Végéphyl – Association for Plant Health
COMMISSION FOR BIOLOGICAL TRIALS
&
RÉSEAU MIXTE TECHNOLOGIQUE
(RMT)[1]

GENERAL PRINCIPLES FOR TESTING PLANT BIOSTIMULANTS

General principles for testing plant biostimulants

GENERAL METHOD N° MG 15

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The following method was implemented by the members of the Biological trials commission (CEB) of the French association Végéphyl and the members of the RMT on fertilization and the environment.

These organisations include specialists:

- having, as trustees, the Ministry of Agriculture: the INRA institute, The Plant Protection Service, Anses;
- Professional Agricultural organisations;
- Plant Protection Industry
- Industry for plant fertilization and nutrition

This method may be subject to updates by the Commission, on account of the evolution of experimental methods and agricultural techniques.

In its present state, it is to be considered as a recommended method for studying the properties of products.

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[1] RMT in French is a Joint technological network (TN)

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INTRODUCTION

In this document, the definition of biostimulants was inspired by the European project 2016/0084 (COD) (appendix 4):
http://www.europarl.europa.eu/oeil-mobile/fiche-procedure/2016/0084(COD)

The term "plant biostimulants" defines any substance (chemical, natural extracts, microorganisms...) or any combination of such substances, applied on plants (above-ground plant surfaces, seeds or root environments) on cultivated soil or on any crop medium intending to stimulate the natural processes of plants or their environment, with a view to improve one or any other of the following characteristics, regardless of the nutrient contents of the biostimulant:

- bioavailability, absorption,
- valorisation of nutrients in plants,
- growth and the development of plants,
- tolerance to abiotic stress,
- crop quality and of their production

Examples of biostimulants identified as part of a study for the MAAF[1], depending on their nature and origin (non exhaustive):

- plant and algae extracts,
- microorganisms and their derived extracts,
- aminoacids and hydrolysed proteins,
- humic substances or assimilated (such as humic acids, fulvic acids, lignosulfonates),
- non-nutritive mineral substances,
- biomolecules (eg.: enzymes, vitamins, antioxydants).

This document is only concerned with the study of the effects of biostimulants. The main effects claimed in terms of stimulator categories are described in Appendix 1.

In this document, the term biostimulant applies to products having no impact on bioagressors. A biostimulant product must show its stimulatory activity (its mode of action).

From a scientific point of view, some substances can at the same time induce resistance to bioagressors and better tolerance to abiotic stresses as well. However, as there is undoubtedly a clear *continuum* between PDA [2,3] and plant biostimulants, it is necessary to distinguish these two notions. The question of experimenting on products called PDA, which induce plant responses to biotic stress, is covered in another CEB document, the General Method n° 14 (MG 14).

1 Ministry of Agriculture, Agri-Food and Forestry

2 Plant defense stimulators

3 Elicitors or stimulators of plant defense responses (SDP) induce defense response mechanisms in plants against biotic stress

From a regulatory perspective, implementing a biostimulant on the market comes under regulations relating to fertilizing matter and crop medium and not to plant protection.

Furthermore, subtances and growth regulators are products which have: "an effect on the vital processes of plants, other than that of nutrients, with an impact on their growth". For this reason, they fall within the scope of the (CE) regulation n° 1107/2009. These first and foremost concern products of which the active substances are phytohormones (natural or synthetic). These products claiming such use are addressed in separate CEB methods (MG10…).

1. THE OBJECTIVES OF THE METHOD

The objective of this document is to define the experimental conditions (controlled, semi-controlled conditions or in full field environments) required for the assessment of biostimulants, claiming the above-defined points, on a crop or on a given crop group. This method is recommended for R&D trials as well as for trials concerned with marketing procedures.

Experiments are carried out in trials which will make it possible to:

- characterize the stimulatory activity of the product,
- define the intrinsic efficacy of the product,
- clarify and justify the claims to products use.

Other studies can compete on the full evaluation of the efficacy of a biostimulant product:

- the study of crop susceptibility,
- the non-intentional effects of the product and the potential impacts of biostimulants on the quality of plants or of treated plant products,
- the study of adverse or unsought side effects.

These studies are subject to particular methods and are not taken into account in this method. Refer to CEB documents and standardized methods (XPU44-166 et 167).

2. CONTEXTUAL IMPLEMENTATION RELATED TO THE DESCRIPTION OF THE PRODUCT UNDER STUDY

In a context where society's expectations on the sustainability of agricultural systems are increasingly higher, stimulatory products are of growing interest to the agricultural community.

Through their original modes of action, biostimulants aim to improve soils and plants functioning, or the interaction between soils and plants, by stimulating biological processes. Biostimulants can be seen as a means to promote or maintain an efficient agriculture, by improving plant nutrition, hence reduce fertilizer inputs.

The stimulating activity of a product must be demonstrated to justify the claims category(ies) specified in Appendix 1.

Stimulation activities can be demonstrated either in controlled conditions or in natural field conditions. Controlled conditions concern any type of crops (greenhouses, climate chambers, air conditioned chambers) where it is possible to simplify and reduce to a miniature scale the production conditions of a plant of agricultural interest. It is essential to describe the conditions used: *in vitro/in planta*, plant species, organs, temperature, light (duration, intensity), hygrometry, nutrients... These trials may make it possible to determine the action mode(s).

It is advisable to confirm claims made on a product in field trials and/or controlled conditions, of which the methodology is provided in chapter 3.

3. EXPERIMENTAL CONDITIONS

Objectives in efficacy trials are to demonstrate the relevance of biostimulants, by providing data according to the activities claimed.

A study carried out on biostimulants is conducted in different types of trial. It is advisable to perform trials in controlled conditions, before a full field experiment is carried out, to characterize the type of activity of the product under study and demonstrate its stimulatory activity on plants. The advantages of such trials are to apprehend its/their mode(s) of action(s), the most appropriate dosage levels, the recommended modes and periods for inputs and eventually the best suited formulations or combinations of biostimulants.

Please refer to appendix 2 for examples of analysable parameters for the purpose of highlighting the effects claimed (non-exhaustive list).

Depending on the claim category, experimental conditions must be adapted, in order to improve the way the effects are brought to light.
Please refer to the table in Appendix 3.

The choice of a plant or plant organ used in controlled conditions must be justified and consistent with the expected effect.

3.1. The choice of the area

This choice is indifferent in the case of controlled conditions.

With regard to field trials, select regions where pedoclimatic conditions facilitate the expression of the benefits claimed and where the targeted crops are representative.

3.2 Choice of location for implementation purpose

The trial should be implemented on a site or on a batch of plants treated homogeneously.

As to field experiments, it is advisable to know and take into consideration the soil history of the plot where the trial is to be implemented. It may be necessary to repeat the experiment on several crop cycles on the same plot, particularly when the effect claimed emerges only on a long term basis and if the crops in the rotation system will allow it.

In the case of field testing of abiotic stress, the very nature of the stress (hydric, thermal, pH,

salinity...) must be defined, as well as its intensity, its duration and its period of application in relation to the phenological stages of the crop. Exposition to these types of stress may require the experiments to be done in controlled conditions.

3.3. The choice of crop

Experiments on efficacy should be carried out on representative categories concerned with the crops claims being held. The petitioner is to select the most relevant crop and variety and to measure the parameters best likely to demonstrate the biostimulant effects being claimed. In the case where the crop is not representative, this choice is to be justified.

3.4 Untreated check

Information with regard to the laying out of untreated check or control in the design can be found in DT method N° 4 of the CEB. Non treated check are required.
In some cases, [(such as a biostimulant containing one or more nutrients)], an input equivalent in terms of its level, must be applied on the untreated check, so as to specifically evaluate the effect of the biostimulant.
Note that 100% mineral inputs equivalent in quantity to organic molecules will have no comparative efficacy. Indeed, a provision of nutrient elements from organic molecules does take time and rarely reaches 100% within the one season.

When a claim is made on the effect of a biostimulant with regard a particular type of stress, it will be appropriate to apply to untreated plots, and generally to all conditions, the same type of stress as that applied to the experimental condition. In the case where the design contains a non-stressed treated condition, a non-treated non-stressed condition should be added.

With regard to field experiments, untreated plots are generally included. Depending on the crop heterogeneity level, or the type of biostimulant (in particular microorganisms), a/some control(s) may be excluded, intertwined or adjacent.
For example:

- excluded, in the case of microorganisms on account of spreading risks,
- intertwined, in the case of anti-stress (abiotic) products,
- adjacent, in the case of products stimulating plant growth.

In the case of evaluation under hydric stress, a "control treated with water" is advised. By "control treated with water", is meant a control condition that receives the same total amount of water as is sprayed on the treated condition, so as to determine the impact of hydric inputs on the effect claimed.

3.5 Experimental design

The experimental design is to be adapted to the objectives of the study and to the claim category, and be justified. The number of blocks should be adapted to a consistent degree of freedom. It must be designed in such a way as to allow for statistical data analysis.

It is advisable to reach a minimum number of degrees of freedom = 12 (adequate statistical power). Please refer to CEB DT 26 as well as to other documents pertaining to the statistical analysis of agricultural experiments.

For example:

- for 1 site: 4 blocks and a minimum of 5 conditions OR 3 blocks and a minimum of 7 conditions,
- for 4 sites: 3 blocks and 3 conditions.

With regard to field experiments, select plots as homogeneous as possible and/or use adequate experimental designs (Latin square, split-plot...)
For instance, the split plot design is particularly adapted to trials on biostimulants involving different levels of fertilization (taking into account the fertilization gradient as a technical requirement).

3.6 Plot size

Plot size and sample size should be adapted to the type of crop and to the product, taking into account the number of observations, the sample size (whether destructive or not), yield and border effects.

3.7 Plot layout

No particular specificity

4. INPUT (TREATMENT)

4.1 Basis for comparison

It is advisable to select a basis for comparison with the same type of effect sought as the preparation under study.

4.2 Doses to experiment on

The doses to be experimented on are determined in preliminary trials.
Choose a range of doses which includes the dose supposed to be efficient.

4.3 Time and frequency of input

The periods, as well as the conditions, for applications are determined in preliminary trials.
Crop parameters should be studied precisely, in that they should allow the best expression of efficacy in terms of the effect claimed: the phenological stage of the plant, its physiological state, its exposure to stress and any other parameter likely to influence the efficacy of the product.

4.4 Implementation of inputs

The modes of application are defined according to biostimulants characteristics.

For instance, when evaluating microorganisms, ensure the integrity/feasibility of the population is reinforced.

To this purpose:

- if applied by means of spraying, select nozzles and appropriate pressure settings,
- if applied to the soil surface, pay attention to input procedures (location, incorporation...),
- consider the biological and chemical parameters which condition the survival of microbial strains: soil PH and its organic status, its nutrients levels, its susceptibility to plant production products, its photosensitivity...

4.5 Treatment for crop management

Select a crop itinerary adapted to biostimulants efficacy (see Appendix 3).

Some fertilizers, growth regulators or plant protection products might interfere with the efficacy of biostimulants and specifically microorganisms. It is therefore crucial to make a note of all the treatments made, including the dates of application, for the purpose of analysing the results. Any intervention made, including inputs, must strictly be identical on all plots concerned.

5. ASSESSMENTS AND RECORDINGS

5.1 Preliminary assessments

These assessments, undertaken before trial implementation, are meant to ensure crops homogeneity.

5.2 Main assessments.

The petitioner determines the most pertinent variables to demonstrate the expected effects. In the case of implementation of trial networks, the same variables will be observed on all the plots concerned with all the network trials.

5.2.1 Organs and observed variables

Depended on the effects claimed, it may be useful to observe the following parameters: (Non-exhaustive list, see appendixes 1 & 2)

5.2.1.1 At the plant level

- Emergence and location
- Density, crop tillering...
- Quantitative and qualitative effects on above-ground development:
  - size and dimension of organs and/or plants,
  - biomass (fresh matter and/or dry according to a methodology normalized throughout),
  - mineral components,
  - plant habit (crop),
  - colour and appearance (deficiency …) of foliage, foliar surface …,
  - yield components.

- Quantitative and qualitative effects on above-ground development:
    - biomass (fresh or dry matter),
    - offshoots, length...,
    - root appearance.

- Nutrition index plant vitality (chlorophyllian index, etc.).

- The development speed of a plant at the appropriate phenological stages for the crop concerned (germination, flowering...).

5.2.1.2 Observation of the crop / production

Depending on the claim categories, it may be useful to collect the quantitative and/or qualitative data of the crop, or at the post-crop stage: weight, grade, number, organoleptic quality, and/or nutritional quality, and/or depending on commercial criteria, aptitude to preservation and/or transformation...

5.2.2 Assessment timing and frequency

Frequency and periods of assessment vary depending on the crops, the expected effects and the experimental objectives.

5.3 Assessment of direct effect on plant (Phytotoxicity)

A first approach on crops susceptibility to products is gathered from further observations carried out in efficacy trials. However, this approach needs to be complemented by specific crop susceptibility trials when phytotoxicity symptoms appear in efficacy trials. In the laboratory environment, phytotixicity can be evaluated on the basis of XPU 44-166 and XPU 44-167 norms. In the field context, please refer to the Végéphyl CEB general method MG12 « General method for testing crop susceptibility to herbicides, fungicides and insecticides ».

5.4 Assessments of the effects of inputs on practical conditions

Due to the diversity of biostimulant products and their modes of action, the efficacy of stimulation products is sometimes difficult to demonstrate, particularly when efficacy trials are an intrinsic feature of the product in a full field context. It seems therefore essential to promote an approach which allows the integration of biostimulants in the full programs of agricultural production.

These trials complement efficacy trials by taking into account all technical, and eventually technico-economic, implications of a product used in conditions close to its future agronomic application.

Their goals are to:

- confirm their practical relevance, if this demonstration has not already been done completely in full field efficacy trials,
- define the products conditions of use, eventually up to harvest and justify claims on product use.

To this effect, it may be useful to:

- define the programs,
- refine the conditions for use and/or application,
- suggest the combination of some biostimulants between them or with other inputs (cf. Appendixes 2 and 3).

For the purpose of implementing these trials, refer to the Végéphyl CEB general method "General principles for testing the practical value of plant protection products" N° MG 13.

5.5. Assessments of the adverse effects of inputs

The evaluation of adverse effects, including effects on non-targeted organisms, as well as the incidence of products with stimulating activities on the quality of plants, or plant products, are subject to specific methods (please see the list of regularly updated CEB methods).

There is no particular specificity attached to this method.

5.6. Recording of meteorological and edaphic data

During the trial it is advisable to acquire as much information as possible so as to later use these data as explanatory co-variables for the variations in observed efficacy. This may involve, for instance, estimating stresses and their intensity, recording symptoms of plant growth delays or any other crop defect.
With regard to full field experiments, proceed with any relevant measure (climatic data, soil profile, soil analysis...) to help define stress intensity and duration.

In the case of cereal straw for instance, the main types of stress to be checked are water and nitrogen stress.

6. STATISTICAL ANALYSIS OF VARIABLES AND INTERPRETATION OF RESULTS

6.1. The development of variables

State how variables were collected calculated from observed variables.

6.2. Statistical analysis

6.2.1. For an individual trial (see Végéphyl DT 26)

6.2.2. Grouping trials (see Végéphyl DT 26)

7. PRESENTATION OF RESULTS

7.1. For an individual trial

It is adapted in accordance with the main assessments undertaken.

In the case of assessments concerned with quantitative and qualitative crop data, the results from control conditions are presented in absolute values and results from treated plots are presented in relative values (efficacy percentages) or in absolute values.

In cases where assessments are carried out on different dates, a graphic representation will be useful and recommended.

7.2. Grouping trials

Results can be expressed in the form of tables or graphic representations, including, for instance:

- the periods of assessment,
- the crop stages, the organs observed,
- the number of trials, the number of product applications.

The results from (the) control(s) condition(s) are presented in absolute values.
Results from conditions are presented in the form of relative values or mean absolute values, indicating values from measures of dispersion.

APPENDIX 1

Details of agricultural claims associated with plant growth-stimulating products, according to the final report: Plant growth-stimulating products in agronomy aiming to improve the biological functions of soils and plants. ("Produits de stimulation en agriculture visant à améliorer les fonctionnalités biologiques des sols et des plantes." - TN) BIO by Deloitte (BIO) et RITTMO Agroenvironnement (RITTMO)- December 2014).
Non-exhaustive and expurgated list of claims on plant protection products (PPPs)

Claims	**List of effects claimed**
Resistance to abiotic stress	- Increased tolerance to cold (including frost) or heat - Tolerance to salinity - Tolerance to oxidative stress (of which UV, ozone) - Increased tolerance to drought and excess water
Growth and development	- Increase of germination rate - Increased precocious germination - Positive action on tillering and magnification of seeds - Stimulation of the number of inflorescences - Boosted development of buds - Stimulation of plant growth - Stimulation of plant hormones production beneficial to growth - Increase in foliar biomass - Stimulation of root development in terms of density and depth - Reinforcement of the root system - Improvement of photosynthetic efficiency - Increase in chlorophyll content
Better nutrient absorption	- Improvement in plant nutrition - Increase in the bioavailability of mineral elements - Solubilisation of mineral elements - Improvement of mineral elements - Reinforcement of plants capacity to absorption of water and nutrients - Optimisation of nutrient release - Improvement of the physical structure of soils - Formation of mycorrhizae - Stimulation of nitrate reductase activity - Production of auxins through microflora - Stimulation of soil microbial activity - Stimulation of organic matter degradation - Increase in soil microbial activity and diversity
Better harvest quality	- Organoleptic (sugar content and other molecules) - Nutritional (vitamin, protein, sugar contents, etc.) - Visual (fruit colour) - Technical (better tolerance to storage or to manipulation) - Improvement of fruit firmness for storing purposes

Claims (Cont'd)	List of effects claimed
Plant growth-stimulating products :	
Economic benefits (of which increase in crop yield)	- Improvement in the size of fruits - Increase in the quality of seeds - Increase in the quality of fruits - Improvement in the efficacy of fertilizers to hence reduce their quantity
Environmental benefits	- Improvement in the efficacy of fertilizers to hence reduce their quantity - Increase in soil microbial diversity - Reduction in water use

APPENDIX 2

Examples of analysable parameters for the purpose of highlighting the effects claimed (non-exhaustive list):

Trials in controlled conditions	Full field trials
Evaluation of major technical, organoleptic and/or nutritional parameters specific to the crop concerned.	Evaluation of major technical, organoleptic and/or nutritional parameters specific to the crop concerned.
	Evaluation of potential drop in fertilizer input
Analyses of soluble forms depending on the methods available in the NPKS laboratory	Analyses of soluble forms of nitrogen No method for any other element; methods are available for major elements. Analyses of the levels of mineral elements + biomass measurements Isotope methods
Estimating vigour, colour, plant behaviour	Estimating vigour, colour, plant behaviour
Evaluation of above-ground and/or root biomass (fresh or dry matter). Evaluation of root architecture (offshoots, length,..) Evaluation of above-ground architecture (size and dimension of organs, plant habit...)	Evaluation of above-ground biomass and/or root biomass (fresh or dry matter) Sample preparation for the analysis of dry matter and the analysis of mineral elements must be thorough. (*)

(*) To this effect, remove excess soil from the plants and clean the parts to be analysed. **Cleaning must be done with distilled or demineralised water.**

APPENDIX 3

Advice for highlighting the effects of biostimulants

<table>
<tr><th colspan="2">claim category</th><th>Advice</th></tr>
<tr><td colspan="2">Indirect nutritional effect (bioavailability / absorption / mobilization)</td><td>o Facilitate the control of major and minor elements to avoid any experimental conditions which may prevent the detection of expected effects (at least with regards elements N, P and K). The global and integrated management of the fertilization program depending on target crop.
o Implement the experiment in such conditions as for fertilizer inputs (as in technical itineraries), at sub-optimum and optimum fertilization doses, according to crops needs. Indeed, these substances will stimulate growth or make some nutrients available without directly feeding the plant. A certain level of fertilization is necessary, without producing over nutrition states detrimental to highlighting the effects of the product being tested. Imprecise methods used to a priori determine doses of major elements require the use of a response curve to avoid cases of over-fertilization.
o When testing with a view to evaluate plant mineral content, make sure that the data measured are not distorted by the presence of these elements which may have remained on the surface of the organs being studied.</td></tr>
<tr><td rowspan="3">Tolerance to abiotic stress</td><td>Hydric stress</td><td>o Give preference to controlled hydric conditions (possibility of regulating quantity and/or input periods). Give particular attention to the nature and depth of the soil, to be linked to the level of hydric supply.
o An a posteriori diagnosis of water availability needs to be planned to evaluate the hydric stress on the site (giving at least records accurately programmed, with perhaps a setup of sensors measuring the hydric state of the soil [tensiometer, capacitive electrodes])...
o Control treated with water (an amount of water made available by the spray volume of the treated condition, so as to evaluate the impact of water input of the volume applied on the condition concerned)</td></tr>
<tr><td>Salt stress</td><td>o Give preference to sites irrigated by means of sprinkling or misting (increases stress)</td></tr>
<tr><td>Therma stress</td><td>o Control treated with water (and not a control with no application)</td></tr>
<tr><td colspan="2">Production quality: impact criteria on production quality (among other yield factors, organoleptic properties, etc.)</td><td>Cf. methods and adapted benchmarks, if available</td></tr>
</table>

APPENDIX 4

Definitions and references pertaining to the document

Official definition of plant biostimulants, according to the European project 2016/0084 (COD):

A plant biostimulant shall be a EU fertilising product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant:
a) nutrient use efficiency,
b) tolerance to abiotic stress, or
c) quality traits.

(Un biostimulant des végétaux est un produit qui stimule les processus de nutrition des végétaux indépendamment des éléments nutritifs qu'il contient, dans le seul but d'améliorer une ou plusieurs des caractéristiques suivantes des végétaux :
a) l'efficacité d'utilisation des éléments nutritifs,
b) la tolérance au stress abiotique, ou
c) les caractéristiques qualitatives du végétal cultivé.)

References:

- CEB: Experiments on natural products stimulating plant vitality - Végéphyl N° **DT 20.**
- OEPP: Design and analysis of efficacy evaluation trials – PP1/152 (4)
- Final report: Plant growth-stimulating products in agronomy aiming to improve the biological functions of soils and plants. ("Produits de stimulation en agriculture visant à améliorer les fonctionnalités biologiques des sols et des plantes." - TN) BIO by Deloitte (BIO) et RITTMO Agroenvironnement (RITTMO). Final report-December 2014.
- 2016/0084 draft reference