SLOVENSKI STANDARD
SIST-TS CEN/TS 17713:2023

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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: **CEN/TS 17713:2022**

ICS:

65.080 Gnojila Fertilizers

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TECHNICAL SPECIFICATION
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ICS 65.080

English Version

Plant biostimulants - Determination of *Azospirillum* spp.

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

Pflanzen-Biostimulanzien - Bestimmung von *Azospirillum* spp.

This Technical Specification (CEN/TS) was approved by CEN on 3 January 2022 for provisional application.

The period of validity of this CEN/TS is limited initially to three years. After two years the members of CEN will be requested to submit their comments, particularly on the question whether the CEN/TS can be converted into a European Standard.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
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CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

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European foreword

This document (CEN/TS 17713:2022) has been prepared by Technical Committee CEN/TC 455 “Plant biostimulants”, the secretariat of which is held by AFNOR.

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

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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 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 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 biostimulants in agriculture based on live microorganisms belonging to the genera *Azosprillum*.

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 biostimulants

Increase biodiversity
By improving soil microorganism quality/quantity
Reinforce biological regulation and interactions
By reinforcing plant-microorganism interactions
- symbiotic exchanges i.e. *Mycorrhizae*
- symbiotic exchanges i.e. *Rhizobiaceae/Faba*
- 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 *Azospirillum* spp. in plant biostimulant products in accordance to the Regulation of EU fertilising products [1].

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

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: CEN/TS 17724:2021, term 3.2.2.1]

4 Enumeration of *Azospirillum* spp.

4.1 General

The goal of the method is the enumeration of *Azosprillium* spp. (CFU/g) in the given bio-stimulant/formulation [2].

4.2 Sampling

Sampling is not part of the method specified in this document (see the specific European Standard dealing with the product concerned). If there is no specific International or European Standard, it is recommended that the parties concerned come to an agreement on this subject.

It is important that the laboratory receives a sample which is representative and has not been damaged or changed during transport or storage.

4.3 Preparation of the sample

4.3.1 Sample preparation

A representative sample of the product to be analysed (see CEN/TS 17702-1) will be prepared according to following procedure which takes into consideration the different formulations of biostimulant based products:

4.3.2 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 [4].

4.3.3 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 speed [4].

4.3.4 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 speed [4].

4.3.5 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 [4].

4.3.6 Solid – Pellets, granules, microgranules (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 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 [4].

4.3.7 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 speed [4].

4.4 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.

4.5 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 ensures the quality of the media they supply [5]. Under this condition, the user/laboratory need not run additional testing on such media, but shall ensure the storage conditions are in accordance with 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 should be evaluated [5].

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.

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 or –ve for the presence of sub-surface pellicle and consider for the purpose of calculation.

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 +ve, 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.

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