SLOVENSKI STANDARD
SIST EN 17718:2025

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Nadomešča:
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Rastlinski biostimulanti - Določanje 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: **EN 17718:2024**

ICS:

65.080	Gnojila	Fertilizers

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EUROPEAN STANDARD
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Supersedes 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 European Standard was approved by CEN on 26 August 2024.

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

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Ref. No. EN 17718:2024 E

Contents

Page

European foreword

This document (EN 17718:2024) has been prepared by Technical Committee CEN/TC 455 “Plant Biostimulants”, the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 2025, and conflicting national standards shall be withdrawn at the latest by May 2025.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN shall not be held responsible for identifying any or all such patent rights.

This document supersedes CEN/TS 17718:2022.

EN 17718:2024 includes the following significant technical changes with respect to CEN/TS 17718:2022:

— the European foreword has been updated;

— the Introduction has been updated;

— the Bibliography has been re-numbered;

— in Annex A, Buffered Peptone Water has been added as diluent for the enumeration;

— in 4.5 a general description of the colonies of the different genera has been included;

— in Clause A.5, the recipe of Phosphate Buffered Saline has been modified;

— Annex ZA has been added.

This document has been prepared under a standardization request addressed to CEN by the European Commission. The Standing Committee of the EFTA States subsequently approves these requests for its Member States.

For the relationship with EU Legislation, see informative Annex ZA, which is an integral part of this document.

Any feedback and questions on this document should be directed to the users’ national standards body. A complete listing of these bodies can be found on the CEN website.

According to the CEN-CENELEC Internal Regulations, the national standards organisations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Republic of North Macedonia, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Türkiye and the United Kingdom.

Introduction

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 [1] 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 relevant amendments, also contributes to the Communication on "Innovating for Sustainable Growth: A Bio economy for Europe". 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 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.

In accordance with Regulation (EU) 2019/1009 of the European Parliament and of the Council [1], only *Rhizobium* spp. is required.

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 *Rhizobiaceae* (*Rhizobium* spp., *Mesorhizobium* spp., *Ensifer* spp., *Bradyrhizobium* spp.).

This document is applicable to the blends of fertilizing products where a blend is a mix of at least two of the following component EU fertilising products categories: Fertilizers, Liming Materials, Soil Improvers, Growing Media, Plant Biostimulants and where the following category Plant Biostimulants is the highest percentage in the blend by mass or volume, or in the case of liquid form by dry mass. If Plant Biostimulants is not the highest percentage in the blend, the European Standard for the highest percentage of the blend applies. In case a blend of fertilizing products is composed of components in equal quantity or in case the component EU fertilising products used for the blend have identical formulations[1], the user decides which standard to apply.

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 17702-1:2024, *Plant biostimulants — Sampling and sample preparation — Part 1: Sampling*

EN 17724:2024, *Plant biostimulants — Terminology*

EN ISO 11133:2014[2], *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media (ISO 11133:2014)*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN 17724:2024 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 Rhizobiaceae, where the most relevant genera are *Rhizobium*, *Mesorhizobium*, *Ensifer* and *Bradyrhizobium* [2] [3] [4] [5] [6]

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

Note 2 to entry: 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 65 % of the total fixed nitrogen. Those microorganisms were originally 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

[1] An example of such a blend is a product with 2 claimed functions consisting of a non-microbial plant biostimulant and an organic fertiliser composed of 1 kg/kg of plant biostimulant from seaweed.
[2] As impacted by EN ISO 11133:2014/A1:2018 and EN ISO 11133:2014/A2:2020.

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 [9], 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 on the sequence of the 16S rDNA sequence comparison and physiological and biochemical properties. Considering that taxonomy and phylogeny of bacteria are in continuous evolution and considering that any current classification scheme is subject to future revision and considering moreover that most of the species in the phylum *Pseudomonadota,* class Alphaproteobacteria, family *Rhizobiaceae* are in the genera *Rhizobium*, *Mesorhizobium*, *Ensifer* and *Bradyrhizobium*, for the purpose of this document we will consider the above-mentioned genera as referring to the *Rhizobiaceae* 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 *Betulaceae* and *Casuarinaceae* families. Such bacteria should be included in the general wording of "*Rhizobium*" according to the terms of this document.

[SOURCE: EN 17724:2024, 3.2.2.14]

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 17702-1:2024 shall be prepared according to the following procedure, which takes into consideration the different formulations of plant biostimulants.

4.2.2 Liquid (water-based) formulations

25 ml of sample (or more for low concentrated products) in 225 ml of sterile diluent (see Annex A) maintained at room temperature in a flask and shaken for 10 min or more until the distribution is optimal, with a magnetic stirrer at half of maximum speed [7].

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

25 ml of sample (or more for low concentrated products) shall be dispensed in 225 ml of sterile diluent (see Annex A) maintained at room temperature in a flask and shaken for 10 min or more until the distribution is optimal, with a magnetic stirrer at half of maximum speed [7].

4.2.4 Solid wettable powder (WP) formulations

25 g of sample (or more for low concentrated products) shall be dispensed in 225 ml of sterile diluent (see Annex A) maintained at room temperature in a flask and shaken for 20 min or more until the distribution is optimal, with a magnetic stirrer at half of maximum speed [7].

4.2.5 Solid water dispersible granules (WDG) formulations

25 g of sample (or more for low concentrated products) shall be dispensed in 225 g of sterile diluent (Annex A) maintained at room temperature in a flask and shaken 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 (150 µm sieve corresponding to a 100 mesh sieve) the particles and resuspended them in the same suspension [7].

4.2.6 Solid pellets, granules, microgranules - slow release - formulations

25 g of sample (or more for low concentrated products) shall be dispensed in 225 g of sterile diluent (see Annex A) maintained at room temperature in a sterile bag and dispersed using a magnetic stirrer for 40 min at half of maximum speed and then sieved in a 150 µm sieve corresponding to a 100 mesh sieve and, if there is still sample material in the sieve, repeat the process for a maximum of three times. Pay attention to all the buffer used to make the exact final calculation [7].

4.2.7 Solid substrate

25 g of sample (or more for low concentrated products) shall be dispensed in 225 g of sterile diluent (see Annex A) maintained at room temperature in a flask and shaken for 20 min or more until the distribution is optimal, with a magnetic stirrer at half of maximum speed [7].

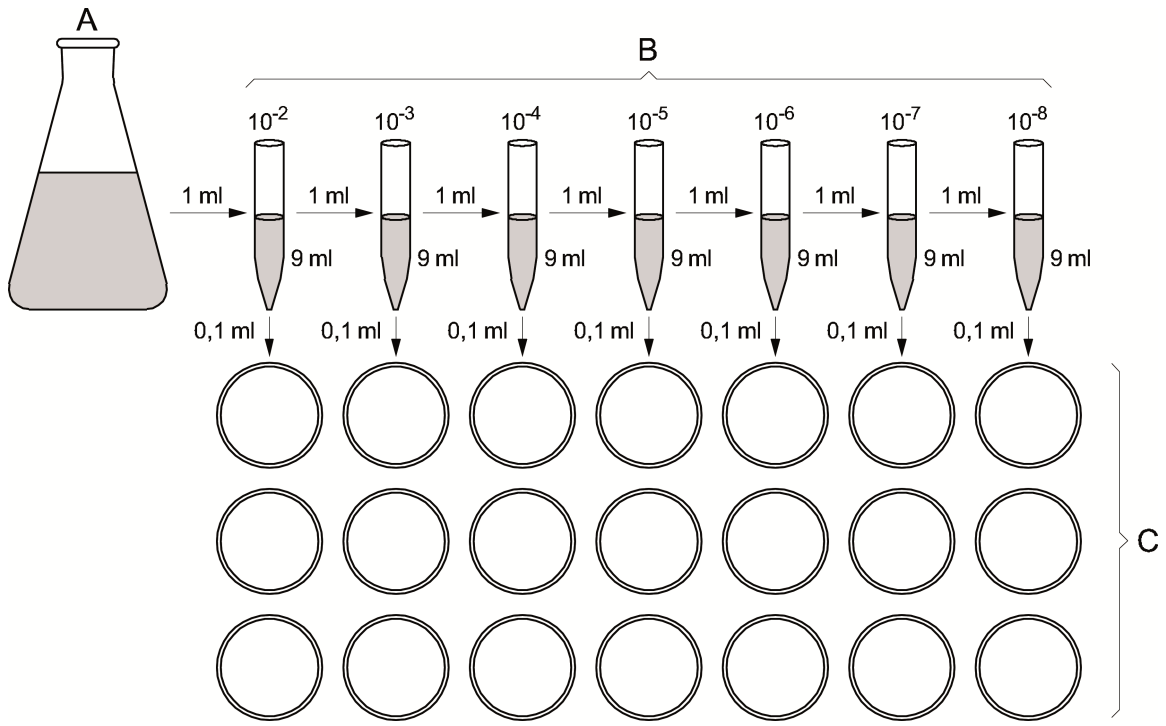
4.3 Serial dilution

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, colonies that grow on plates from single cells, or cells estimated through the plant-infection technique (with the principle that a single cell can multiply to initiate an infection). Serial dilutions may be applied to all kinds 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 of the culture media is described in Annex A. The preparation and performance testing of culture media are fundamental steps to ensure the integrity of microbiological examination. When ready-to-use media are used, the manufacturers of these available media should have a quality program that ensures the quality of the media they supply, according to EN ISO 11133:2014[2]. 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 shall be evaluated according to EN ISO 11133:2014[2].



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 or BPW)) which represents the first level of dilution. This is then serially diluted with a sample at each level of dilution directly plated [10].

4.5 Spread plate counting

The choice of medium is made according to the genus researched by referring to Table 2.

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 ± 2 °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 the plates where colonies are well separated. If colony numbers are low, the 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 per plate to give statistical robustness.