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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PP 1/319(1)

Efficacy Evaluation

PP 1/319 (1) General principles for efficacy evaluation of plant protection products with a mode of action as plant defence inducers

Specific scope

This Standard describes the conduct of trials for the efficacy evaluation of plant protection products based on plant defence inducers (PDIs) when applied to plants to induce defence responses against pests (including bacteria, fungi, nematodes, viruses and insects). The scope is limited to products where the PDI is the main mode of action. Products based on mild viruses or pathogens strains that work through gene silencing are considered to be outside of the scope of the Standard.

This Standard does not cover plant biostimulants.[1]

Specific approval and amendment

First approved in 2020–09.

Introduction

Plant defence inducers (PDIs, also known as plant defence elicitors) include any substance (products of synthetic or natural origin or micro-organisms) which, when applied to a plant, can induce a state of local and/or systemic resistance against biotic stress. This effect should be significantly higher when compared to an untreated control. PDIs are perceived by plants as a signal of danger and do not target the pest directly. They act to develop or implement different defence mechanisms, leading to increased plant resistance to pests.

If the PDI is a low-risk product, reference should also be made to EPPO Standard PP 1/296 *Principles of efficacy evaluation for low-risk plant protection products*, in particular for information on the number of trials. In addition, see relevant pest-specific EPPO PP1 Standards depending on the use for which the Plant Protection Product (PPP) is tested.

The efficacy demonstration should be based on:

- Preliminary studies of the level of direct activity against the targeted pest;
- Efficacy trials allowing the level of efficacy of the PDI (stand-alone products) to be evaluated

In practice, PDIs are often recommended to be applied in mixtures or as a component of an integrated pest management (IPM) programme. In the case of a specific label claim (e.g. complementarity of efficacy in mixtures or as a component of an IPM programme) efficacy trials allowing the level of efficacy of the PDI to be evaluated when it is a component of a programme should be carried out.

1. Preliminary evaluation of the level of direct activity against the targeted pest

Prior to carrying out field trials to evaluate efficacy, the absence of a significant direct effect of the PDI on the claimed pest(s) should be demonstrated.

Tests should be set up preferably under controlled conditions (e.g. laboratory, greenhouses, climate chambers, air-conditioned rooms) allowing the pest to develop. The experimental conditions should be described (e.g. *in vitro/in planta,* plant species, observed plant parts, growing stages, temperature, light (duration, intensity), hygrometry, irrigation, nutrients).

Experiments should include a range of doses or concentrations expected under practical use conditions and be

[1]Please refer to the definition of 'plant biostimulant' in article 47 of Regulation (EU) 2019/1009 laying down rules on making EU fertilizing products available on the market and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003.

compared with an untreated control and a reference product with known direct effect on the pest. For example, in the case of bacteria or fungi, *in vitro* tests can be carried out on solid or liquid media. Direct activity can be evaluated at the different development stages of the pathogen (spore germination, mycelium and bacterial growth, sporulation, etc.).

Additional information on the demonstration of the PDI mode of action is available in Appendix 1.

2. Efficacy trials

2.1. Experimental conditions

2.1.1. Test organism, selection of crop and cultivar

The trial should be performed on the species and/or representative cultivars of crops specified for the intended use and the plants used should be of known and certified origin. Efficacy should be tested on several cultivars. Artificial inoculation (infestation) is a possible option.

2.1.2. Trial conditions

Cultural conditions (e.g. soil type, fertilization, under-cropping) should be uniform for all plots of the trial and should conform to local agricultural practices. To evaluate the efficacy of the tested PDI, treatments that may interfere with the PDI effect by actions on the crop or the pest should be avoided.

The trials should form part of a series carried out in different regions with distinct environmental conditions and preferably in different years or growing seasons (see EPPO Standard PP 1/181 *Conduct and reporting of efficacy evaluation trials, including good experimental practice* and PP 1/226 *Number of efficacy trials*).

2.1.3. Design and layout of the trial

Treatments: The test product(s), reference product(s) and untreated control should be arranged in a suitable statistical design.

Plot size (net): Should be adapted according to the type of crop and sample size for assessments. See the specific EPPO Standards related to the claimed uses where available.

Replicates: At least 4.

For further information on trial design, see EPPO Standard PP 1/152 *Design and analysis of efficacy evaluation trials.*

2.2. Application of treatments

2.2.1. Test product(s)

The product(s) under investigation should be the named formulated product(s) and should be applied as specified for the intended use (e.g. with an adjuvant), see EPPO Standard PP 1/181 *Conduct and reporting of efficacy evaluation trials, including good experimental practices.*

2.2.2. Untreated control

The untreated control should be used for the evaluation of the efficacy of the test product and for statistical analysis.

2.2.3. Reference product(s)

Reference product(s) should be used to validate the trial but not necessarily for direct comparison with the PDI. The reference product should be a product known to be satisfactory in practice under the agricultural, plant health and environmental (including climatic) conditions in the area of intended use.

2.2.4. Mode of application

Applications should comply with good standard practices. Particular attention should be paid to the water volumes applied as for PDIs higher spray volumes may be necessary than for conventional pesticides.

2.2.5. Type of application

The type of application (e.g. spray) should be as specified for the intended use.

2.2.6. Type of equipment

Application(s) should be made with suitable equipment providing an even distribution of product on the whole plot or accurate directional application where appropriate. Factors that may affect efficacy (such as operating pressure, nozzle type, volume rate) should be chosen in relation to the intended use.

2.2.7. Time and frequency of application

The number and date of applications should be as specified for the intended use and should be recorded. For this type of product, it is recommended to take into account the possible delay in plant response, the physiological state of the crop and the persistence of action of the product and its potential cumulative effect.

2.2.8. Dose and volumes

The product should normally be applied at the dosage specified for the intended use. Doses higher or lower than the intended dose may be tested to determine the margin of effectiveness and crop safety, respectively (see EPPO Standard PP 1/225 *Minimum effective dose* and, if relevant, PP 1/296 *Principles of efficacy evaluation for low-risk plant protection products*).

Full details on doses and volumes and how to convert between main country dose expression methods in three-dimensional crops are given in EPPO Standard PP 1/239 *Dose expression for plant protection products.*

Deviations from the intended dosage should be noted.

2.2.9. Data on other plant protection products

If other plant protection products (or any biocontrol agents) have to be used they should be applied uniformly to all plots, separately from the test product and reference product. Possible interference with these should be avoided (e.g. products suspected to have physiological effects).

2.3. Mode of assessment, recording and measurements

2.3.1. Meteorological and edaphic data

2.3.1.1. Meteorological data. On the days before and after application (e.g. 7 days before and 7 days after), meteorological data should be recorded which are likely to affect the development of the crop and/or pest and the activity of the PDI. This normally includes data on precipitation and temperature.

All data should preferably be recorded on the trial site but may be obtained from a nearby meteorological station. Its location and distance from the trial site should be noted.

On the date of application, meteorological data should be recorded which are likely to affect the quality and persistence of the treatment. This normally includes at least precipitation (time between treatment and start of precipitation, and amount in millimetres), wind speed and direction (at the trial site during application), temperature (average, maximum, minimum in °C), relative humidity and, if possible, cloud cover and light intensity. Any significant change in weather should be noted.

Throughout the trial period, extreme weather conditions, such as severe or prolonged drought, heavy rain, late frosts, hail, etc., which are likely to influence the results should also be reported. All data concerning irrigation should be recorded as appropriate.

2.3.1.2. Edaphic data. Depending on the nature of the PDI or the pest, the following characteristics of the soil could be recorded: pH, organic matter content, soil type (according to a specified national or international standard), moisture (e.g. dry, wet, waterlogged) and fertilizer regime see EPPO PP1 Standards available for specific crops and pests.

2.3.2. Type, time and frequency of assessment

The BBCH growth stage of the crop at each date of application and assessment should be recorded.

For type, time and frequency of assessment, see the specific EPPO Standards related to the claimed uses where available.

Type, time and frequency of assessment have to be adapted to take into account the specific mode of action of the PDI. For example, in case of repeated applications, assessments after each application are recommended.

2.3.3. Direct effects on the crop

The crop should be examined for the presence of phytotoxic effects. In addition, any positive effects should be noted. The type and extent of such effects on the crop should be recorded and, if there are no effects, this fact should also be recorded. Phytotoxicity should be recorded as follows:

(1) If the effect can be counted or measured, it should be expressed in absolute figures.
(2) In other cases, the frequency and degree of damage should be estimated, for example by comparing treated with untreated plots to estimate percentage phytotoxicity.

In all cases, unintended effects on the crop should be accurately described (stunting, chlorosis, deformation, delay in emergence, etc.).

For further details, see EPPO Standard PP 1/135 *Phytotoxicity assessment*, which contains sections on individual crops.

2.3.4. Effects on other organisms

Any observed effects, positive or negative, on the incidence of pests, on naturally occurring or introduced pollinators or on natural enemies should be recorded. Any other environmental effects should also be recorded, especially effects on wildlife.

2.3.5. Quantitative and qualitative recording of yield

Quantitative and qualitative recording of yield may need to be done. See EPPO PP1 Standards available for specific crops and pests.

3. Additional efficacy evaluation

In addition to the evaluation of PDI efficacy when applied alone (compared to the untreated control), evaluation of the practical uses may be performed in the case of specific claims (e.g. complementarity of efficacy in mixtures or as a component of an IPM programme). These trials will enable evaluators to assess the practical value of such products, especially if their inherent performance is limited and to determine the conditions of use of the PDI in the framework of a crop protection programme which may extend up to harvest.

The following trial types are suggested as examples.

3.1. Efficacy in mixtures

The purpose is to demonstrate the value of the tank mixture (PDI and product (P) with well-known level of activity). In this case, the aim is to evaluate if the PDI can lead to a reduction in the dosage of product P. Thus, the experiment will consist of several ratios of the mixture, see examples below:

Untreated control
PDI (N1) + P (0.75 N2) compared to the product (P) applied alone P (0.75 N2)
PDI (N1) + P (0.50 N2) compared to the product (P) applied alone P (0.50 N2)
PDI (N1) + P (0.25 N2) compared to the product (P) applied alone P (0.25 N2)
P (1.00 N2)
With: N1 = PDI optimal dose used on its own
N2 = product P registered dose

The product (P) applied alone at the reduced dose rates and at full dose rate is essential to highlight the added value of the PDI in the mixture. These trials should include an untreated control.

3.2. Efficacy of alternating treatments in programmes

The aim of evaluation is to demonstrate the relevance of including a PDI in a programme including one or more products with known efficacy. In this case, the aim is to show that the PDI can reduce the number of applications of conventional products in a programme.

As an example, it can be assumed that a PDI can replace a first treatment based on a product P in a programme which consists of four applications (1–4) carried out every 10 days. This reference programme is then compared in the following minimum programmes and to untreated control:

a.	PDI	P2	P3	P4
b.	–	P2	P3	P4
c.	PI	P2	P3	P4
d.	Untreated control			

If it is not known when the PDI could best be applied in a programme, the following protocol is suggested:

a.	Untreated control			
b.	PDI	P2	P3	P4
c.	–	P2	P3	P4
d.	P1	PDI	P3	P4
e.	P1	–	P3	P4
f.	P1	P2	PDI	P4
g.	P1	P2	–	P4
i.	P1	P2	P3	PDI
j.	P1	P2	P3	–
k.	P1	P2	P3	P4

These trials include an untreated control as well as equivalent programmes without PDI.

4. Results

The results should be reported in a systematic form and the report should include an analysis and evaluation. Original (raw) data should be available. Statistical analysis should normally be done using appropriate methods, which should be indicated. If statistical analysis is not done, this should be justified (see EPPO Standard PP 1/152 *Design and analysis of efficacy evaluation trials).*

Appendix 1 – Demonstration of plant defence inducer mode of action

1. General information on PDI mode of action

A PDI modifies plant responses by activating or priming defence mechanisms. In the case of priming agents, the demonstration of a PDI effect may require that defences are measured following the action of a stimulus triggered by the pest or by any product able to mimic its action.

Considering the potential specificities of a PDI with regard to a given plant/pest pair, it is advisable to carry out tests on the plant on which the product is intended to be used. However, it is still possible to carry out tests on one or several other plants. It has to be kept in mind that at this point that the objective is not to demonstrate efficacy *per se*, but to show an induction of plant defences.

Defence reactions can be measured at different levels, whether they are activated directly or through priming, for example:

- Measurements of early events occurring several minutes up to a few hours after treatment or stimulations, e.g. changes in cell membrane polarisation, changes in ion flows, remobilization of calcium, production of reactive oxygen (H_2O_2) or nitrogen (NO) species. These changes are not systematically correlated with the mobilization of efficient defence systems. Rather, they represent indicators.
- Measurements of later events occurring several hours up to a few days after treatment or stimulations. These events involve signalling, such as protein phosphorylation by kinases/dephosphorylation by phosphatases and regulation of the transcription of many genes. These genes can encode defence proteins that are pathogenesis-related proteins or others involved in secondary metabolism (phenylpropanoid, terpenes, fatty acids and modified amino acid pathways) or in parietal adjustments (hydrolases, synthases, peroxidases). All this leads to changes in enzymatic activities, metabolite production and structural changes, especially at cell wall level. These changes may represent markers of defence or resistance response.

2. Demonstration of PDI mode of action

The mechanisms used by plants to protect themselves against pests are multiple, complex and sometimes antagonistic.

These tests are generally set up in laboratory.

Experiments will use a range of doses compatible with those expected to be used in field conditions. It is highly advisable to use an untreated control and the intended product formulated without PDI active substance.

Many methods can highlight the activation of plant defence mechanisms. Among these possible approaches are the following:

- Transcriptional analyses: These consist of measuring the expression of genes involved in defence, RT-real-time PCR, macro- and micro-arrays, and next-generation sequencing. Observing the induction of several genes coding for different functions is recommended, for example by using RNA-Seq. Studying one sole gene cannot achieve a satisfactory result with respect to a PDI effect.
- Protein analyses: Some nontargeted approaches do exist through the analysis of proteins, e.g. two-dimensional

gels or identification and quantification with a coupling method using liquid chromatography/mass spectrometry (LC/MS). Other targeted studies aim to measure specific proteins by visual inspection (SDS-PAGE gel, Western blot, high-performance liquid chromatography), by ELISA tests, or by measuring enzymatic activity (peroxidases, PAL, chitinase, protease, etc.)

- Metabolic analyses: These are mainly concerned with secondary metabolites produced in plants by means of qualitative and quantitative analysis (LC/MS, gas chromatography/mass spectrometry, colorimetric and chromatographic techniques, e.g. gas chromatography, high-performance liquid chromatography and thin layer chromatography. As an example, there are phytoalexins that are antimicrobial agents (sesquiterpenes in *Solanaceae*, pterocarpans in *Fabaceae*, stilbenes in grapevine, sulphur indoles in *Brassicaceae*).
- Modifications at the cellular level: Histochemical techniques are used to visualize changes at the cell wall: thickenings, impregnations by polyphenols, detection of callose or other polymers, etc.
- Chromatin modifications: DNA methylation/demethylation and histone modifications.

No specific markers for resistance (full protection) are known at this stage. In general, the defence mechanisms activated by a plant do not allow a reliable prediction of whether a plant will have better resistance against a pest. Several markers should therefore be studied.