



SLOVENSKI STANDARD
oSIST prEN 17713:2023
01-maj-2023

Rastlinski biostimulanti - Ugotavljanje prisotnosti in števila 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: prEN 17713

<https://standards.iteh.ai/catalog/standards/sist/2849ce45-6dec-481b-b68e-0de283dd50a1/osist-pren-17713-2023>

ICS:

65.080 Gnojila Fertilizers

oSIST prEN 17713:2023 **en,fr,de**

EUROPEAN STANDARD
NORME EUROPÉENNE
EUROPÄISCHE NORM

DRAFT
prEN 17713

April 2023

ICS 65.080

Will supersede CEN/TS 17713:2022

English Version

Plant biostimulants - Determination of *Azospirillum* spp.

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

Pflanzen-Biostimulanzien - Bestimmung von
Azospirillum 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.

This draft European Standard was established by CEN in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

CEN members are the national standards bodies of 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 United Kingdom.

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.

Warning : This document is not a European Standard. It is distributed for review and comments. It is subject to change without notice and shall not be referred to as a European Standard.



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

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

Contents	Page
1 Scope.....	7
2 Normative references.....	7
3 Terms and definitions	7
4 Enumeration of <i>Azospirillum</i> spp.	7
4.1 General.....	7
4.2 Sample preparation	8
4.2.1 General.....	8
4.2.2 Liquid -based water- formulations.....	8
4.2.3 Liquid - oil based (emulsifiable concentrate -EC) formulations	8
4.2.4 Solid - Wettable Powder (WP) formulations.....	8
4.2.5 Solid - Water dispersible granules (WDG) formulations.....	8
4.2.6 Solid - Pellets, granules, microgranuls (slow release) formulations.....	8
4.2.7 Solid - substrate	8
4.3 Requirements (apparatus)	8
4.4 Serial dilution.....	9
4.5 Preparation of the culture media.....	9
4.6 Preparation of serial dilution for MPN count	9
4.7 Incubation of tubes.....	9
4.8 Counting	9
4.9 Method for estimating MPN count	10
5 Species Determination of <i>Azospirillum</i> spp. via genetic analysis.....	10
5.1 General.....	10
5.2 Preparation of the sample for the genomic DNA extraction	10
5.2.1 Isolation and preparation of the microorganism.....	10
5.2.2 Sample concentration.....	10
5.2.3 DNA extraction and storage	11
5.2.4 Partial PCR Amplification of the 16S rRNA Genes	11
Annex A (normative) Formula of the culture media	13
A.1 0,1 M Phosphate Buffer Saline (PBS)	13
A.2 Composition of Nfb semisolid medium	13
A.2.1 Ingredients	13
A.2.2 Method to prepare Trace element solution.....	13
A.2.3 Method to prepare Vitamin solution.....	14
A.2.4 Sterilization and preparation of MPN tubes.....	14
A.3 Nutrient agar	14
A.4 Nutrient broth	14
Annex B (normative) Table of MPN values [9]	15
Annex C (informative) Repeatability and reproductibility data.....	17
C.1 General.....	17
C.2 Microbial analysis-Materials used in the interlaboratory comparison study	17
C.3 Microbial analysis-Interlaboratory comparison results	18
C.4 Genetic analysis-Materials used in the interlaboratory comparison study	19

C.5	Genetic analysis- Interlaboratory comparison results	20
Annex ZA	(informative) Relationship of this European Standard and the essential requirements of Regulation (EU) 2019/1009 making available on the market of EU fertilising products aimed to be covered	21
Bibliography.....		22

iTeh STANDARD PREVIEW
(standards.iteh.ai)

[oSIST prEN 17713:2023](https://standards.iteh.ai/catalog/standards/sist/2849ce45-6dec-481b-b68e-0de283dd50a1/osist-pren-17713-2023)

<https://standards.iteh.ai/catalog/standards/sist/2849ce45-6dec-481b-b68e-0de283dd50a1/osist-pren-17713-2023>

prEN 17713:2023 (E)

European foreword

This document (prEN 17713:2023) 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 17713:2022.

In comparison with the previous edition, the following technical modifications have been made:

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.

iTeh STANDARD PREVIEW
(standards.iteh.ai)

<https://standards.iteh.ai/catalog/standards/sist/2849ce45-6dec-481b-b68e-0de283dd50a1/osist-pren-17713-2023>

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 fertilizing 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". The Working Group 5 "Labelling and denominations", was created to develop a work program as part of this Standardization 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 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 plant, 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 genera *Azospirillum*.

The Table 1 below summarizes many of the agro-ecological principles and the role played by 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. <i>mycorrhize</i>
- symbiotic exchanges i.e. <i>rhizobiaciae/fava</i>
- secretions mimicking plant hormones (i.e. <i>trichoderma</i>)
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.

iTeh STANDARD PREVIEW (standards.iteh.ai)

[oSIST prEN 17713:2023](https://standards.iteh.ai/catalog/standards/sist/2849ce45-6dec-481b-b68e-0de283dd50a1/osist-pren-17713-2023)

<https://standards.iteh.ai/catalog/standards/sist/2849ce45-6dec-481b-b68e-0de283dd50a1/osist-pren-17713-2023>

1 Scope

This document provides the methodology for the enumeration and determination of *Azospirillum* spp. 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.

prEN 17702-1:—¹, *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:

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

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* 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 details, however most likely they constitute a coherent group.

Note 2 to entry: They are aerobic non-fermentative chemoorganotrophs, vibroid, 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: prEN 17724:—¹, 3.2.2.1]

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 [6].

¹ Under preparation

4.2 Sample preparation

4.2.1 General

A representative sample of the product to be analysed as per the requirements of prEN 17702-1:—¹ will be prepared according to following procedure, which takes into consideration the different formulations of Plant 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 of maximum speed [7].

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 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 [7].

4.2.4 Solid - Wettable Powder (WP) formulations

Dispense 25 gr 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 of maximum speed [7].

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 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 [7].

4.2.6 Solid – Pellets, granules, microgranuls (slow release) formulations

Dispense 25 g (or more for low concentrated products) 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 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 [7].

4.2.7 Solid - substrate

Dispense 25 g (or more for low concentrated products) of sample in 225 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 of maximum speed [7].

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 temperature, equipped with a thermometer calibrated to read up to 250°C and with vents suitably located to ensure prompt and uniform heating;
- Autoclave/Steam sterilizer;
- Incubator;
- Hand Tally or Mechanical counting Device;
- pH meter.

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.

The diluent is the PBS (see Annex A, 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 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 programme that ensure the quality of the media they supply according to EN ISO 11133:2014² [6]. Under this conditions, the user/laboratory 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 11133:2014² [6].

4.6 Preparation of serial dilution for MPN count

Prepare the sample as described in 4.3. Make serial dilutions up to 10⁻⁸ dilution. Pipette out 1 ml aliquots of 10⁻⁴ to 10⁻⁸ dilution and deliver it to screw cap tubes or test tubes containing N-free semi solid Nfb media. 5 replicates are necessary.

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 have developed typical white sub-surface pellicle.

Count the tubes as +ve (positive) or -ve (negative) for the presence of sub-surface pellicle and consider for the purpose of calculation.

² As impacted by EN ISO 11133:2014/A1:2018 and EN ISO 11133:2014/A2:2020).