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Microbial reference materials in seed health test standardization

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Summary

Harmonization and standardization of seed health testing require tools, such as reference materials, to determine the value and to verify the performance of the test methods. Reference materials (RMs) for bacteria testing are defined as encapsulated, preserved bacterial preparations containing a specified quantity of viable bacterial cells of a well described pathogenic strain. In a project financially supported by the European Union, protocols to obtain stable and homogeneous RMs have been developed for four priority seed-transmitted bacterial diseases. An interlaboratory study with 13 seed testing laboratories participating showed the usefulness of the prepared RMs for quality control in seed health testing, e.g. as a check on the quality of commonly applied semi-selective media. In addition, the results indicated that RMs may contribute to determining the accuracy of quantitative seed health testing. Ongoing research aims at further improvement of the quality of the RMs. The value of the RMs for use as standards in actual seed test protocols will be determined in a second interlaboratory test, which will involve testing the performance of RMs in seed extracts with normal microflora background. Besides supporting the establishment of quality assurance systems and the standardization of test methods, RMs will be useful tools for laboratory accreditation bodies.

Introduction

Disease-free planting material is essential for an economically profitable and environmentally safe crop production. Production and selection of healthy seed can be ensured by testing seed for the presence of unwanted plant pathogens. In order to harmonize seed health testing all over the world, standardization and validation of test methods is needed. To achieve standardized and validated seed health test methods, tools to determine the value and to verify the performance of the test methods are required. Reference materials (RMs), defined as encapsulated, preserved bacterial preparations containing a specified quantity of viable bacterial cells of a well described pathogenic strain, are an example of such useful tools (Van den Bulk and Taylor, 1997). The use of RMs of known contamination level is recommended in the analytical quality control of microbial analyses as well (Lightfoot and Maier, 1998).

In 1996, a project under the FAIR Programme of the European Union was started with as main objective to help provide standards for seed health testing in the EU by: 1) developing candidate reference materials, and 2) determining the value of the reference materials developed in intercomparative studies with 12 European laboratories. In the project, candidate reference materials are developed for four seedborne bacteria, i.e. *Clavibacter michiganensis* subsp. *michiganensis* (causing bacterial canker of tomato), *Erwinia (Pantoea) stewartii* (causing Stewart's bacterial wilt of maize), *Pseudomonas syringae* pv. *phaseolicola* (causing halo blight of beans) and *Xanthomonas campestris* pv. *campestris* (causing black rot of cabbage). The chosen bacteria are all agriculturally important organisms for questions about seed legislation and are either quarantine organisms or fall under the general phytosanitary regulations.

Preservation method

In the first two years of the project, work focussed on testing of preservation techniques, especially with regard to the stability of the reference material. Stability is an important

prerequisite for a reference material, in order to recover in time the same number of viable cells of the target bacterium as specified by the producer of the reference material. The preservation techniques tested included freeze-drying, freeze-drying on paper disks, spray-drying and storage on silicagel. Particularly for the spray-drying and the silicagel method, both involving raised temperatures during the drying process, all bacterial strains tested showed a sharp decrease in recoverable cell numbers immediately after the preservation procedure. Strain effects were noted for both preservation techniques, emphasising the importance of testing various strains before selecting those from which to prepare the reference materials. In general, recovery from spray dried and silicagel dried preparations was most stable at low storage temperatures, e.g. -20 °C. Preservation by freeze drying, with or without paper disks, was the most stable over a range of storage temperatures . Also, the number of viable cells was higher with both freeze drying methods when compared to the spray-drying and silicagel methods. Overall it was concluded that the freeze dried paper disk system was the best method from the point of view of stability and practicality.

Optimising the production method

For each target organism, strains were selected on the basis of stability of recovery over time. In this respect the spray drying and silicagel methods showed greater differentiation between strains. Selected strains were also tested for pathogenicity. The initially selected *E. stewartii* strain was replaced by another when it failed to show the expected level of pathogenicity. Comparison of homogeneity of reference materials from the same production batch showed variation in recovery between preparations to be dependent on organism, strain and preservation method used. Improving homogeneity of the reference materials needs further attention, since homogeneity is the second essential requirement for a reference material.

The freeze dried paper disk procedure was optimised by adding a pre-drying step, omitting the pre-freezing step, and improving the composition of the preservation medium. Because the recovery of viable cells is a known critical step in the procedure, the best resuscitation fluid (0.1% peptone broth) and most optimal disk soaking time (2 hours) were established. Recovery experiments on semi-selective media confirmed that on some media suppression of growth occurs. The results suggested that isolate effects might occur as well. However, since the recovery on semi-selective media was reproducible, a slight inhibition of growth is acceptable. In practice, a known reduction in recovery may be useful to verify the correct composition of the media.

Physical representation

The physical representation of the developed reference materials is a 2-ml vial, 4 cm high and 14 mm in diameter, containing a 6-mm diameter paper disk inoculated with 20 µl of bacterial suspension. After a pre-drying step and over-night freeze drying, the vials are stoppered under vacuum with a rubber stopper, capped with an aluminium seal and stored at 4 °C. An alternative storage condition for freeze dried disks, i.e. under nitrogen instead of in a vacuum, is currently being tested.

Test batches

In the third project year, test batches of reference materials were shown to be rather stable in recovery up to 12 months of storage at 4 °C (Figure 1).

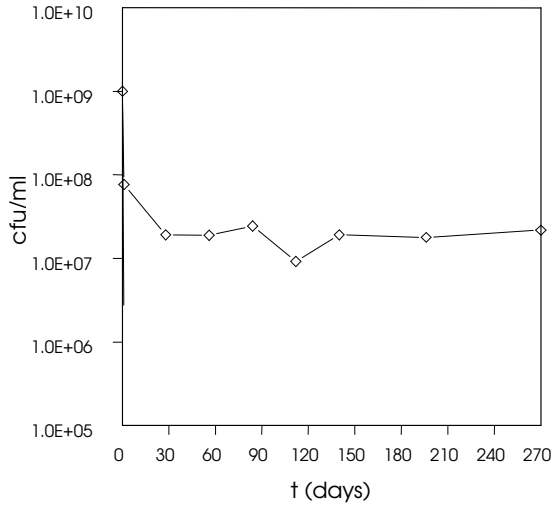

Figure 1. Stability of recovery in time for a test batch of *Clavibacter michiganensis* subsp. *michiganensis* reference materials.

Homogeneity tests of *X. campestris* and *C. michiganensis* test batches showed that the variance between reference materials was larger than the variance between the replicates in the plating procedure. Both the heterogeneity of the reference materials as well as the method used to recover them contributed to the variance. Exposure of reference materials to short-term elevated temperatures, up to 50 °C, showed a slight decrease of recovery for *C. michiganensis*. Based on temperatures recorded during shipment of reference materials through the postal system and temperatures anticipated to occur, it seems therefore reasonable to send reference materials in cooled packages.

Interlaboratory test

An interlaboratory test was organised to determine whether the materials developed provided effective reference materials. Reference materials of each of the four pathogens were despatched and tested in 13 laboratories, with each laboratory following a precisely defined protocol for recovery, diluting, plating and recording. Statistical analysis of the results obtained for each pathogen/isolation medium combination indicated that the overall reproducibility, including both within and between laboratory variance, was at an acceptable level. In general, within laboratory variance and between-laboratory variance contributed to the same extent to the reproducibility variance. Results for *X. campestris* and *P. syringae* were particularly good, with similar levels of reproducibility for both selective and non-selective media. Results for *E. stewartii* and *C. michiganensis* were not as good, due mainly to poorer reproducibility on the selective media. However, this is probably more an indication that the selective media are not adequate rather than an indication that the reference materials *per se* were less satisfactory. The variability for *X. campestris* and *P. syringae* reference materials was much lower than for *E. stewartii* and *C. michiganensis* reference materials. A major question which remains, however, is what constitutes an acceptable level of variability. Overall, this interlaboratory test showed that reference materials were valuable tools for quality control of media used in seed health testing.

Future actions

Research to monitor the long term stability of reference materials is ongoing. Future work will especially focus on the testing of candidate reference materials in an second inter-laboratory study, to determine the value of the reference materials for use as standards in actual seed

test protocols. This involves testing the performance of the reference materials in an environment with a 'live background' (seeds with their normal microflora).

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