SLOVENSKI STANDARD
oSIST prEN 17718:2023

01-maj-2023

Rastlinski biostimulanti - Ugotavljanje prisotnosti in števila Rhizobium spp.

Plant biostimulants - Determination of Rhizobium spp.

Pflanzen-Biostimulanzien - Bestimmung von Rhizobium spp.

Biostimulants des végétaux - Détermination de Rhizobium spp.

Ta slovenski standard je istoveten z: **prEN 17718**

ICS:

65.080 Gnojila Fertilizers

oSIST prEN 17718:2023 **en,fr,de**

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

DRAFT
prEN 17718

April 2023

ICS 65.080

Will supersede CEN/TS 17718:2022

English Version

Plant biostimulants - Determination of *Rhizobium* spp.

Biostimulants des végétaux - Détermination de *Rhizobium* spp.

Pflanzen-Biostimulanzien - Bestimmung von *Rhizobium* spp.

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

If this draft becomes a European Standard, CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration.

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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 (prEN 17718:2023) 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 CEN enquiry.

This document will supersede CEN/TS 17718:2022.

This document has been prepared under a Standardization Request given to CEN by the European Commission and the European Free Trade Association, and supports essential requirements of EU Directive(s) / Regulation(s).

For relationship with EU Directive(s) / Regulation(s), see informative Annex ZA, which is an integral part of this document.

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 standardization request, presented as SR M/564 and M/564 Amd1, also contributes to the Communication on “Innovating for Sustainable Growth: A Bio economy for Europe”. Working Group 5 “Labelling and denominations”, was created to develop a work program as part of this standardization request.

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 plant 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 plant 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 plant biostimulants, and will promote and support commercialisation of the European biostimulant industry.

Plant 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 Fertilising Products Regulation.

This document is applicable to all plant 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 plant biostimulants.

Table 1 — Agro-ecological principles and the role played by plant 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
— e.g. 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 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.

EN 17702-1:—[1], *Plant biostimulants — Sampling and sample preparation — Part 1: Sampling*

EN 17724:—[2], *Plant biostimulants — Terminology*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN 17724:—[2] and the following apply.

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

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

3.1
Rhizobium
beneficial bacteria belonging to the group named Rhizobia, where the most relevant genera are Rhizobium, Mesorhizobium, Ensifer and Bradyrhizobium [2] [3] [4] [6] [7]

Note 1 to entry: Rhizobium belonging to this group are *Rhizobium* spp., *Mesorhizobium* spp., *Ensifer* spp., *Bradyrhizobium* spp.

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 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* spp. group.

[1] Under preparation
[2] Under preparation

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: EN 17724:—[2], 3.2.2.7]

4 Enumeration of *Rhizobium* spp., *Mesorhizobium* spp., *Ensifer* spp., *Bradyrhizobium* spp.

4.1 General

This procedure is meant to determine the number of colony-forming units (CFU) of the above-mentioned bacteria, 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 EN 17702-1:—[1] shall be prepared according to following procedure, which takes into consideration the different formulations of plant biostimulants based products.

4.2.2 Liquid - Water based 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 of maximum speed [9].

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

Dispense 25 ml of sample (or more for low concentrated products) in 225 ml of sterile 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 of maximum 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 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 of maximum speed [9].

4.2.5 Solid - Water dispersible granules (WDG) formulations

Dispense 25 g of sample (or more for low concentrated products) in 275 g of sterile 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 of maximum speed. If required, help the dispersion of the formulations with other apparatus such as a laboratory paddle blender 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 of sample (or more for low concentrated products) in 225 g of sterile PBS maintained at room temperature, in a sterile bag and disperse them using a magnetic stirrer for 40 min at half of maximum speed and then sieve in a 100 mesh sieve and, if material remains 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 of sample (or more for low concentrated products) in 275 g of sterile 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 of maximum 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 may 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 may be adopted [8].

The diluent is the PBS (see Annex A, A.5).

4.4 Preparation of the culture media

The preparation and the culture media is described in Annex A. The preparation and performance of culture media is a fundamental step to ensure the integrity of microbiological examination. When ready-to-use media are used, the manufacturers of this available media should have a quality program that ensure the quality of the media they supply, according to EN ISO 11133:2014[3] [15]. Under these conditions, the user/laboratory does not need to run additional testing on such media but shall ensure the storage condition according to the ones recommended by the manufactures. For diluents and media prepared by the user/laboratory directly from commercially available dehydrated formulations and/or from basic individual components, the performance of these diluents/media should be evaluated according to EN ISO 11133:2014[3] [15].

[3] As impacted by EN ISO 11133:2014/A1:2018 and EN ISO 11133:2014/A2:2020