Végéphyl – Association for plant health

COMMISSION FOR BIOLOGICAL TRIALS

GENERAL PRINCIPLES FOR STUDYING THE FIELD EFFICACY OF PRODUCTS CONTAINING PHEROMONES AND OTHER SEMIOCHEMICALS INTENDED TO CONTROL PESTS BY BEHAVIOURAL DISRUPTION OF ADULTS

GENERAL METHOD No. MG09
Compatible with the EPPO PP 1/264(2) guideline
Not compatible with the EPPO PP 1/314(1) guideline for grapevine moths
Compatible with the EPPO 1/314(1) guideline for the codling moth and oriental fruit moth
No corresponding EPPO guidelines for other Lepidoptera or Coleoptera
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This method/document was drawn up by the members of the Biological Trials Commission (CEB) of Végéphyl, an association for plant health.

The CEB is made up of specialists from:
- public bodies: INRAE, DGAL, ANSES;
- professional agricultural organisations;
- the plant protection products industry;
- service providers in the area of crop protection.

This method/document may be revised by the CEB considering the evolution of experimental methods and agricultural techniques.

In its current state, it should be regarded as a recommended method/document for studying the properties of a preparation (or a macro-organism where appropriate).

A CEB method is considered to be compatible with an EPPO guideline when it is at least as demanding as the guideline with regard to plot size, number of replicates, type and number of observations, sample size, etc.

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CONTENTS

Additions and changes in the 2023 issue

- Change to the last paragraph of the foreword

FOREWORD

For optimal understanding and implementation of this method/document, reference should be made to all or part of the following technical documents and general methods:

CEB: *Rôle et implantation des témoins sans traitements dans les essais de produits phytosanitaires* [Role and implementation of untreated controls in trials of plant protection products]. Végéphyl No. **DT 04**

CEB: *Principes d'appréciation des effets des produits phytosanitaires dans les essais de plein champ* [Principles for assessing the effects of plant protection products in field trials]. Végéphyl No. **DT 05**

CEB: *Expérimentation des préparations naturelles à activité directe ou indirecte contre les champignons phytopathogènes ou les insectes ravageurs* [Testing of natural preparations with direct or indirect activity against plant pathogenic fungi or insect pests]. Végéphyl No. **DT 18**
This document can be used to supplement and adapt specific CEB methods when testing a preparation containing natural active substances.

CEB: *Les tests statistiques – leur utilisation en expérimentation des préparations destinées à la protection des plantes* [Statistical tests: their use in product trials for the purpose of plant protection]. Végéphyl No. **DT 26**

CEB: *Principes généraux d'étude en conditions de culture de l'efficacité pratique de préparations ou de macro-organismes destinés à protéger les plantes et les produits végétaux contre les ravageurs* [General principles for studying the practical efficacy of preparations or macro-organisms designed to protect plants and plant products from pests under cultivation conditions]. Végéphyl No. **MG 01** (*)

CEB: *Principes généraux d'étude en conditions de culture de l'efficacité pratique de préparations destinées à protéger les plantes et les produits végétaux contre les maladies* [General principles for studying the practical efficacy of preparations designed to protect plants and plant products from diseases under cultivation conditions]. Végéphyl No. **MG 02** (*)

CEB: *Principes généraux d'étude en plein champ de l'efficacité pratique des herbicides destinés au désherbage sélectif des zones cultivées ou non cultivées* [General principles for studying the practical efficacy of herbicides designed for selective weed control in cultivated or non-cultivated areas in open fields].
Végéphyl No. **MG 03** (*)

CEB: *Principes généraux d'étude de l'efficacité pratique des substances de croissance sur les végétaux* [General principles for studying the practical efficacy of plant growth regulators]. Végéphyl No. **MG 10** (*)

CEB: *Principes généraux d'étude de la sensibilité des cultures, vis-à-vis d'une préparation herbicide, fongicide ou insecticide* [General principles for studying the susceptibility of crops to a herbicide, fungicide or insecticide preparation]. Végéphyl No. **MG 12**

CEB: *Principes généraux d'étude de la valeur pratique d'une préparation herbicide, régulateur de croissance, fongicide, insecticide ou acaricide* [General principles for studying the practical value of a herbicide, growth regulator, fungicide, insecticide or acaricide preparation]. Végéphyl No. **MG 13**

The assessment of unintended effects, including effects on non-target organisms, as well as the assessment of the impact of plant protection products on the quality of plants or plant products and on processing, are covered by specific methodologies (see list of regularly updated CEB methods). It should be noted that some of the observations relating to these effects, described in the specific methods, can be made as part of efficacy trials.
This method is suitable for studying chemical or natural preparations and living organisms.

(*) Depending on the type of preparation studied, in the vast majority of cases.

1. PURPOSE OF THE GENERAL METHOD

The aim of this general method is to study the efficacy of products that disrupt crop insect pests behaviour using semiochemicals.

Testing concerns a product defined as a formulation containing a set of semiochemicals and its dispenser. This method applies to all currently known modes of application. Appendix II of OECD (2017)[1] lists known examples of plant protection products containing semiochemicals.

Semiochemicals are substances emitted by plants, animals and other organisms that evoke a behavioural or physiological response in individuals of the same or other species (OECD, 2017).

Semiochemicals are divided into two groups:

- pheromones, which promote intraspecific interactions,
- allelochemicals, which promote interspecific interactions:
    - kairomones: benefit the receptor species,
    - allomones: benefit the emitting species,
    - synomones: benefit both species.

There are various types of pheromones: sex, aggregation, alarm, trail, territorial and epideictic.

Sex pheromones enable the receiver to locate individuals of the opposite sex and attract them for mating.

The aim of pest control by mating disruption is to disrupt this phase of courtship by emitting into the atmosphere all or some of the components of the sex pheromone produced by females, or analogues thereof. It is not an usual insecticide-based control of pests but a disruption of the life cycle before the appearance of the damaging stage (mostly larvae).

Kairomones enable a receiver of one species to locate emitters belonging to another species, in particular host plants in the context of this method. Broad beans, for example, emit volatile organic compounds that attract pests such as the broad-bean weevil. Although kairomones are mainly used for trapping (attractant), they could also potentially be used for disruption purposes (egg-laying sites, baited food sources).

For all semiochemicals, it is important to define the range of activity.

For most of the families of insect pests considered in this method, the larvae that emerge from the eggs are what cause the crop damage observed.

2. IMPLEMENTATION CONTEXT

Control by disruption influences the behaviour of insects by emitting substances into the air. This has major repercussions on the experimental design, which differs significantly from conventional testing methods (size and layout of plots, observations, interpretation of results, etc.). Behavioural disruption products should be tested in large plots (see 3.6).

[1] OECD, Guidance Document on Semiochemical Active Substances and Plant Protection Products, Series on Pesticides No. 93, 2017, ENV/JM/MONO(2017)33.

The specific biology of each pest as well as its behavioural patterns (adult travel distances, mating and egg-laying sites, spatial structuring of populations, etc.) should be considered. When kairomones are used, the physiology of the host plant should also be considered.

3. EXPERIMENTAL CONDITIONS

To verify the intrinsic efficacy of a product, part of the trials must, as far as possible, enable the efficacy of the product tested alone. However, the need to conduct these trials in large plots can result in high economic costs in the event of crop losses (depending on the harmfulness of the considered pest). It can therefore be difficult to carry out trials with the product alone.
To get as close as possible to practical conditions, it is also advisable to test the product with supportive foliar applications (if necessary). These trials will be referred to as "practical value trials" (see 4.6 and Table 1).

These two types of trials are complementary. Depending on the pest studied, its harmfulness and the conditions of use recommended by the applicant, the proportion of intrinsic efficacy trials and practical value trials may vary. The reasons for carrying out mainly (or exclusively) one or other of these types of trials should be justified.

For pests that are likely to cause significant crop losses, practical value trials will sometimes be the only ones economically feasible. Intrinsic efficacy trials can be carried out under conditions of low infestation, with the possibility of converting them into practical value trials if infestation turns out to be higher than expected (see 4.6).

All the recommendations in this document apply to both intrinsic efficacy trials and practical value trials.

3.1. Choice of region

A region where the pest is usually found should be chosen. Trials carried out in several regions can take into account possible variations in the number and duration of pest generations, as well as differences in the level of infestation.

3.2. Choice of varieties

It is preferable to conduct trials with varieties that are representative of the market and susceptible (or palatable) to the pest(s) under study.

It is important to be able to assess the product's efficacy on every claimed generation of each pest, by selecting suitable varieties. For pests with a multi-annual cycle, it is necessary to conduct testing over the entire cycle and, when appropriate, on the following generation.

For perennial crops, it is not mandatory to have single-variety plots; but ideally all the varieties should be present in each observation zone, if possible, in comparable proportions. Different varieties with comparable characteristics (palatability, harvest date) can be used.

3.3. Choice of implementation site

The shape of the site, its topography and the technical plan (planting distance, density and depth of sowing, volume of vegetation, presence of an anti-hail net, etc.) can have a significant impact

on the behaviour of the insects as well as on the diffusion of the semiochemical and its stability in the air. Other influencing factors include the immediate environment of the experimental plot(s): neighbouring crops, area with host or refuge plants for the pest, buildings, light sources, windbreaks and shelterbelts, etc.

It is important to ensure that the product is effective in the widest possible range of growing situations.

For perennial crops, these should be crops in full production (except in the specific case of pests such as the leopard moth that tend to attack young trees).

In orchards equipped with anti-hail nets, it is strongly recommended to avoid any direct contact with the dispenser given that the nets could potentially be impregnated with the semiochemicals.

3.4. Untreated control

The control is essentially characterised by the absence of any treatment constituting the aim of the trial. However, it is subject to all the interventions that are applied consistently across the trial, in particular growing techniques and measures to protect against pests not under study.

Its role is usually to:

- confirm the presence, nature, level and evolution of an infestation (trapping and degree of crop damage),
- allow the results of the analysis to be expressed and represented, thus helping with their interpretation and understanding,
- possibly provide a point of comparison for the different treatments.

For behavioural disruption trials, as an excluded control is used, it only serves to evaluate the infestation (the first point mentioned above). It cannot be a point of comparison for the different treatments, except in the specific case where no reference product is available (see 4.1). In this case, it replaces the "reference product" and is used as a point of comparison. Under no circumstances it can be used to calculate efficacy.
However, the untreated control can be included when studying annual crops, leaving a buffer zone (see 3.6).

The untreated control is also used to identify the pest responsible for the damage observed, for example to distinguish between the codling moth (*Cydia pomonella*) and the oriental fruit moth (*Cydia molesta*) in the event of damage to fruit, or between species of corn borers (European corn borer and Mediterranean corn borer).

Wherever possible, the use of a control is highly advisable. It should be located sufficiently far from the influence of the semiochemicals. The distance should be defined according to the area of influence of the test product and the reference product; if there is no known minimum distance, 100 metres may constitute a satisfactory precaution (for the codling moth).

The control generally covers a smaller area than the treatments treated via behavioural disruption, as it is not subject to the same size constraints associated with the diffusion of the semiochemical in the crop. Nevertheless, it should be large enough to show a representative level of damage to the crop. This can be achieved by making observations for samples of a size

at least equivalent to that recommended in the specific methods for the target pest (or a pest with comparable behaviour and biology).

3.5. Experimental design

Because the area of influence of the disruption process extends beyond the trial plot, it is necessary to establish buffer zones between gross plots (zones protected by disruption and/or an insecticide programme – see 3.6 and Figure 1).
For the same reason, it is recommended to maintain a minimum distance between observation areas, e.g. 100 metres for codling moths. This distance should be adjusted depending on the pest targeted, the direction of the prevailing winds, any potential sources of infestation in the vicinity of the plots, and the estimated range of the disruption device.

There can be some heterogeneity within a plot for a given treatment and between plots for the treatment(s) of comparison (agronomic conditions, varieties, management methods, age of plants, history of populations and plant protection, etc.). This increases with the distance to be maintained between the plots.

Due to constraints associated with this type of trial (large plot size and heterogeneity within plots), a block design cannot be used, even if the blocks are broken up. Similarly, a design with total randomisation cannot be adopted, as this would require a very large number of plots to compensate for this heterogeneity. There can therefore only be one plot per treatment, with no replication.

The delimitation of several observation areas within a treatment does not constitute relevant statistical replication, as the areas are neither independent nor randomised. As a result, they do not meet the conditions for an analysis of variance.
Due to the lack of replication of experimental units, multiple trials should preferably be carried out to compensate for this bias. However, it is difficult to achieve a large number of trials, for practical and economic reasons (large-plot trials). this can be compensated for by increasing the sample size (e.g. number of organs observed). This size should then be increased in relation to the size of the samples observed in the small-plot trials carried out for the crop/pest pair in question (see CEB methods specific to the target pest and Table 2).
To confirm the presence of the pest and estimate the level of infestation (this is essential for designs where there is no control), it is necessary to organise monitoring of the population(s) by trapping, within the plots and/or in the vicinity (depending on the configuration of the site and the immediate environment). This can be achieved using sex, food or light traps, depending on the type of semiochemical being studied (see 5.2.1). Regional epidemiological surveillance (*Bulletin de Santé du Végétal* (BSV), technical institutes, Chambers of Agriculture, etc.) can also help. These are all essential for interpreting the trial.

3.6. Plot sizes

Some definitions:

- Gross plot: Total surface area of the experimental unit, and in the specific case of semiochemicals, of the treatment.

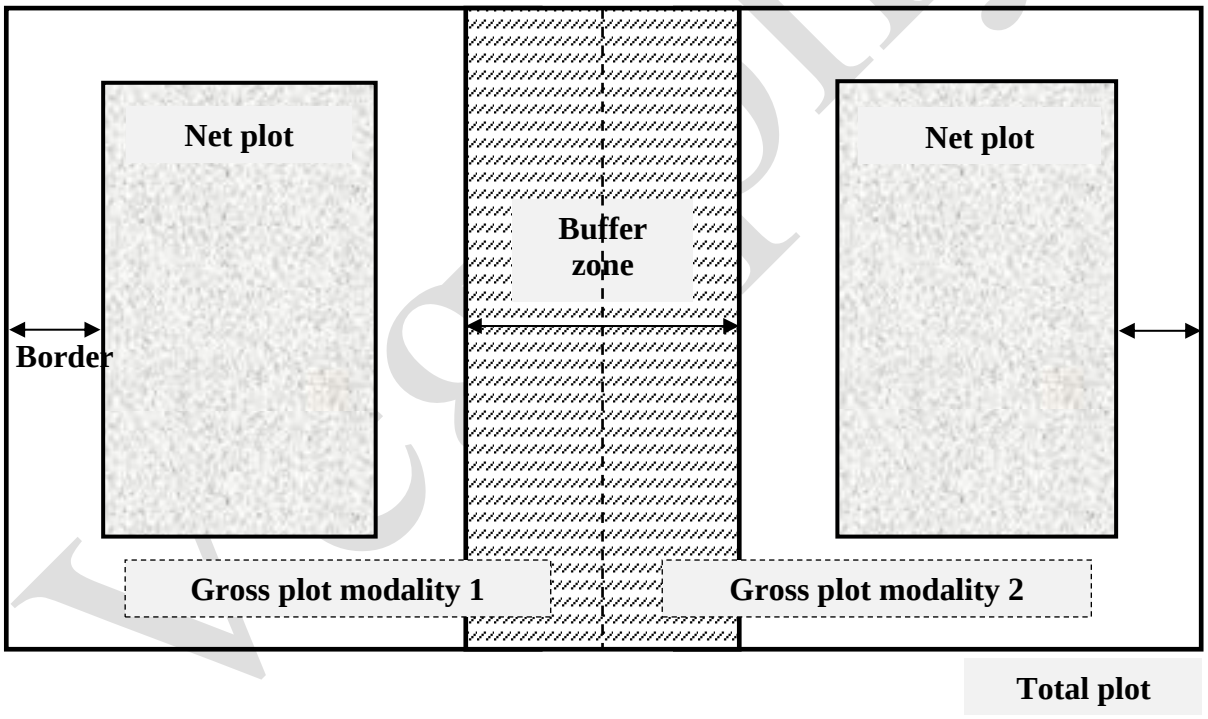
- Net plot: Main observation area, included in the gross plot. This is the gross plot minus the buffer zone(s) and border.

- Total plot: All the gross plots (when contiguous).

- Border: Edge of each net plot. It is an area at risk of infestation with the target pest(s). Its width will depend on the biology of the pest, the type of the crop and the immediate environment around the gross plot (prevailing winds, artificial lighting, neighbouring crops, windbreaks, etc.). Its protection can be reinforced if necessary. Specific observations are highly recommended in this area.

- Buffer zone: Area between the gross plots and around the net plots. Its purpose is to prevent interactions between the different treatments and with the immediate environment of the total plot. No observations are carried out in this zone. Between two treatments, it can be part of the total plot, land for another crop, or any non-cultivated area (path, road, home, windbreak, etc.). If it includes the support crop or a host crop for the target pest(s), the buffer zone may need to be protected to prevent any significant increase in pest populations. The width of this buffer zone should, for example, be at least 100 metres for pheromones targeting fruit-boring moths in arboriculture and vineyards. This size should be adapted depending on the pest, the type of crop (open field, under shelter) and the estimated range of the disruption product(s).

Figure 1. Simplified view of gross plots, net plots, borders and a buffer zone



In general, by way of example, gross plot sizes (net + borders) of around 2-3 ha are recommended. The shape of the plot should be as compact as possible, close to a square.
It may be possible to reduce the size of the gross plot, for example for special cases of compact and/or single-variety plots, certain pests (wireworms), or certain disruption products. For crops grown under shelter in particular, a shelter (a more or less enclosed greenhouse or tunnel) should constitute an experimental unit.

For a comparison treatment protected by an insecticide programme (biocontrol or otherwise), this plot is not subject to the same size constraints. However, its general characteristics (crop

species, variety, age, orientation of trees/vine stocks, topography, etc.) should be comparable to those of the semiochemical treatment plots

3.7. Plot layout

Refer to sub-section 3.5 Experimental design.

4. TREATMENTS

4.1. Point of reference

The reference plot should be protected by products that are authorised for the target use. These should be chosen from among those most widely used and should have recognised qualities for this use. It may be protected by:

- a behavioural disruption product, used alone,
- a behavioural disruption product, used in addition to supportive applications, if necessary (Table 1), especially in cases of heavy infestation,
- a programme based solely on insecticide preparations (conventional or biocontrol preparations),
- or the control, if no reference product is available (in the event of an emerging pest or orphan use).

The main role of the reference product is to call into question the validity of a trial if it shows unexpected results. It serves as a point of comparison with the other treated plots.

4.2. Doses to be tested

It is essential to have information enabling the dose to be expressed as an amount of semiochemical per hectare and per application (border reinforcement to be specified separately), as well as information on practical application conditions (number of dispensers, aerosols, sprays, etc.).

To limit the decrease in the concentration of a semiochemical due to exposure to the wind, it is often necessary to reinforce the protection of the borders (same semiochemical). The nature, shape and size of these border zones should be specified. The additional dose applied to these borders should be mentioned.

4.3. Treatment times

Treatments should be applied on timings specific to each preparation and the pest. The generation(s) targeted and therefore the duration of protection per application should be specified.

4.4. Treatment application

As these are large-plot trials, all treatments can be applied using the farmer's own equipment and/or labour, in accordance with Good Agricultural Practice.

4.5. Maintenance applications

Cultivation operations and protective measures other than those studied, applied separately, should be carried out throughout the trial, if possible under similar conditions (doses, dates, etc.), so as to not cause any uncontrolled disturbance to the development of the pest or the crop. Maintenance applications can be applied using the farmer's equipment, in accordance with Good Agricultural Practice.
If these maintenance applications differ from one treatment to another, the reason should be provided (longer application time given potential differences in the surface areas to be treated).

4.6. Summary: intrinsic efficacy trials versus practical value trials

In the specific case where semiochemical-based products are used, intrinsic efficacy trials are not always economically feasible depending on the level of infestation and the harmfulness of the pest. Therefore, a large part (or even all) of the trials carried out for certain pests may be practical value trials. Point 3 on "Experimental conditions" and Table 1 below describe the situations in which this type of trial can be set up, as well as the treatments compared.
With large-plot trials, the purpose of supportive insecticide applications* targeting the pest is to prevent overpopulation that could result in economically unbearable damage.

In this case, as far as possible, these applications should be made in the same way in all the treatments. Where possible, a likelihood treatment testing the programme of supportive applications alone (without behavioural disruption) should be implemented**. When a control has been included in the experimental design, it may receive the supportive treatment and become a likelihood treatment.

Table 1: Treatments for an intrinsic efficacy trial and a practical value trial

	Untreated control	**Likelihood treatment**	**Reference treatment**	**Tested treatment**	**Other tested treatments where applicable**	**Comments**
Intrinsic efficacy trial	Recom-mended	-	Point of reference	Tested semio-chemical	Tested semio-chemical (different dose, different product, etc.)	Possibility of low infestation (economically bearable damage). If the level of infestation turns out to be higher than expected, the trial can be converted into a practical value trial.
Practical value trial	-	Supportive application(s)* alone, if possible**	Supportive application(s)* + Point of reference	Supportive application(s)* + Tested semio-chemical	Supportive application(s)* + Tested semio-chemical (different dose, different product, etc.)	A trial may be planned from the beginning as a practical value trial (when the economic losses caused by a pest are too high), or it may become one in the course of an efficacy trial, when supportive applications* have proven necessary. In this case, the control may undergo the supportive applications* and become a likelihood treatment.

* Supportive applications: when necessary, insecticides effective on the target pests should be applied to reinforce the behavioural disruption process. These supportive applications should be identical (active substances, dose, etc.) in the different treatments tested in the same trial, including in terms of timing (close dates), ensuring that there is no leaching in the event of a slight timing difference. If there is leaching, the application considered as having leached should be reapplied.
The number of applications and/or the persistence of action of the products chosen should be low to allow the added value of the reference treatment to be expressed.
Indeed, to validate the trial results, the reference treatment must differ from the likelihood treatment. If necessary, refer to the published regional or national technical requirements.

** It is not always possible to set up a likelihood treatment in situations of heavy infestation, over a surface area equivalent to that of the disrupted plots. In this case, a smaller plot can be used and salvage applications made once the lack of efficacy of the supportive treatments has been demonstrated.
In the absence of a likelihood treatment, reasons should be provided as to why the supportive applications are necessary but not sufficient to maintain satisfactory protection for the duration of the trial.

5. OBSERVATIONS AND RATINGS

A minimum distance must be maintained between the observation areas of two different treatments (see 3.6 Plot sizes and Figure 1).

5.1. Preliminary observations

The site's history of infestation with the target pest should be checked beforehand with the farmer/technician/crop manager: extent of damage, treatment schedule, trapping monitorings from previous years, etc.

In cases where the product is not applied on the first generation, damage or pest incidence should be rated beforehand to assess the level of infestation before the product is applied.

5.2. Main observations

The efficacy of the control method should be assessed by observing the incidence and severity of damages and possibly by comparing trapping data. These observations apply to all the treatments.

5.2.1. Verifying the establishment of disruption and monitoring populations

It is necessary to verify the quality of distribution of the semiochemicals and the effective implementation of behavioural disruption, as well as the presence of the target pest.

At present, there are no simple methods for directly measuring atmospheric concentrations of semiochemicals around crops. The only means of verification is indirect: absence or reduction of insects caught in traps using the same semiochemical as the one under study. The same type of trap (including attractant) should be used simultaneously for all the treatments.

In addition, to prove the presence of the pest and the level of infestation, it is necessary to organise the monitoring of populations within and/or in the vicinity of the plots.

All the trapping techniques capable of achieving these objectives are detailed in Table 2 below.

Table 2: Verifying the establishment of disruption and monitoring populations

Target pest	Type of trap	Attractant	Location	Target pest stage	Monitoring frequency	Total monitoring duration	Objective	Minimum number of traps
Codling moth (*Cydia pomonella*) Oriental fruit moth (*Cydia molesta*) Leopard moth (*Zeuzera pyrina*) Red-belted clearwing (*Synanthedon myopaeformis*) European grapevine moth (*Lobesia botrana*) European grape berry moth (*Eupoecilia ambiguella*) Tomato leafminer (*Tuta absoluta*) Striped rice stem borer (*Chilo suppressalis*)	Delta	Sex pheromone	In the centre of each net plot under disruption	Adult males	Weekly	Until the end of the last generation's flight period	Check for the establishment of sex disruption (catch inhibition)	One per treatment
			Outside gross plots under disruption and at a distance that prevents any interference	Adult males	Weekly	Until the end of the last generation's flight period	Prove the presence of the pest in the immediate environment and monitor population dynamics to position any additional treatments	One for the entire test site
		Sex pheromone + kairomone (if available)	In the centre of each net plot under disruption	Sex pheromone: adult males Kairomone: adult females and possibly adult males depending on the kairomone	Weekly	Until the end of the last generation's flight period	Prove the presence of the pest in the disrupted plot and monitor population dynamics to position any additional treatments	One per treatment
European corn borer (*Ostrinia nubilalis*) Mediterranean corn borer (*Sesamia nonagrioides)* Cotton bollworm (*Helicoverpa armigera*)	Cage or delta	Sex pheromone	In the centre of each net plot under disruption	Adult males	Weekly	Until the end of the last generation's flight period	Check for the establishment of sex disruption (catch inhibition)	One per treatment
			Outside gross plots under disruption and at a distance that prevents any interference	Adult males	Weekly	Until the end of the last generation's flight period	Prove the presence of the pest in the immediate environment and monitor population dynamics to position any additional treatments	One for the entire test design

Target pest	Type of trap	Attractant	Location	Target pest stage	Monitoring frequency	Total monitoring duration	Objective	Minimum number of traps
Western corn rootworm (*Diabrotica virgifera virgifera*)	PAL pheromone	Sex pheromone	In the net plot	Adult males	Weekly	Two months	Check for the establishment of sex disruption in year n (catch inhibition)	Four per treatment
	Pherocon AM chromatic	Colour	In the net plot	Adult males and females	Weekly	Two months	Prove the presence of the pest in the immediate environment and monitor population dynamics	Six per treatment
Box tree moth (*Cydalima perspectalis*) Pine processionary moth (*Thaumetopoea pityocampa*)	Funnel	Sex pheromone	In the centre of each net plot under disruption	Adult males	Weekly	Until the end of the last generation's flight period	Check for the establishment of sex disruption (catch inhibition)	One per treatment
			Outside gross plots under disruption and at a distance that prevents any interference	Adult males	Weekly	Until the end of the last generation's flight period	Prove the presence of the pest in the immediate environment and monitor population dynamics to position any additional treatments	One for the entire test site
Wireworms/click beetle (all species)	Soil sorting	None	In the net plot	Larvae	Once a year (when the larvae rise to the surface)	-	Prove the presence of the pest and measure the abundance of larvae (identify them if necessary)	16 per treatment
	Kirfman-Chabert (see Annex M248)	Wheat and maize seeds	In the net plot	Larvae	Once a year (when the larvae rise to the surface)	-	Prove the presence of the pest and measure the abundance of larvae (identify them if necessary)	10 per treatment
	Barber pots	Passive	In the net plot	Adult males and females	Weekly	2.5 months	Prove the presence of the pest and measure the abundance of adults (identify them if necessary)	Three per treatment (nine is optimum)
	Pitfall	Sex pheromone	In the net plot	Adult males	Weekly	2.5 months	Prove the presence of the pest and measure the abundance of adults (identify them if necessary)	One per treatment (increase if this variable is used to compare compositions/doses, due to highly heterogeneous catches)

5.2.2. Organs observed: depending on the crop and pest (shoots, fruits, etc.)

The main observations (damage, larvae, etc.) must be made in the net plot (excluding the buffer zone and borders).
In addition, it is highly recommended to make specific observations in the border zones of the plot. These can provide information about potential localised outbreaks of infestation with the target pest and should be kept separate from the rest of the observations.

Random observations must be made of plants spread out over the entire net plot (change of trees/vine stocks/plants or rows with each observation, except for soil pests with a multi-annual cycle) so as to cover the whole plot and take account of the heterogeneous distribution of damage.

Table 3 gives some examples and recommendations for a number of crop/pest pairs. In particular, sub-samples (per tree, per m², per linear metre, etc.) have been introduced so that the results can be statistically processed.

5.2.3. Observation timings

Observations must be made in the period when the harmful stages of each of the target generations are present, and also at harvest (if relevant depending on the pest). For wood-boring pests, observations need to continue beyond harvest until the end of the pest's period of activity in the crop.
Recommended observation timings are detailed in Table 3.

5.2.4. Observed variables

During the field observations, the data recorded are grouped into sub-samples (hereinafter referred to as "locations") for each variable observed, to obtain enough values for the statistical analyses (see 6.2. Statistical analyses).

Most of the time, damage incidence assessments are the most relevant, particularly when a single attack on fruit is enough to make it non-marketable (e.g. with the codling moth). In some cases, the severity of the damage may also be relevant, for the red-belted clearwing and wireworms for example (see Table 3).

Rating methods are generally non-destructive (for example, fruits are rated on the tree, without being detached), except in the case of harvesting, or if dissection is required to determine the pest (roots, aerial parts), and for grapevine moths.

When several pests can be responsible for the same type of damage, it is important to distinguish which species is involved.

Table 3: Recommendations concerning the main observations, for some crop/target pest pairs (variables, sampling method, sample size, observation frequency, etc.)

Crop observed	Target pest	Variables observed by treatment	Sampling method	Minimum sample size (large-plot design)/net plot	Observation frequency (minimum)	Vegetation observation area	Comments*
Apple (trees in production)	Codling moth (*Cydia pomonella*)	- Average % of fruits attacked per tree and per location (observed on trees), - Number of attacked fruits on the ground per tree and per location.	- Trees randomly selected in the net plot and spread over a minimum of five rows (change rows or trees with each rating), observation of fruits without sampling, - Counting of all the attacked fruits on the ground, under each tree observed.	- Fruits in trees: 1000 fruits at a rate of 20 fruits per tree x 50 trees, - fruit counting on the ground per tree (showing symptoms of attack by lepidopterans) x 50 trees.	Two ratings per generation (including one at the end of the generation), maximum 10 to 14 days apart, from 50% hatching.	Per tree: 20 fruits in total, including 10 on each side of the row (not necessarily on the same trees), with half in the upper third of the canopy and half at breast height. Fruits on the ground, under the side of the tree observed.	If there are any doubts about the pest (e.g. oriental fruit moth) or if it is difficult to observe symptoms (attack at eye level on pears), dissection can be carried out. If the trial continues through to harvesting of the variety(-ies) present, the final observation should be made just before harvest for each variety.
Walnut (trees in production)	Codling moth (*Cydia pomonella*)	- Average % of fruits attacked per tree and per location (observed on trees), - Average % of attacked fruits on the ground per hoop.	- Trees randomly selected in the net plot (change rows or trees with each observation). Observation of fruits in a cage (without sampling), - Counting of all the fruits on the ground in the hoop (randomly thrown throughout the net plot).	- 1000 fruits at a rate of 50 fruits per tree x 20 trees, - fruit counting on the ground: 50 x 1 m² hoop throws for the rating at harvest.	- One observation at the end of each generation, - One observation at harvest.	Per tree: 50 fruits in total, with half in the upper third of the canopy and half in the remaining two thirds. Observations are made in equal proportions on two sides of the trees (not necessarily the same trees) (e.g. 10 observations on the south side and 10 on the north side). Fruits on the ground (at harvest).	If there are any doubts about the pest (e.g. locust bean moth), dissection can be carried out. If the trees are too tall, observations should be made at the maximum height accessible to the operator.

Crop observed	Target pest	Variables observed by treatment	Sampling method	Minimum sample size (large-plot design)/net plot	Observation frequency (minimum)	Vegetation observation area	Comments*
Peach (trees in production)	Oriental fruit moth (*Cydia molesta*)	- Average % of shoots attacked per tree and per location, - Average % of fruits attacked per tree and per location, - Number of attacked fruits on the ground, per tree.	- Trees randomly selected in the net plot and spread over a minimum of five rows (change rows or trees with each observation), observation of fruits without sampling, - Counting of all the attacked fruits on the ground, under each tree observed (from 15 days before harvest).	- 1000 terminal shoots at a rate of 40 shoots per tree x 25 trees, - 500 fruits at a rate of 20 fruits per tree x 25 trees, - fruit counting on the ground (showing symptoms of attack by lepidopterans) per tree x 25 trees.	- One rating of shoots at the end of the first two generations, from one month before harvest to harvest, - Three ratings of fruits every 14 days from one month before harvest, - Fruits on the ground (from 15 days before harvest).	Shoots: 40 terminal shoots in the upper quarter of the tree. Fruits, per tree: 20 fruits, including 10 on each side of the row (not necessarily on the same trees), with half in the upper third of the canopy and half at breast height. Fruits on the ground.	In the event of high risk, an additional rating of shoots may be useful for the first and second generations. From the third generation, generations overlap and no distinction can be made between them. If there are any doubts about the pest (e.g. peach twig borer), dissection can be carried out.
Arbori-culture (young trees)	Leopard moth (*Zeuzera pyrina*)	- Average % of shoots (for the year) attacked per tree and per location, - Total % of shoots attacked per tree and per location (regardless of the year of the attacks).	Locations randomly selected in the net plot (change locations with each observation).	20 locations with 10 trees each (one rating per tree).	- One observation in mid-July to confirm the presence of the pest (and provide for a supportive treatment as needed). - One observation in late August.	All the shoots on the tree.	In regions where the larval cycle of the leopard moth is multi-annual, trials should be organised over several years for the same plot(s).
Arbori-culture (trees in production)	Red-belted clearwing (*Synanthedon myopaeformis*)	- Average number of empty pupal skins per tree and per location, - Average % of trees attacked per location.	Locations randomly selected in the net plot (change locations with each observation).	20 locations with 10 trees each (one rating per tree).	- Empty pupal skins: One observation in mid-June, - Trees attacked: One observation in mid- to late September.	Entire tree, observation of old wood only.	Given that the larval cycle is multi-annual, trials should be organised over several years for the same plot(s).
Grapevine (vineyard in production)	European grapevine moth (*Lobesia botrana*) European grape berry moth (*Eupoecilia ambiguella*)	- Frequency and average number of glomeruli per 100 bunches and per location (first generation), - Average % of perforations per bunch and per location (G2 and G3), - Average % of live larvae per bunch and per location (G2 and G3).	25 locations randomly selected in the net plot, made up of three or four consecutive vine stocks, spread over at least five rows (change locations with each observation).	G1 glomeruli: 10 bunches observed for each location with three or four vine stocks, i.e. 25 batches of 10 bunches (for a total of 250 bunches). G2 and G3: 10 bunches sampled for each location with three or four vine stocks, i.e. 25 batches of 10 bunches (for a total of 250 bunches).	- One observation of glomeruli at the end of the first generation, - One observation of perforations and larvae for the second and third generations: observations to be made 14 to 21 days after the occurrence of the first damage.	Bunches distributed over all the vine stocks. In G2 and G3, spread out sampling of the 10 bunches on both sides of the row (five bunches per side).	The grape dissection method is recommended to count the number of live larvae per bunch. Sampling by brining is still possible (see CEB method M222 Grapevine moths).

Crop observed	Target pest	Variables observed by treatment	Sampling method	Minimum sample size (large-plot design)/net plot	Observation frequency (minimum)	Vegetation observation area	Comments*
Tomato under shelter	Tomato leafminer (*Tuta absoluta*)	- Average % of fruits attacked per plant and per location, - Average % of leaves attacked per plant and per location, - Average number of mines per plant and per location.	20 locations with five plants. Locations randomly selected in the net plot (change locations with each observation).	- 1000 leaflets at a rate of 10 leaflets per plant x 100 plants, i.e. 50 leaflets per location. - 300 fruits at a rate of three fruits per plant x 100 plants, i.e. 15 fruits per location.	Two leaflet observations per generation. Two observations as soon as the first full-sized fruits appear.	Observation of the fully developed leaflets below the apex. Observation of the full-sized fruits, regardless of ripeness.	
Rice	Striped rice stem borer (*Chilo suppressalis*)	First-generation damage: - Average number of attacked tillers (withered flag leaf) per location. Second- and third-generation damage: - Average number of stems attacked per m^2 and per location (dry stem with perforation sometimes visible), - Average number of sterile panicles per location (or white panicles).	Quadrats of at least 0.25 m^2 (50 cm x 50 cm). 20 locations randomly selected in the net plot (change locations with each observation).	10 quadrats per location x 20 locations (i.e. a total of 200 quadrats, or 50 m^2 observed), with observation of all plants.	- First observation of tillers (before the flight period of the second generation, first half of July). - Observation of stems and panicles, before healthy straw starts to dry out.	See column 3 (variables observed).	Position the final observation to observe as much damage as possible (including late damage). Observing too early can lead to damage being underestimated, as the latest attacks have not yet resulted in straw wilting.

Crop observed	Target pest	Variables observed by treatment	Sampling method	Minimum sample size (large-plot design)/net plot	Observation frequency (minimum)	Vegetation observation area	Comments*
Maize	European corn borer (*Ostrinia nubilalis*) Mediterranean corn borer (*Sesamia nonagrioides)* Cotton bollworm (*Helicoverpa armigera*)	- Average number of larvae per plant and per location, - % of plants attacked per location.	Dissection of plants in the locations.	If there is a single generation, 20 locations with 10 consecutive plants, at the end of the generation. If there are two generations, 20 locations with 20 consecutive plants at the end of the first generation, then 20 locations with 10 plants at the end of the second generation.	One or two ratings depending on the number of generations of the target pest in the area.	Whole plants (European corn borer, Mediterranean corn borer) or ears only (cotton bollworm). Refer to specific method M210.	For semiochemicals other than sex pheromones, the sampling plan can be adapted based on the positioning of the treatment in relation to the cultivated plot.
	Western corn rootworm (*Diabrotica virgifera virgifera*)	- Average severity of larval damage per plant and per location.	Removal of plants (destructive).	20 locations with two consecutive plants per net plot.	Once a year, around flowering (generally in the second half of July).	Roots.	Observations in year n+1 only (n = year of application, multi-annual trials are possible).
		- Average % of lodged plants per location.	Counting of lodged plants in randomly selected locations.	20 locations with four consecutive plants per net plot.		Plants.	
		- Abundance of adults.	- Chromatic trap.	Six traps per net plot.	Once a week for two months.	-	Observations in year n (year of application) and year n+1 to assess the efficacy of the control method in reducing populations.
Box tree~~wood~~	Box tree moth (*Cydalima perspectalis*)	- Average number of larvae per location, - Average % of leaves attacked per location.	Quadrats of at least 0.04 m² (e.g. 20 cm * 20 cm). 20 locations randomly selected in the net plot (change locations with each observation).	Five quadrats per location x 20 locations (i.e. a total of 100 quadrats)	One observation every 14 days from the first generation.	Depending on the height and shape of the boxwood, the choice of quadrat positioning may differ. For example, for pruned boxwood, the entire rim and height of the pruned surface, as well as the top side, should be sampled.	

Crop observed	Target pest	Variables observed by treatment	Sampling method	Minimum sample size (large-plot design)/net plot	Observation frequency (minimum)	Vegetation observation area	Comments*
Pine trees	Pine processionary moth (*Thaumetopoea pityocampa*)	- Average number of winter nests resulting from mating in year n-1, per tree and per location, - Average number of nests in year n+1, per tree and per location.	20 locations randomly selected in the net plot (change rows or trees with each observation).	100 trees per net plot, spread over 20 locations with five trees each.	One observation per year (n and n+1) between January and March.	Whole canopy.	
All annual crops	Wireworms (all species)	- Abundance and identification of larvae in soil (if necessary).	Soil sorting in the spring of years n+1 to n+x (depending on the length of the target species' life cycle), and/or Kirfman-Chabert traps (see Annex M248).	16 soil sorting operations and/or 10 Kirfman-Chabert traps per net plot.	Once a year (when the larvae rise to the surface)	Soil.	Apply semiochemicals before the adults start to emerge every year, for three to five years (depending on the length of the target species' life cycle). Experimental design: one 30 x 30 m net plot, located in the centre of a gross plot (surface area to be defined according to product characteristics), per treatment. This design requires that the same crop plan (including the cultivated species) be followed for all the gross plots and for each year of testing. It is not mandatory to use an 'indicative' crop (on which damage can easily be observed) every year, but it may be appropriate to assess efficacy from the third year of the study on an indicative crop. Observations can be made for several crops in the same experimental year (e.g. maize followed by wheat).
		- Average frequency and/or severity of larval attacks per location, for an 'indicative' crop (maize, potato, carrot, etc.).	Counting in randomly selected locations in areas with damage.	20 locations measuring 0.5 (e.g. carrot) to 5 (e.g. maize) linear metres per net plot, to be adapted depending on the species (different sowing/planting densities).	One to four ratings depending on the crop (see CEB M248). For kinetics (several ratings), only the final rating will be statistically analysed.	Area indicative of damage (whole plant for maize, daughter tubers for potato, roots for carrot, etc.).	
		- Abundance of adults.	Monitoring of passive (Barber pots) and sex pheromone traps.	Three passive traps (optimum of nine traps) and one sex pheromone trap per net plot (increase if this variable is used to compare compositions/doses, due to highly heterogeneous catches).	Weekly surveys for 2.5 months.	-	

* Site monitoring: In general, it is important to ensure that the health status of the entire site remains under control for the target pest.

5.3. Observations of the direct effect on the plant (phytotoxicity)

An initial estimate of the crop's susceptibility to the products should be obtained from additional observations carried out during the efficacy trials.

Specific trials will only be required if any symptoms of phytotoxicity appeared in the efficacy trials. For their implementation, reference should be made to the following document:
- *Principes généraux d'étude de la sensibilité des cultures, vis-à-vis d'une préparation herbicide, fongicide ou insecticide* [General principles for studying the susceptibility of crops to a herbicide, fungicide or insecticide preparation] (MG 12).

If there is no direct contact between the semiochemicals and vegetation (when passive dispensers are used), these observations are not necessary.

5.4. Observations of the effect of treatments under practical conditions

Refer to sub-section 4.6. Summary: intrinsic efficacy trials versus practical value trials, and Table 1.

5.5 Observations of unintended effects of treatments

The assessment of unintended effects, including effects on non-target organisms, as well as the impact of plant protection products on the quality of plants or plant products and on processing, are covered by specific methodologies (see list of regularly updated CEB methods).

If there is no direct contact between the semiochemicals and vegetation (when passive dispensers are used), these observations are not necessary.

However, if relevant, observations of these effects can be carried out during efficacy (or practical value) trials as described in this general method.

5.6. Recording of weather and soil data

The recording of data on weather (temperature, humidity, artificial lighting, etc.) and soil (soil type, growing medium, etc.) during the trial can generate explanatory variables for the behaviour of the pests and disruption products.

5.7 Additional observations

In order to optimally interpret the trial network's results, it is necessary to have diffusion kinetics based on the weight loss of the dispenser as a function of time. These apply only to the semiochemical-based product studied and are intended to verify the quality and duration of emission into the atmosphere under practical conditions (where possible).

The measurement methodology depends on the method of diffusion used:

- With passive dispensers (solid matrix + semiochemical, retrievable), weighing can be implemented (generally, a sample of 10 dispensers are individually weighed and numbered). Weighing should be regularly repeated throughout the diffusion period, at intervals in line with the expected duration of diffusion: for example, from every week for diffusion periods of a few weeks, to every 15 days for diffusion periods lasting several months. The dispensers should be placed in the same conditions (in vegetation) as used in the intrinsic efficacy or practical value trials. This weighing should be carried out in different years and regions.

- For other types of formulations, if these studies under practical conditions are not feasible, this information can be obtained under controlled conditions.

For passive dispensers, the diffusion kinetics over time should be checked at least once by chromatographic analysis, under conditions similar to those encountered in practice (in vegetation, outdoors). These analyses should be performed with five dispensers (destructive analyses) at each sampling time (for example, once a month).

The results should be presented in the form of a diffusion kinetics curve (amount of semiochemical as a function of time). These additional studies enable the presumed useful diffusion time of the tested design to be validated.

6. STATISTICAL ANALYSIS OF VARIABLES AND INTERPRETATION OF RESULTS

6.1. Calculation of variables

See Table 3.

6.2. Statistical analyses

6.2.1. For an individual trial

As part of the experimental design associated with the disruption technique, the results cannot be analysed using an analysis of variance followed by a multiple comparison of means test.

However, as the goal is generally to compare the results from an experimental plot (P1), treated with a semiochemical, with the results from a reference plot (P2), one option is to use Welch's t-test, which is an adaptation of Student's t-test (for more details, see Annex 1). This avoids the assumption of equal variances (estimates of variances in populations), given that if the sample size is large enough (minimum 20 organs/groups of organs observed/trees, etc.), the distribution of the populations is asymptotically normal. For a design with more than two treatments, the treatments should be compared in pairs.

6.2.2. For a group of trials

Each group relates to the same variable.
It can be useful to group together trials with comparable protocols and treatments (number of application, crop stage, mode of application, etc.).

After potential transformation, the variables resulting from a measurement or visual estimation should be subject to an analysis of variance. This analysis can be followed by a Dunnett test to compare the studied treatments with the reference treatment, the likelihood treatment or the control, and then by a Newman-Keuls test to compare the treatments with each other.

The advantage of this analysis is that it uses more powerful tests than Welch's t-test and significantly increases the degrees of freedom and therefore the ability to highlight significant differences between the two populations being compared.

6.3. Interpretation of results

6.3.1. Catch inhibition

If behavioural disruption is implemented correctly and if the semiochemical is evenly distributed throughout the crop, pest catches are much lower in the disrupted treatments than in the non-disrupted plots or in the traps located outside the experimental design, without the influence of any source of semiochemicals.

6.3.2. Damage analysis

See Table 3. The damage analysis is the primary basis for assessing the efficacy of the method tested.

6.3.3 Trial validation

Trial validation depends on two conditions:

- The level of infestation must be sufficient in the control or in the likelihood treatment. If it is insufficient in either one, it must be possible to quantify the level of infestation with the pest studied via trapping (see 5.2.1) and based on local information concerning the presence of the pest (e.g. *Bulletins de Santé du Végétal*),
- The reference treatment must show a behaviour as expected in the context of the trial (to be explained).

When these conditions are met, the results of the different treatments should be compared in pairs (see Welch's t-test, described in Annex 1).

6.3.4 Trial interpretation

The result of Welch's t-test should be considered in light of the homogeneity of infestation in the two plots corresponding to the treatments being compared.

When Welch's t-test shows a significant difference for the variable studied, it can be concluded that the "treatment factor" effect investigated in the experimental design is probably the cause of this significant difference. However, the power of the test should be checked using the formula in section 6.4 of DT 26. In this case, the value of the delta parameter should be estimated on an empirical basis according to the values generally accepted for the pest being studied.

However, it should be considered that the variable measured could have been influenced by other uncontrolled factors interfering with the treatment factor. For example, a pest that is less present in a plot can be an interfering factor, which is not always easy to understand, and can artificially increase or reduce the perceived efficacy in the plot in question, regardless of the efficacy of the treatment.

To compensate for this, trends should be verified in several trials, to confirm the effects seen in the individual trials, with the aim of attributing them unreservedly to the treatment factor studied.

7. PRESENTATION OF RESULTS

7.1. For an individual trial

In all cases, the infestation should be characterised, and the crop stage and generation(s) of the pest specified during each observation.

For an excluded control (or excluded likelihood treatment), the level of efficacy cannot be calculated. The results should therefore be expressed solely in terms of % damage and/or the number of pests, for example.

If observations have been made on several dates, a graph showing changes in pest populations (weekly trap counts, if possible) or damage is desirable.

As these are trials that can be complex to interpret, it is particularly important to describe the specific conditions of the trial: infestation levels, positioning of peak periods for flight/emergence of adults in relation to the treatments applied, weather events, insecticide treatments used, spatial distribution of damage, area of infestation with the pest as observed during the trial, etc. For each individual trial, the results obtained should be commented on in light of the trial conditions.

7.2. For a group of trials

A group of trials is only appropriate if it includes trials with comparable protocols (and therefore similar treatments). In other cases, it is preferable to present the trials individually, or in small groups of comparable trials.

damage levels cannot be compared if they result from different infestation levels and protection programmes.

ANNEX 1: DETAILS CONCERNING WELCH'S T-TEST

Samples of size "n1" and "n2" respectively are randomly observed in each net plot. The size of these samples may vary, for example, when attacked fruits that have fallen to the ground are counted.

"X1" and "X2" are the means of these two samples.

The test of significance of the difference "X1 - X2" is a Welch's t-test with a 5% type-I error risk.

The following formula is then used to calculate the Welch's t-statistic ("t_{obs}"):

$$t_{\text{obs}} = \frac{|X1 - X2|}{\sqrt{\frac{SCE1 + SCE2}{n1 + n2 - 2}\left(\frac{1}{n1} + \frac{1}{n2}\right)}}$$

In the formula,

- "X1" and "X2" are the means of two datasets,
- "n1" and "n2" are the sizes of the two samples,
- "SCE1" and "SCE2" are the sums of the squared deviations.

This "t_{obs}" should be compared with the "theoretical $t_{0.975}$" (0.975, to take account of the confidence interval) found in the t-tables in the Excel document attached to the method.

If the two samples are the same size ("n1" = "n2"), the formula is simplified:

$$t_{\text{obs}} = \frac{|X1 - X2|}{\sqrt{\frac{SCE1 + SCE2}{n(n-1)}}}$$

A pre-filled Excel "t-test for MG 09" table is attached to the method, to make the test easier to use.