SLOVENSKI STANDARD
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Plant biostimulants - Determination of Rhizobium spp

Biostimulanzien für die pflanzliche Anwendung - Bestimmung von Rhizobium spp

Biostimulants des végétaux - Détermination de Rhizobium spp.

Ta slovenski standard je istoveten z: FprCEN/TS 17718

ICS:

65.080	Gnojila	Fertilizers

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English Version

Plant biostimulants - Determination of Rhizobium spp

Biostimulanzien für die pflanzliche Anwendung - Bestimmung von Rhizobium spp

This draft Technical Specification is submitted to CEN members for Vote. It has been drawn up by the Technical Committee CEN/TC 455.

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Recipients of this draft are invited to submit, with their comments, notification of any relevant patent rights of which they are aware and to provide supporting documentation.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

This document (FprCEN/TS 17718:2021) has been prepared by Technical Committee CEN/TC 455 “Plant Biostimulants”, the secretariat of which is held by AFNOR.

This document is currently submitted to the Vote on TS.

This document has been prepared under a Standardization Request given to CEN by the European Commission and the European Free Trade Association.

Introduction

This document was prepared by the experts of CEN/TC 455 'Plant Biostimulants'. The European Committee for Standardization (CEN) was requested by the European Commission (EC) to draft European standards or European standardization deliverables to support the implementation of Regulation (EU) 2019/1009 of 5 June 2019 laying down rules on the making available on the market of EU fertilising products ("FPR" or "Fertilising Products Regulation"). This request, presented as SR M/564, also contributes to the Communication on "Innovating for Sustainable Growth: A Bio economy for Europe". The Working Group 5 "Labelling and denominations", was created to develop a work program as part of this request.

The technical committee CEN/TC 455 'Plant Biostimulants' was established to carry out the work program that will prepare a series of standards. The interest in biostimulants has increased significantly in Europe as a valuable tool to use in agriculture. Standardization was identified as having an important role in order to promote the use of biostimulants. The work of CEN/TC 455 seeks to improve the reliability of the supply chain, thereby improving the confidence of farmers, industry, and consumers in biostimulants, and will promote and support commercialisation of the European biostimulant industry.

Biostimulants used in agriculture can be applied in multiple ways: on soil, on plants, as seed treatment, etc. A microbial plant biostimulant consists of a microorganism or a consortium of microorganisms, as referred to in Component Material Category 7 of Annex II of the EU Fertilizing Products Regulation.

This document is applicable to all biostimulants in agriculture based on live microorganisms belonging to the group Rhizobia.

Table 1 summarizes many of the agro-ecological principles and the role played by biostimulants.

Table 1. — Agro-ecological principles and the role played by biostimulants

Increase biodiversity
By improving soil microorganism quality/quantity
Reinforce biological regulation and interactions
By reinforcing plant-microorganism interactions
— symbiotic exchanges i.e. *mycorrhize*
— symbiotic exchanges i.e. *rhizobiaciae/fava*
— secretions mimicking plant hormones (i.e. *trichoderma*)
By regulating plant physiological processes
— for ex growth, metabolism, plant development
Improve biogeochemical cycles
— improve absorption of nutritional elements
— improve bioavailability of nutritional elements in the soil
— stimulate degradation of organic matter

WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably trained staff.

1 Scope

This document provides the methodology for the enumeration and determination of *Rhizobium sp., Mesorhizobium sp., Ensifer sp.*, or *Bradyrhizobium sp.*) in plant biostimulant products in accordance with the Regulation (EU) 2019/1009 of the European Parliament and of the Council [1].

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

FprCEN/TS 17724, *Plant Biostimulants – Terminology*

FprCEN/TS 17702-1, *Plant Biostimulants - Sampling and sample preparation – Part 1: Sampling*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in FprCEN/TS 17724 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at https://www.electropedia.org/
- ISO Online browsing platform: available at https://www.iso.org/obp

3.1
rhizobium
beneficial bacteria belonging to the group named Rhizobia, where the most relevant genera are Rhizobium, Mesorhizobium, Ensifer and Bradyrhizobium

Note to 1 entry: Rhizobium belonging to this group are *rhizobium sp., mesorhizobium sp., ensifer sp., bradyrhizobium sp..*

Note to entry 2: Legumes (Leguminosae or Fabaceae) are considered the second most cultivated crop, covering 14 % of the total cultivated land worldwide and providing an important source of food for human beings via direct consumption or indirect consumption via animal feed. Leguminosae can ensure high quality protein-rich food and feed due to a special symbiosis they have with specific microorganisms present in the soil that can fix in the rhizosphere, atmospheric nitrogen. Those microorganisms can account for a 65 % of the total fixed nitrogen. Those microorganisms have originally been called Rhizobium. The word 'rhizobium' is actually derived from two Greek words 'rhizo' meaning root and 'bium' meaning home. Since the late nineteenth century, all legume root-nodule bacteria were placed in the genus 'Rhizobium'. Gradually it was realized that they were rather diverse. A few slow-growing rhizobia were split off into a new genus 'Bradyrhizobium'. In the 1984 edition of Bergey's Manual of Systematic Bacteriology, all rhizobia were placed in the family Rhizobiaceae which included Bradyrhizobium and Rhizobium. Since then, the number of bacterial genera representing rhizobia has increased rapidly; Rhizobia are plant root nodule inhabiting, associative symbiotic, nitrogen fixing bacteria. Today the classification of the different Rhizobia species is based the sequence of the 16S rDNA sequence comparison and physiological and biochemical properties. Considering that taxonomy and phylogeny of bacteria is in continuous evolution and considering that any current classification scheme is subject to future revision and considering moreover that most of the Rhizobial species in the alpha-proteobacteria class of phylum proteobacteria in Rhizobiaceae family are in either the Rhizobium, Mesorhizobium, Ensifer, or Bradyrhizobium genera, for the purpose of this document we will consider the above mentioned genera as referring to the Rhizobium sp. group.

Note 3 to entry: Other nodule-forming bacteria belong to the genus Frankia and interact with non-leguminous species, including woody species of the families Betulaceae and Casuarinaceae. Such bacteria should be included in the general wording of "Rhizobium" according to the terms of this document.

[SOURCE: FprCEN/TS 17724, 3.2.2.7]

4 Enumeration of Rhizobium sp., Mesorhizobium sp., Ensifer sp., Bradyrhizobium sp.)

4.1 General

This procedure is meant to determine the number of colony-forming units (CFU) of the above mentioned bacteria «Pathogen», per gram or per millilitre. The method, in order to be fast, cheap and repeatable, is based on serial dilutions and plating.

4.2 Sample preparation

4.2.1 General

A representative sample of the product to be analysed as per the requirements of FprCEN/TS 17702-1 will be prepared according to following procedure, which takes into consideration the different formulations of biostimulants based products.

4.2.2 Liquid (based water) formulations

Dispense 25 ml of sample (or more for low concentrated products) in 225 ml of sterile Phosphate Buffer Solution (PBS) maintained at room temperature, in a flask and shake for 10 min or more until the distribution is optimal, with a magnetic stirrer at half speed [9].

4.2.3 Liquid - based oil, emulsifiable concentrate (EC) formulations

Dispense 25 ml of sample (or more for low concentrated products) in 225 ml of sterile Phosphate Buffer Solution (PBS) maintained at room temperature, in a flask and shake for 10 min or more until the distribution is optimal, with a magnetic stirrer at half speed [9].

4.2.4 Solid - Wettable Powder (WP) formulations

Dispense 25 g of sample (or more for low concentrated products) in 225 ml of sterile Phosphate Buffer Solution (PBS) maintained at room temperature, in a flask and shake for 20 min or more until the distribution is optimal, with a magnetic stirrer at half speed [9].

4.2.5 Solid - Water dispersible granules (WDG) formulations

Dispense 25 g (or more for low concentrated products) of sample in 275 g of sterile Phosphate Buffer Solution (PBS) maintained at room temperature in a flask and shake for 40 min or more until the distribution is optimal, with a magnetic stirrer at half speed. If required help the dispersion of the formulations with other apparatus such as a stomacher after having sieved (100 mesh sieve) the particles and resuspend them in the same suspension [9].

4.2.6 Solid – Pellets, granules, microgranules - slow release - formulations

Dispense 25 g (or more for low concentrated products) g of sample in 225 g of sterile Phosphate Buffer Solution (PBS) maintained at room temperature, in a sterile bag and disperse them using a magnetic stirrer for 40 min at half speed and then sieve in a 100 mesh sieve and if material remain in the sieve repeat the process for a maximum of three times. Put attention to all the buffer used to make the exact final calculation [9].

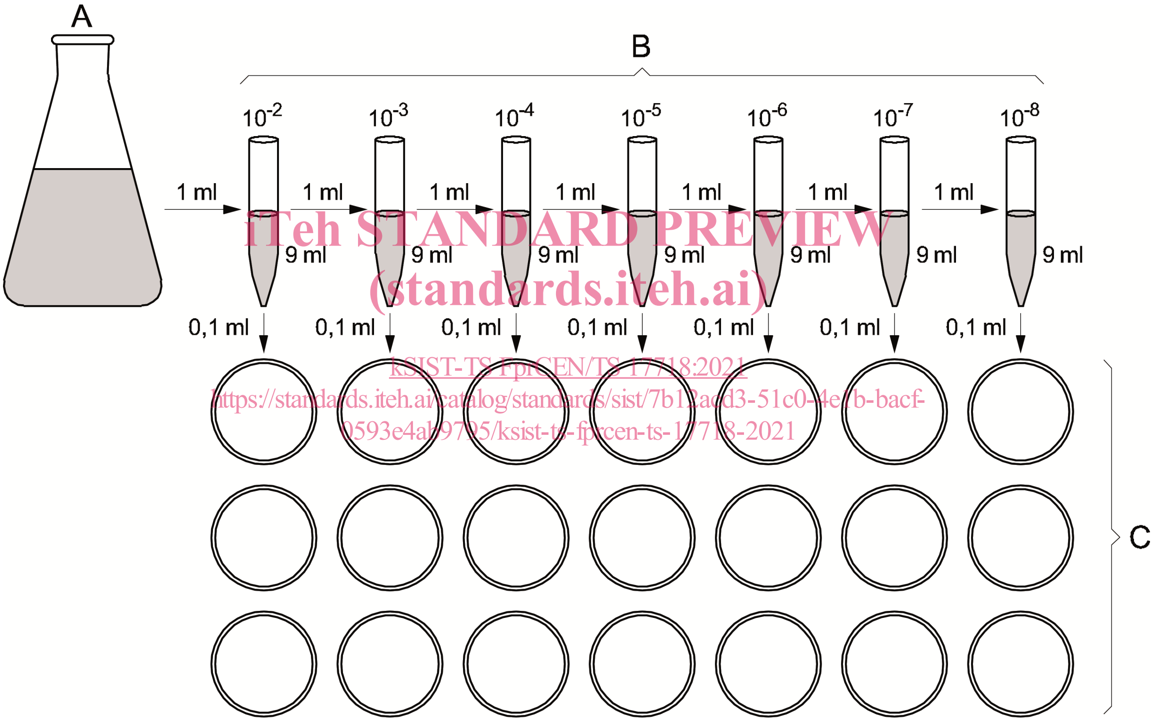
4.2.7 Solid - substrate

Dispense 25 g (or more for low concentrated products) g of sample in 275 g of sterile Phosphate Buffer Solution (PBS) maintained at room temperature in a flask and shake for 20 min or more until the distribution is optimal, with a magnetic stirrer at half speed [9].

4.3 Serial dilution

The principle in counting bacteria by dilution is to serially dilute them to reduce the bacterial density to the level where individual cells can be differentiated. This may be, for example, as live cells under the microscope, as colonies that grow on plates from single cells, or estimated in the plant-infection technique (with the principle that a single cell can multiply to initiate an infection). Serial dilution can be applied to all kind of formulations. A 10-fold serial dilution is most often used (Figure 1) but if the number of rhizobia is expected to be low then a lower number of dilutions can be adopted [8].

The diluent is the Phosphate Buffer Solution (PBS) (see A.5)



Key

A Suspension of the sample

B Serial dilutions

C Petri plates

Figure_1 — Scheme of serial dilutions

A sample of the product is shaken in a bulk diluent (PBS) which represents the first level of dilution. This is then serially diluted with a sample at each level of dilution directly plated

4.4 Plate counts of rhizobia in sterile diluent

The counting of microorganisms on plates, following dilution, is also called direct counting. Counts only plates where there are between 30 to 300 colonies.

4.5 Spread-plate counting

1) Inoculate 0.1 mL of the serial dilutions desired (e.g. 10^{-5}, 10^{-6} and 10^{-7}) on the surface of the culture medium in Petri dishes (Figure 1).

2) Spread the 0,1 mL aliquot over the culture medium with a sterilized L-shaped glass spreader (or equivalent, e.g. a Drigalski loop).

3) There should be at least three separate replicate plates for each dilution.

4) After inoculation and absorption of the inoculum into the agar, the plates are placed in an incubator at approximately 28°C for a period of two to eight days (according to the growth rate of the species; see Table 2.

5) Count the number of colonies on plates where colonies are well separated. If colony numbers are low, variation between plates and errors may be large. If colony numbers are too high, overcrowding may result in an underestimation of numbers. Many texts recommend counting between 30 and 300 CFU (Colony Forming Units) per plate to give statistical robustness.

4.6 Growth Media

For the different Rhizobia species use different Growth Media according to Table 2: YT media for *Rhizobium* spp. (alternatively use AYEM media), YT media for *Mesorhizobium* spp., YT media for Ensifer spp. And R2A media for *Bradyrhizobium* spp.

Table 2 — Requirements Media and Incubation

Genus	Media	Bibliographic Reference for the Media	Conditions	Time of incubation	Temperature
Rhizobium spp	YT	(14)	Aerobic	7–8 days	28°C ± 2 (11)
	AYEM (alternative media)	(13)	Aerobic	7–8 days	28°C ± 2
Mesorhizobium sp.	YT	(10)	Aerobic	7–8 days	28°C ± 2
Ensifer sp.	YT	(11)	Aerobic	7–8 days	28°C ± 2
Bradyrhizobium	R2A	(12)	Aerobic	7–8 days	28°C ± 2

4.7 Calculation

Multiply the average number of CFU on the three Petri dishes by the inverse of the dilution that gave the reading in the range 30 to 300 CFU by 10 (to correct for the 0.1 mL or gr used).

EXAMPLE Assuming that the average of three plates was 75 CFU and that the dilution that gave this reading was 10^{-6}, calculate:

Average = 75.

Correction factor = 10.

Dilution of the suspension = $10^{-6.}$

CFU = 75 X 10 X 10^{6} ⇒7,5 X 10^{8} CFU/mL or gram.