SLOVENSKI STANDARD
SIST-TS CEN/TS 17715:2023

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Rastlinski biostimulanti - Ugotavljanje prisotnosti Shigella spp.

Plant biostimulants - Detection of Shigella spp

Pflanzen-Biostimulanzien - Nachweis von Shigella spp.

Biostimulants des végétaux - Recherche de Shigella spp.

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Plant biostimulants - Detection of *Shigella* spp.

Biostimulants des végétaux - Recherche de *Shigella* spp.

Pflanzen-Biostimulanzien - Nachweis von *Shigella* spp.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
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European foreword

This document (CEN/TS 17715:2022) has been prepared by Technical Committee CEN/TC 455 “Plant Biostimulants”, the secretariat of which is held by AFNOR.

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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 commercialization of the European biostimulant industry.

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 microbial biostimulants in agriculture.

The table 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 [1]

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 a method for verifying that the pathogen *Shigella* spp. is not present in microbial plant biostimulants in a concentration that exceeds the respective limits outlined in the EU Regulation on Fertilising Products.

The detection method for *Shigella* pathogens is not sensitive and quantification is rarely performed. Detection is usually performed using an enrichment medium followed by subculturing onto a variety of selective media.

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.

CEN/TS 17708, *Plant biostimulants — Preparation of sample for microbial analysis*

CEN/TS 17724, *Plant Biostimulants — Terminology*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in CEN/TS 17724 and the following apply.

3.1
***Shigella* spp.**
microorganisms which form typical colonies on solid selective media described and which display the morphological, physiological and biochemical characteristics described when the analysis is carried out in accordance with this document

3.2
detection of *Shigella* spp.
determination of the presence or absence of these microorganism in a particular mass of product, when tests are carried out in accordance with this document

4 Principle

4.1 General

Detection of *Shigella* spp. will be conducted according to the sections specified in EN ISO 21567:2004 and with the following four successive stages (see Annex A, Figure A.1).

4.2 Enrichment in selective liquid medium

A test portion is inoculated into Shigella broth containing 0,5 µg/ml of novobiocin, then incubated anaerobically at (41,5 ± 1) °C for 16 h to 20 h.

4.3 Plating out and identification of colonies

From the enrichment culture obtained, three selective differential media are inoculated: MacConkey agar with low selectivity; XLD agar with moderate selectivity; Hektoen enteric agar with the greatest selectivity. All are incubated at 37 °C for 20 h to 24 h.

4.4 Biochemical and serological confirmation

Typical and suspect colonies are selected from each of the three selective agars. The colonies are purified on nutrient agar, then biochemical and serological characterizations are carried out using the tests described.

5 Culture media, reagents and antisera

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

See Annex B for descriptions of all media, reagents and antisera.

Commercially available dehydrated media should give more consistent results than media prepared from their component parts in the laboratory. Follow the manufacturer's instructions exactly, as small changes in preparation can significantly change the performance of selective media. Excessive heating of the selective agars used in this document by autoclaving, storage and then re-heating for use may result in loss of selectivity.

6 Apparatus and glassware

Disposable equipment is an acceptable alternative to re-usable glassware if it has suitable specifications.

Usual microbiological laboratory equipment according to CEN/TS 17708 shall be used.

7 Sampling

Sampling is not part of the method specified in this document (see the specific European Standard dealing with the product concerned).

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

8 Procedure

8.1 General

The presence or absence of *Shigella* pathogens in at least 25 g or 25 ml of the product under test will be evaluated.

8.2 Test portion

See the appropriate part of CEN/TS 17708.

8.3 Enrichment

8.3.1 General

A representative sample of the product to be will be prepared according to following procedure, which takes into consideration the different formulations of biostimulant based products.

8.3.2 Liquid formulations

Dispense 25 g of sample in 225 ml of sterile *Shigella* broth containing 0,5 µg/ml of novobiocin (B.2.2). Soon after take the entire suspension and proceed then with the incubation under anaerobic conditions at (41,5 ± 1) °C for 16 h to 20 h.

8.3.3 Solid formulations

Dispense 25 g of sample in 225 ml of sterile *Shigella* broth containing 0,5 µg/ml of novobiocin (B.2.2). Soon after, take the entire suspension and process them in a stomacher for 2 min at highest speed.

Incubate the *Shigella broth* under anaerobic conditions at (41,5 ± 1) °C for 16 h to 20 h.

8.4 Plating out and colony selection

8.4.1 Using the culture obtained in 8.3, gently mix the contents by hand and allow the larger particles to settle.

Inoculate, by means of a loop, the surface of the following selective agars to obtain well-isolated colonies: MacConkey agar (B.3.1) with low selectivity; XLD agar (B.3.2) with moderate selectivity; and Hektoen enteric agar (B.3.3) with a greater selectivity.

8.4.2 Incubate the plates at (37 ± 1) °C for between 20 h and 24 h.

The appearance of different *Shigella* species can vary on the same selective agar. See Annex C for a description of *Shigella* colonies on the different selective agar used.

Mark any typical or suspect colonies found on each plate.

If no typical colonies are seen and the growth of the other microorganisms is weak, re-incubate the plates for a further 24 h. Examine them again for the typical *Shigella* colonies.

Carry out the confirmation procedure described in 8.5.

8.5 Confirmation of colonies

8.5.1 General

Identification kits (currently commercially available) that have been proven by the user to be reliable for the identification of the different species of *Shigella* may be used. Follow the manufacturer's instruction precisely.

For confirmation, sub-culture from each dish of each selective medium (see 8.4) five marked typical or suspect colonies.

If on one dish there are fewer than five typical or suspect colonies, take all the marked colonies for confirmation.

Use pure cultures for biochemical and serological confirmation.

8.5.2 Purification of colonies

Streak the selected colonies onto the surface of nutrient agar plates (B.4) so as to gain well-isolated colonies. Incubate the plates at (37 ± 1) °C for 18 h to 24 h.

If the cultures on nutrient agar are mixed, sub-culture the suspect colony onto a further plate of nutrient agar and incubate at (37 ± 1) °C for 18 h to 24 h to obtain the pure culture.

Shigella sonnei can give two colony types on the same agar plate: a smooth round domed colony (phase 1), and a flat irregular colony with a mat surface (phase 2).

NOTE It is possible to first test the most characteristic colony from each selective agar plate. If positive, it is not necessary to test other colonies. If negative, progress through the other selected colonies until either all are negative or a positive is found.

8.5.3 Biochemical confirmation

8.5.3.1 General

By means of an inoculation needle, inoculate the media specified in 8.5.3.2 to 8.5.3.9 respectively with each of the cultures selected in 8.5.2 and record all the results.

8.5.3.2 Triple sugar iron agar (TSI slopes) (B.5)

Stab the butt and streak the agar slope.

Incubate at (37 ± 1) °C for (24 ± 3) h.

Interpret the changes in the medium as shown in Table 2.

Table 2 — Interpretation of triple sugar iron agar test

Area of slope	Appearance	Indication
Butt	Yellow Red or unchanged Black Bubbles or cracks	Glucose fermented: positive Glucose not fermented: negative Formation of hydrogen sulfide: positive Gas formation
Slant surface	Yellow Red or unchanged	Lactose and/or sucrose utilized: positive Lactose and sucrose not utilized: negative

Typical *Shigella* cultures show a yellow butt (acid formation) and no gas bubbles, there is no change in the colour of the slant (no utilization of lactose or sucrose) and no hydrogen sulfide production (see Table 3).

8.5.3.3 Semi-solid nutrient agar for motility tests (B.6)

Stab the semi-solid nutrient agar with a colony using an inoculation needle.

Incubate tubes at (37 ± 1) °C for 18 h to 24 h.

Examine the line of inoculation for spreading growth. Non-motile microorganisms will give a discrete line; motile strains will give diffuse growth away from the inoculum line.

All *Shigella* species are non-motile.

8.5.3.4 Urea agar (B.7)

Streak the agar surface.

Incubate at (37 ± 1) °C for (24 ± 3) h and examine at intervals.

If urea is hydrolysed, a rose-pink to deep cerise colour develops from the release of ammonia by the decomposition of the urea with a change in the colour of the pH indicator. There is no change in colour of the agar with a negative reaction.

Shigella species do not hydrolyse urea.

8.5.3.5 L-Lysine decarboxylase medium (B.8)

Inoculate below the surface of the liquid broth. Incubate at (37 ± 1) °C for (24 ± 3) h.

Turbidity and a purple colour after incubation indicate a positive reaction; yellow indicates a negative result.

Shigella species do not decarboxylate lysine.

NOTE The use of a paraffin overlay in the tubes can help to ensure anaerobic conditions.

8.5.3.6 L-Ornithine decarboxylase medium (B.9)

Inoculate below the surface of the liquid broth. Incubate at (37 ± 1) °C for (24 ± 3) h.

If a purple colour develops, the test is positive; a yellow colour means a negative result.

Shigella sonnei decarboxylates ornithine, but other *Shigella* species do not (see Table 3).

8.5.3.7 Detection of indole formation (B.10)

Inoculate a tube containing 5 ml of tryptone/tryptophan medium (B.10.1) with the pure culture.

Incubate at (37 ± 1) °C for (24 ± 3) h.

After incubation, add 1 ml of Kovac's reagent (B.10.2).

The formation of a red ring within 10 min indicates indole formation, and a yellow/brown colour indicates a negative reaction.

Shigella sonnei is negative whilst other strains give variable reactions (see Table 1).

8.5.3.8 Detection of β-galactosidase (B.11)

Suspend a loopful of the purified culture from the nutrient agar into 0,25 ml of saline solution (B.13) in a screw cap bottle or test tube.

Add one drop of toluene and shake to mix well.

Put the tube in an incubator set at 37 °C and leave for several minutes. Add 0,25 ml of the complete reagent and mix.

Replace in the incubator set at 37 °C and leave for (24 ± 3) h, examining at intervals.

A yellow colour indicates the formation of β-galactosidase, which can occur in as little as 20 min.

Sigella sonnei is positive. *S. dysenteriae* and *S. boydii* give variable reactions and *S. flexneri* is negative. (see Table 1).

8.5.3.9 Utilization of carbohydrates (B.12)

Inoculate each of the prepared carbohydrate broths with a small inoculum.

Incubate at (37 ± 1) °C for (24 ± 3) h.

A positive reaction when carbohydrate is utilized gives a change in the pH indicator from purple to yellow.

See Table 3 for the reactions of different *Shigella* species.

8.5.3.10 Interpretation of biochemical results

Strains within some *Shigella* species vary in their biochemical reactions (see Table 3), therefore interpretation based only on biochemical results is difficult and serotyping is essential to establish identity.

Shigella are Gram-negative bacilli, 2 µm to 4 µm by 0,5 µm in size, but often show a tendency to shorter cocco-bacillary forms and typically do not produce gas from glucose. They are non-motile, do not produce hydrogen sulfide or decarboxylate lysine, and are lactose negative at 24 h. The other tests described above give variable reactions or differing reactions according to the species.

Within the genus *Shigella*, mannitol discriminates *Shigella* dysenteriae (negative) from other species and L-ornithine decarboxylase differentiates *Shigella sonnei* (positive) from other species.