SLOVENSKI STANDARD
SIST EN 17713:2025

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Rastlinski biostimulanti - Določanje Azospirillum spp.

Plant biostimulants - Determination of Azospirillum spp.

Pflanzen-Biostimulanzien - Bestimmung von Azospirillum spp.

Biostimulants des végétaux - Détermination d'Azospirillum spp.

Ta slovenski standard je istoveten z: **EN 17713:2024**

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65.080	Gnojila	Fertilizers

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Plant biostimulants - Determination of *Azospirillum* spp.

Biostimulants des végétaux - Détermination d'*Azospirillum* spp.

Pflanzen-Biostimulanzien - Bestimmung von *Azospirillum* 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

CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

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European foreword

This document (EN 17713:2024) 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of an identical text or by endorsement, at the latest by May 2025, and conflicting national standards shall be withdrawn at the latest by May 2025.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 17713:2022.

EN 17713:2024 includes the following significant technical changes with respect to CEN/TS 17713:2022:

— 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;

— the recipe of Phosphate Buffered Saline (A5) 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.

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 *Azospirillum* spp. in microbial plant biostimulants.

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*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological 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
***Azospirillum* spp.**
Gram-negative bacteria that belong to the alphaproteobacterial phylum

Note 1 to entry: *Azospirillum* is a Gram-negative, microaerophilic, non-fermentative and nitrogen-fixing bacterial genus. *Azospirillum* spp. are Gram-negative, do not form spores, and have a slightly-twisted oblong-rod shape. *Azospirillum* have at least one flagellum and sometimes multiple flagella. The genus has about 20 species, the relationships between all the species have not been resolved in detail, however they most likely constitute a coherent group.

Note 2 to entry: *Azospirillum* bacteria are aerobic non-fermentative chemoorganotrophs, vibroid, they produce several hormones, mainly auxins (not described for all species yet), and most of them are diazotrophic (fix atmospheric nitrogen gas into a more usable form).

[SOURCE: EN 17724:2024, 3.2.2.1 [2]]

[1] An example of such a blend is a product with 2 claimed functions consisting of a non-microbial plant biostimulant and an organic fertilizer composed of 1kg/kg of plant biostimulant from seaweed.

4 Enumeration of *Azospirillum* spp.

4.1 General

This procedure is meant to determine the number of colony-forming units (CFU) of *Azospirillum* spp., per gram or per millilitre. The method, in order to be fast, cheap and repeatable, is based on serial dilutions and plating [3].

4.2 Sample preparation

4.2.1 General

A representative sample of the product to be analyzed 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

Dispense 25 ml of sample (or more for low concentrated products) in 225 ml of sterile phosphate buffer saline (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 [3].

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 phosphate buffer saline (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 [3].

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 saline (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 [3].

4.2.5 Solid water dispersible granules (WDG) formulations

Dispense 25 g (or more for low concentrated products) of sample in 275 ml of sterile phosphate buffer saline (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 another apparatus such as a laboratory paddle blender after having sieved (150 µm sieve corresponding to a 100 mesh sieve) the particles, and resuspend them in the same suspension [3].

4.2.6 Solid pellets, granules, microgranules (slow release) formulations

Dispense 25 g (or more for low concentrated products) of sample in 225 ml of sterile phosphate buffer saline (PBS) maintained at room temperature in a sterile bag and disperse them using a magnetic stirrer for 40 min at half of maximum speed. Then sieve the suspension in a 100 mesh sieve (150 µm sieve corresponding to a 100 mesh sieve) and if material remains in the sieve, repeat the process for a maximum of three times. Pay attention to the volume of buffer used to make the exact final calculation [3].

4.2.7 Solid substrate

Dispense 25 g (or more for low concentrated products) of sample in 225 ml of sterile phosphate buffer saline (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 [3].

4.3 Requirements (apparatus)

— Graduated pipettes (1 ml and 10 ml);

— Dilution bottles or flasks;

— Petri dishes clear, uniform, flat-bottomed;

— Hot air oven - Capable of giving uniform and adequate temperatures, equipped with a thermometer calibrated to read up to (250 ± 1) °C and with vents suitably located to ensure prompt and uniform heating;

— Autoclave/steam sterilizer;

— Incubator;

— Hand tally or mechanical counting device;

— pH meter, having an accuracy of calibration of ±0,1 pH unit at 25 °C.

4.4 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.

For an example of suitable diluents see A.1.

4.5 Preparation of the culture media

The preparation and the composition of N-free semisolid medium (Nfb) is described in Annex A.

The preparation and quality of culture media is a fundamental step 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[3] [5]. Under these conditions, the user/laboratory does not need to run additional testing on such media but shall ensure storage conditions according to the ones recommended by the manufacturers.

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 may be evaluated according to EN ISO 11133:2014[3] [5].

4.6 Preparation of serial dilution for MPN count

Prepare the sample as described in 4.2. Make serial dilutions in order to cover a range including the concentration expected/given by the manufacturer. Inoculate at least 5 dilutions. Five replicates of each dilution shall be carried out, making it a total of at least 25 tubes per sample. Pipette out 1 ml aliquots of the dilutions and deliver them to test tubes containing 5 ml of Nfb media. The operator or laboratory shall check with the applicant (manufacturer, distributor) of the expected concentration of *Azospirillum* spp.

4.7 Incubation of tubes

Label the tubes and incubate at (36 ± 1) °C for 3 days to 4 days in vertical position in a test tubes stand. Do not disturb the medium during the entire period of incubation.

4.8 Counting

Count the tubes which have turned blue and which have developed the typical white sub-surface pellicle.

Count the tubes as positive or negative for the presence or not presence of sub-surface pellicle respectively, and consider the number of tubes for the calculation (4.9).

4.9 Method for estimating MPN count

To calculate the most probable number of organisms in the original sample, select as P_1 the number of positive tubes in the least concentrated dilution in which all tubes are positive, or in which the greatest number of tubes is positive and let P_2 and P_3 represent the numbers of positive tubes in the next two higher dilutions.

Then find the row of numbers in Table B.1 in Annex B in which P_1 and P_2 correspond to the values observed experimentally. Follow that row of numbers across the table to the column headed by the observed value of P.

The figure at the point of intersection is the most probable number of organisms in the quantity of original sample represented in the inoculum added in the second dilution. Multiply this figure by the appropriate dilution factor to obtain the MPN value.

$$Azospirillum\,count\ per\ g\ of\ carrier = \frac{\text{Value from MPN table X Dilution level}}{\text{Dry mass of product}\left(\text{g}\right)}$$

5 Species determination of *Azospirillum* spp. via genetic analysis

5.1 General

If required, this method shall be used for the determination of *Azospirillum* spp. using amplified rDNA restriction analysis (ARDRA) of the 16S rRNA genes.

5.2 Preparation of the sample for the genomic DNA extraction

5.2.1 Isolation and preparation of the microorganism

— From a positive tube, streak a non-selective nutrient agar in order to obtain typical colonies of *Azospirillum* spp.

— Put 1 g of the product in 100 ml of sterile normal saline.

— Keep the suspension in agitation for 5 min under sterile conditions.

— Under sterile conditions, strike a loop of the suspension in a Petri dish prepared with nutrient agar, in order to isolate a few single colonies.

— Incubate the plate for 48 h to 72 h at (28 ± 1) °C.

— Check for a single typical colony (convex, with margins, colour).

— Under sterile conditions, take a loop of the typical isolated colony in a flask with 100 ml of nutrient broth and keep in agitation at 130 rpm at (28 ± 1) °C for 48 h.

5.2.2 Sample concentration

— Take a 0,5 ml aliquot of the broth and put it in 1,0 ml of sterile saline in a 1,5 ml Eppendorf®[2] and centrifuge at 13 000 rpm for 10 min.

[2] Eppendorf® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.