organisms, host specificity testing of fungal organisms is required to assess their suitability for biological control of target species. Controlled, replicated experiments, under optimal conditions for infection are required. These may need to be carried out in a dew chamber within a quarantine suite (Figure 5) or a phytotron (enclosed research greenhouse).

Before host range testing commences, a thorough understanding of infection parameters of the fungal organism is required in order to consistently achieve optimal infection in all tests.

The following sections briefly describe the key aspects. More detailed methodologies can be found in the literature (e.g., for *Puccinia*: see Tanner et al., 2015; for *Mycosphaerella* see Seier et al., 2018).

6.2.1 | Fungal inoculum

Prior to host range testing, methods should be established on how to prepare and maintain the fungal inoculum which will be used in the testing procedure

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FIGURE 5 Dew chamber (Image courtesy of S. Thomas CABI, GB).

(see Section 4.3). This may be either in vitro or in vivo and is dependent on the fungal organism involved.

6.2.2 | Inoculation technique

The inoculation should follow a proved method. This may include foliar application, stem injection, root dipping or soil drench depending on the fungal organism.

The area of the test plant that is inoculated should be consistent between the replicates. It is important to always include positive control plants (the target plant) and, if applicable, media plates which contain a subset of the spore inoculum, in every test, to ensure viability of the inoculum used. Furthermore, a sufficient number of negative control plants (e.g. a minimum of three plants consisting of the adjuvant/carrier minus fungal spores) should be included in each test.

An extended dew period to safeguard against any delayed infection on target or non-target plants should be considered.

6.2.3 | Assessment of symptoms

All plants should be maintained under optimal conditions and assessments should begin once symptoms begin to develop on the positive controls. Test plants should be maintained beyond the period of symptom expression to ensure any latent infection has developed and can be detected. Symptoms should be assessed both macroscopically and microscopically if appropriate. Macroscopic symptoms can be assessed according to a predetermined scale. An example of such a scale for fungal organisms (which can be amended for a particular BCA) is given below.

0: Immune: no symptoms
1: Resistant: chlorosis and/or limited necrosis (no further symptom development)
2: Weakly susceptible: delayed symptom expression compared to the target, macroscopic symptoms visible (necrosis) but not fully developed compared to the target species
3: Fully susceptible: symptoms and development the same as on the target

Microscopic assessments can be considered to confirm negative results and can be achieved by preparing (staining and clearing) leaf samples and following methods such as Bruzzese and Hasan (1983) staining technique. Assessment of disease symptoms should always be accompanied by re-isolation from or molecular verification of the presence of the applied fungal organism in diseased tissue.

6.3 | Non-target effects

Non-target effects may be seen during the host range testing. Such effects may include feeding damage and egg laying linked to life cycle development of invertebrate organisms and, in the case of a fungal organisms, infection and life cycle development.

Host range testing experiments that show non-target effects, including incidental non-target effects should be repeated to confirm that any feeding and or egg laying, infection or life cycle development is not an isolated case.

If the organism completes its life cycle on a non-target plant, if possible, the resulting generation should be reared on this non-target species to assess whether the individuals are able to maintain and grow a population.

7 | DATA PRESENTATION

Following completion of the host range testing, all data should be presented to provide the National Plant Protection Organization and other relevant authorities with comprehensive information on the host range of the potential BCA. According to the EPPO Standard PM 6/2 (3) *Import and release of non-indigenous biological control agents* (EPPO, 2014), this should include information on:

- Known hosts,
- Organisms tested,
- Procedures used for host range testing,
- Effects on target and non-target plants.

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APPENDIX 1 - TEST PLANT LIST CASE STUDIES

Case study 1: *Trichilogaster acaciaelongifoliae* against the invasive *Acacia longifolia* in Portugal

Adapted from Marchante et al. (2011).

Trichilogaster acaciaelongifoliae is a gall wasp and an Australian BCA used against the invasive plant *Acacia longifolia*. It was initially introduced in South Africa in 1982 (Dennill, 1985) and later in Portugal in 2015 (Marchante et al., 2017) from South African populations. This case study refers to the selection of test plant species relevant to Portugal for host specificity testing of the BCA. Despite the previous testing of the BCA in South Africa which found it to be specific, it was tested again prior to release in Portugal. No non-target effects have been reported in Portugal.

The species to be included in the test plant list were selected according to criteria outlined by Briese (2002) and Briese and Walker (2002), including phylogenetic proximity and morphological similarity (specifically bud structure) to *A. longifolia*. Other factors considered were economic value, conservation importance (e.g., endemic species), and biogeographic and ecological overlap (i.e., plants that are common in sand dunes, the habitat most frequently invaded by *A. longifolia*). The selection included 40 species that fulfilled either one or more of the selection criteria. The final plant list was approved independently by ICNB (Portuguese Institute for Nature & Biodiversity Conservation back then), who had proposed some of the species on the list.

The degree of phylogenetic separation between the listed plant species and *A. longifolia* was established following Judd et al. (1999), mainly to determine higher levels of phylogeny (families, orders and major clades). Congeneric species were not included in the test list, with the exception of *A. melanoxylon*, because: (a) there are no congeneric native species (or any other Mimosoideae) in Portugal or elsewhere in Western Europe; (b) none of the introduced *Acacia* species has major economic value in Portugal; and (c) several *Acacia* species (*A. baileyana*, *A. cyclops*, *A. dealbata*, *A. decurrens*, *A. floribunda*, *A. mearnsii*, *A. melanoxylon* and *A. saligna* were subject to host-specificity tests in South Africa where galls only developed on *A. floribunda*, a recognized host plant of *T. acaciaelongifoliae* in its native range. Besides *A. longifolia*, *A. melanoxylon* was included in the tests to confirm the status of infrequent observations of sporadic gall formation on this plant species in South Africa.

The test species were separated into six categories on the basis of their phylogeny. The groups comprised the target plant *A. longifolia*, and five clades with increasing phylogenetic distance from the target species, including: (1) species from the genus *Acacia*; (2) species from other genera within the family *Fabaceae*; (3) species from other families within the order Fabales, namely Polygalaceae; (4) species from more distant related families within the Rosidae (specifically clade Eurosids I, which includes the Fabaceae), namely Rosaceae, Salicaceae, Rhamnaceae, Ulmaceae, Fagaceae and Myricaceae; and (5) species from distant families outside the Eurosids I. Although some authors (e.g., Heywood, 1993) consider the order Fabales to be monophyletic, including Fabaceae alone, others (e.g., Judd et al., 1999) recognize three families in it, based on morphological characters and rbcL sequences, with the Polygalaceae being the only family with species present in Portugal.

Three annual species (*Vicia faba*, *Pisum sativum* and *Phaseolus vulgaris*) were included on the list even though the wasp needs an entire year to complete its development within its gall, a mismatch which will preclude annual plants as possible hosts. The three species were included because they belong to the same family as *A. longifolia* and because of their importance as economic crops.

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Case study 2: *Puccinia komarovii* var. *glanduliferae* against *Impatiens glandulifer*a in Great Britain

Puccinia komarovii var. *glanduliferae* is a fungal biological control agent collected on *Impatiens glandulifera* from its native range, the Indian Himalayas (Pollard et al., 2021). The rust fungus was first approved for release against *I. glandulifera* in Great Britain in 2014. No non-target effects have been reported in Great Britain.

The test plant list was compiled using the centrifugal phylogenetic method (Wapshere, 1974), and was modified to include the work of Briese (2005) and the recent study on the phylogenetics of the genus *Impatiens* (Janssens et al., 2005). Thus, the initial selection involved closely related plant species from the genus *Impatiens*, which was then expanded to more distantly related species in other families within the same order (Ericales) as the target species.

In addition, Wapshere (1974) advocated the inclusion of species with a similar morphology and biochemical composition to that of the target species. Therefore, species meeting these criteria were chosen, although often these species were already included due to their close relatedness to the target. Finally, a group of safeguard species were included in the testing process. Commercially available species were selected from the Plant Finder tool of the RHS (http://apps.rhs.org.uk/rhsplantfinder/). The nomenclature of species names followed that of Stace (2010), whilst Morgan (2007) was used specifically for *Impatiens* species.

The full test plant list comprised 75 species, including the target species, from 7 orders and 21 families:

Ericales (Balsaminaceae, Actinidiaceae, Clethraceae, Cyrillaceae, Diapensiaceae, Ericaceae, Myrsinaceae, Polemoniaceae, Primulaceae, Sarraceniaceae, Symplocaceae, Theaceae), Apiales (Apiaceae), Asterales (Asteraceae), Brassicales (Brassicaceae, Limnanthaceae), Gentianales (Rubiaceae, Geraniaceae), Lamiales (Lamiaceae), Rosales (Rosaceae, Urticaceae).

In addition, 10 varieties of three widely grown ornamental species in Great Britain were included. Economically important *Impatiens* species that are widely cultivated in Europe, such as *I. walleriana* (five cultivars included) and *I. hawkeri* (four cultivars included), were represented by more than one cultivar. *Impatiens noli-tangere*, the only native *Impatiens* species in Great Britain and of high conservation importance (Hatcher, 2003), was represented by two distinct populations, one from Wales and the other from the English Lake District.

Case study 3: *Aculus crassulae* against *Crassula helmsii* in Great Britain

Adapted from Varia (2020) and Varia et al. (2022).

Aculus crassulae (Acari: Eriophyoidea) is a biological control agent against *Crassula helmsii*. The mite was collected in South-East Australia and was approved for release in England and Wales in 2018. No non-target effects have been reported in England and Wales.

The test plant list was developed with a focus on Great Britain but also applied to Northwest Europe due to the likelihood that the mites, which spread via wind currents, could naturally spread to the continent.

The test plant list was compiled using the centrifugal phylogenetic method (Wapshere, 1974), and was modified to include the work of Briese (2005) and Briese and Walker (2002).

The target plant, *C. helmsii*, belongs to the plant order Saxifragales, which although relatively small, is a morphologically highly diverse group. The order includes annual and perennial herbs, succulents, aquatics, shrubs, vines and large trees (Jian et al., 2008). The test plant list consisted of 40 species and included terrestrial and aquatic species from plant families across the order, as well as unrelated plant species that share a habitat with *C. helmsii*.

Species from the families which make up the Saxifragales were included in the list including from plant families in the 'Woody Clade'; Paeoniaceae, Altingiaceae, Hamamelidaceae, Cercidiphyllaceae and Daphniphyllaceae, and the 'Core Saxifragales' which includes Crassulaceae, Haloragaceae, Iteaceae, Pterostemonaceae, Saxifragaceae and Grossulariaceae. The Crassulaceae family is the largest in the order Saxifragales, and is a family of succulent species. The genus *Crassula*, to which the target plant belongs, is one of over 30 genera within Crassulaceae, many of which are well-known to the public; for example, *Sedum*, *Kalanchoe* and *Sempervivum*. The only two native European *Crassula* species were included in the test plant list: *C. aquatica* and *C. tillaea*.

The Saxifragales order contains several families which are important in the horticultural industry, particularly Paeoniaceae, Crassulaceae and Saxifragaceae. Species belonging to the economically significant genus *Ribes* are abundant in Western Europe and were included in the test plant list.

The *Myriophyllum* genus in the Haloragaceae family contains some native water plants which share a similar habitat to *C. helmsii* and could regularly be exposed to the biological control agent. The non-native aquatic species, *Myriophyllum aquaticum*, was included in the list.

Finally, safeguard species which are unrelated but share a habitat with *C. helmsii* were included in the test list due to the likelihood of contact with *A. crassulae*. Given the target species' different growth forms, both marginal and submerged aquatic plant species from genera such as *Potamogeton* and *Alisma* were included. *Damasonium alisma* populations have suffered significant decline and inhabit a similar environment to *C. helmsii*. This species therefore was also included in the test plant list.

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