SELECTION OF SCREENING TESTS FOR DETECTION OF QUARANTINE BACTERIA OF THE GENUS *Xanthomonas* IN *Citrus* spp.

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ABSTRACT

Early diagnosis is key to reducing the risk of introduction and spread of plant pests and diseases. Through internal validations and interlaboratory comparisons, we obtain data on the performance of tests. As part of the VALITEST research project (Horizon 2020 GA 773139), the National Institute of Biology participated in an interlaboratory comparison study of laboratory tests for the detection of bacteria of genus *Xanthomonas* causing important diseases in citrus production. In a series of blank samples prepared by the study organizer, ANSES Plant Health Laboratory, Unit for Tropical Pests and Diseases, we tested various tests based on LAMP, PCR and real-time PCR methods. Based on the TPS and internal results, we chose an optimal combination of tests to determine bacteria of the genus *Xanthomonas* on citrus fruits that provide fit-for-purpose sensitivity and specificity of screening testing.

Key words: laboratory testing, diagnostics sensitivity, diagnostics specificity, interlaboratory comparisons of tests, *Xanthomonas citri* pv. *citri*, *Xanthomonas citri* pv. *aurantifolii*

IZVLEČEK

IZBOR PRESEJALNIH TESTOV ZA DOLOČANJE KARANTENSKIH BAKTERIJ IZ RODU *Xanthomonas* NA AGRUMIH

Zgodnja diagnostika je ključna za zmanjšanje tveganja vnosa in širjenja rastlinskih škodljivih organizmov. Z validacijami in medlaboratorijskimi primerjavami pridobivamo podatke o zanesljivosti testov, ki se uporabljajo. Nacionalni inštitut za biologijo je v okviru raziskovalnega projekta VALITEST (Horizon 2020 GA 773139) sodeloval v medlaboratorijski primerjalni študiji laboratorijskih testov za določanje karantenskih bakterij *Xanthomonas*, ki povzročajo pomembne bolezni agrumov. V seriji slepih vzorcev, ki jih je pripravil organizator študije, Laboratorij za zdravje rastlin ANSES, Enota za tropske škodljivce in bolezni, smo testirali različne teste na podlagi metod LAMP, PCR in PCR v realnem času. Na podlagi rezultatov študije in rezultatov laboratorija smo

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izbrali optimalno kombinacijo testov za določanje bakterij iz rodu *Xanthomonas* na agrumih, ki zagotavljajo ustrezno občutljivost in specifičnost presejalnega testiranja.

Ključne besede: laboratorijsko testiranje, diagnostična občutljivost, diagnostična specifičnost medlaboratorijska primerjava testov, *Xanthomonas citri* pv. c*itri, Xanthomonas citri* pv. *aurantifolii*

1 INTRODUCTION

Quarantine bacteria of the genus *Xanthomonas* (*Xanthomonas citri* pv. *citri* (Xcc) and *Xanthomonas citri* pv. *aurantifolii* (Xca)) are the causal agents of citrus bacterial canker (CBC) that presents a major risk for the citrus industry in the EU. The Xcc pathogen causes necrotic lesions on leaves, stems and fruits in plants of the family Rutaceae, with *Citrus spp*. the hosts of major economic importance. Severe infections can cause defoliation, badly blemished fruits, premature fruit drop, twig dieback and general tree decline. According to disease severity and host range of bacteria *Xanthomonas citri* pv. *citri*, we distinguish pathotypes of which pathotype A have the greatest economic impact on citrus industry. These strains are distributed worldwide and induce canker symptoms on a broad range of citrus hosts. Strain A* and Aw have restricted host range. *Xanthomonas citri* pv. *aurantifolii* (strains *B* and *C*) causes a minor canker of diminishing importance on a narrow host range.

Citrus bacterial cankers do not occur in Europe and it is important to prevent the introduction of the causative agents. EPPO standards describe some of the tests which can be used for the detection and identification of *Xanthomonas* in *Citrus* (EPPO 7/44). Since its last revision several novel tests were designed to address known issues of the existing tests.

The *Xanthomonas* pathogens of Citrus are introduced to new areas through the movement of infected citrus fruits and seedlings. Upon accidental entry, the success of eradication would depend upon the early detection of a newly established population. While citrus are not of economic importance in Slovenia and are only occasionally grown in limited quantities mainly as ornamentals, Port Koper is an entry point of relevance for citrus fruits. Visual inspections are performed to detect and exclude citrus fruits with lesions with the aim to prevent entry of the disease. Laboratory tests can be used to support visual inspections through detecting bacteria in symptomatic plant material or identification of bacteria in pure cultures. Laboratory tests are also necessary for detecting bacteria in latent infections. The aim of the use of laboratory tests is to provide unequivocal result corresponding to the presence or absence of the pathogens of interest.

2 MATERIALS AND METHODS

The test performance study (TPS) was organized by Anses in 2020/2021. Two sets of 24 blind samples and 2 controls were distributed to each participant for analysis with the provided protocols for real-time PCR, PCR and LAMP. The organizer ensured that the samples (already extracted DNA) used for evaluation of the methods and tests were

sufficiently homogeneous and stable. In addition to the strains *A, A** and *Aw* of *X. c.* pv. *citri* (Xcc), some samples contained strains *C* and *B of X. c.* pv. *aurantifolii* (Xca) and a non-target strain of *X. c.* pv. *bilvae* (Xcb). Samples were prepared in such a way, that they covered a broad concentration range of the target bacteria Xcc (from 2.3 c/mL to 6.3 c/mL of plant extracts) in a background of orange (*Citrus x sinensis*) and lime (*Citrus x aurantifolia*) (Test performance study on *Xanthomonas* on *Citrus*, Final report, Valitest (Horizon 2020 GA 773139)).
The overall diagnostic parameters determined in the TPS were compared to the results obtained by NIB using (where relevant) its own kits, reagents, consumables and instruments. Diagnostic sensitivity (DSE) and diagnostic specificity (DSP) were determined for each test by comparing the obtained results with the true status of the blind samples as reported by the organizer. DSP, a parameter describing the ability of the method not to detect the non-targets was calculated using the following formula: 100 x true negative / (false positive + true negative). Similarly, diagnostic sensitivity was calculated, a parameter describing the proportion of contaminated samples detected with a test. The advantages and disadvantages of tests were taken into account in selection of tests most suitable for screening. Results of the use of tests in a proficiency test are also summarized.

3 RESULTS AND DISCUSSION

The test performance study (TPS) focused on molecular tests including PCR, real-time PCR (qPCR) and LAMP. At NIB, we performed six different conventional PCRs (Hartung *et al.*, 1993; Miyoshi *et al.*, 1998; Cubero & Graham., 2002; Mavrodieva *et al.*, 2004; Park *et al.*, 2006; Robène *et al.*, 2020), three different real-time PCRs (Mavrodieva *et al.*, 2004; Cubero & Graham, 2005; Robène *et al.*, 2020), and one LAMP (Rigano *et al.*, 2010). Each test was applied to a set of blind samples provided by the TPS organizer. The samples included in the TPS contained plant material spiked with target bacteria or non-target bacteria. For each sample, the results of tests were reported as positive or negative. Finally, the results for tests and blind samples were compared to the expected results and the overall results of the TPS.
Of the tests performed three conventional PCR tests (Hartung *et al.*, 1993; Miyoshi *et al.*, 1998; Park *et al.*, 2006) showed subpar performance with regards to their diagnostic sensitivity (data not shown) and are excluded from further analysis here.
The variability in specificity of the available tests is known and has been confirmed in the TPS (Fig. 1). All the tests with satisfactory diagnostic sensitivity (conventional PCRs (Cubero & Graham, 2002; Mavrodieva *et al.*, 2004), real-time PCRs (Mavrodieva *et al.*, 2004; Cubero & Graham, 2005; Robène *et al.*, 2020), and LAMP (Rigano *et al.*, 2010)) were able to detect different pathotypes of *Xanthomonas campestris* pv. *citri* (Xcc) when present in high enough concentrations (A, A* and Aw; Fig. 1). The pathotypes are described and are associated with different geographic distribution and host range. Of the pathotypes the pathotype A is of major importance. It can infect many different rutaceaous plants and is present in Asia, Africa, South America and Oceania. Two groups of Xcc strains related to the pathotype A are described but have more restricted host range with pathotype A* mainly infecting

Mexican lime in Asia and Africa, and pathotype Aw infecting Mexican lime and Alemow, present in Florida.

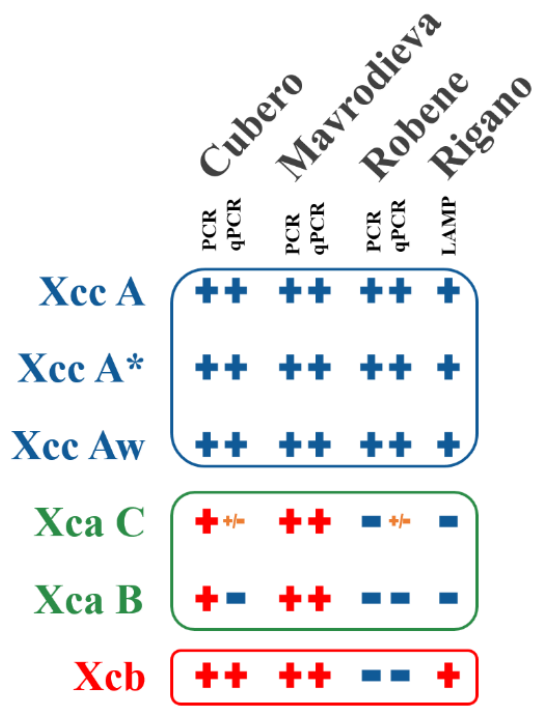

Figure 1: Tests have different specificity with respect to strains of *Xanthomonas* on citrus (target strains A, A* and Aw of X. c. pv. citri (Xcc), non-target strains C and B of *X. c.* pv. *aurantifolii* (Xca), non-target strain *X. c.* pv. *bilvae* (Xcb)). Results considered true (true positive or true negative) in this analysis are shown in blue.

Of the tests with the initial satisfactory performance PCR and real-time PCR (Robène *et al.*, 2020) and LAMP (Rigano *et al.*, 2010) showed higher specificity for the main quarantine pathogen Xcc and exclusivity with regards to *X. citri* subsp. *aurantifolii* (Xca) and *X. c.* pv. *bilvae*. (Xcb) (Fig. 1). Although Xca can cause citrus bacterial canker (CBC) and is regulated within the EU (Annex II part A of the Commission Implementing Regulation (EU) 2019/2072), the latter has not been observed for decades in citrus production or at EU entry points.

As with other diseases, exclusion of false positive results from the diagnosis is crucial to avoid unnecessary economic losses. In the TPS, this was tested by analyzing non-target isolate of *X. c.* pv. *bilvae*. While *bilvae* is also pathogenic to rutaceous species but has a distinct symptomatology (is not known to cause cankers) and does not have a quarantine status (Bui Thi Ngoc *et al.*, 2010). In general, if the tests were able to detect

both quarantine species of *Xanthomonas*, they were also prone to false positive results with the non-target bacteria and/or sometimes challenging interpretation with negative samples containing plant material only.
The differences in analytical specificity of tests lead to higher variability of diagnostic specificity in the overall results of TPS, ranging from approximately 25 to 100 % (Fig. 2). PCR and real-time PCR based on Robène *et al.*, 2020, showed the highest DSE and DSP (88 % and 100 % for PCR and 100 % and 75 % for real-time PCR, respectively; Fig. 2).

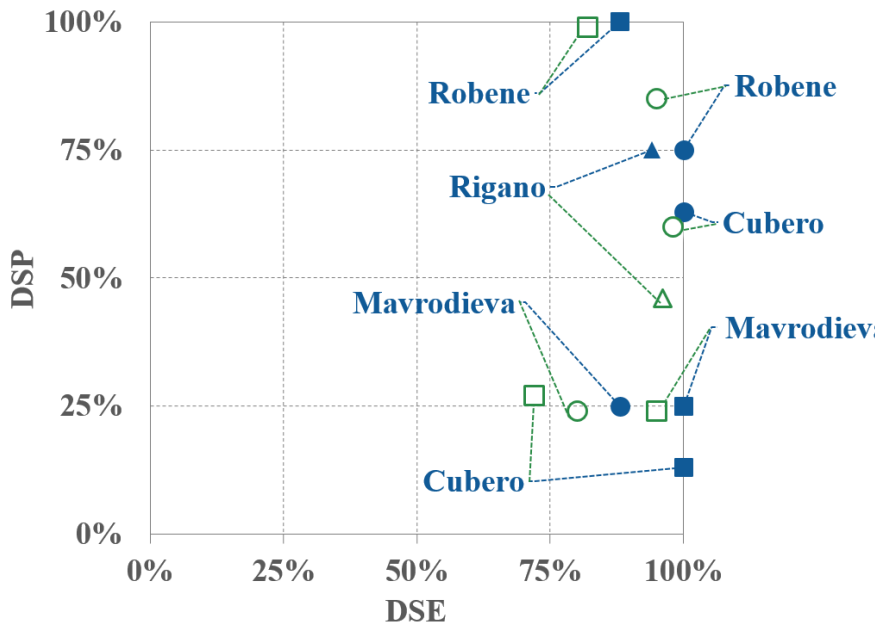

Figure 2: Diagnostic sensitivity (DSE) and diagnostic specificity (DSP) as obtained by NIB (full blue markers) in comparison with the overall DSE and DSP determined in the Valitest TPS (empty green markers); squares = PCR, circles = real-time PCR, triangles = LAMP.

In general, NIB obtained better results in the TPS than those averaged across participating laboratories (Fig. 2). The exception was the diagnostic specificity in Cubero PCR and Robene real-time PCR; these tests could benefit from further optimization adapted to specific laboratory conditions which would prevent false positive signals with plant material and other bacteria, respectively.
Based on the results none of the methods and/or tests used has optimal performance on its own. While tests based on Mavrodieva are promising for both detecting and differentiating target and non-target *Xanthomonas* spp. in Citrus, further optimization is needed to allow for unequivocal interpretation of results obtained from negative plant

material and low concentrations of the target in plant background. With respect to specific and sensitive detection of Xcc only, a combination of real-time PCR (Robène *et al.*, 2020) confirmed with PCR (Robène *et al.*, 2020) provide the most accurate approach. Because of its higher sensitivity real-time PCR may be more suitable for detection of the target bacteria in plant material whereas both PCR and real-time PCR are suitable for identification of bacteria in pure culture.
When the aim is to also detect Xca, PCR (Mavrodieva *et al.*,2004) could be used, taking into account that its positive results need to be differentiated from the potential false positive reactions with non-target bacteria.
The tests described here were further used in the proficiency test organized by CREA, within the activity of the European Union Reference Laboratory for pests of plants on bacteria. The results confirmed that further optimization of tests were needed for 100 % proficiency and that the challenge is mainly differentiation between true positive results at low concentration from the spurious amplification originating from plant material and/or its microflora.

4 CONCLUSIONS

Compared to previously described tests the novel tests PCR Robene and real-time PCR Robene showed improved diagnostic specificity and diagnostic sensitivity for detecting the main quarantine pathogen of Citrus, *X. c.* pv. *citri* (Xcc). The combination of the two tests is thus most suitable for testing a range of samples namely asymptomatic samples, fruit cankers and/or bacterial isolates from fruit cankers. Nevertheless, interpretation of the combined results of the two tests may be challenging for lower concentrations of the target bacteria in plant material as expected e.g. in ornamental Citrus spp. For fruit cankers in which the expected concentration of the pathogen is relatively high also LAMP (Rigano *et al.*, 2010) as an on-site amenable test seems promising.
There is no single test allowing for reliable detection of both quarantine pathogens, Xcc and Xca. Such analysis needs to be combined with further tests to exclude false positive results with non-target bacteria.
Overall, further work may be needed to ensure high diagnostic specificity of the tests on a wider range of naturally occuring isolates of Xcc. However, the benefits of test performace studies to gain experience with testing for pathogens which may not be the main focus of a laboratory can not be overstated.

5 ACKNOWLEDGEMENTS

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6 REFERENCES

Bui Thi Ngoc, L., Vernière, C., Jouen, E., Ah-You, N., Lefeuvre, P., Chiroleu, F., Gagnevin, L., Pruvost, O., 2010. Amplified fragment length polymorphism and multilocus sequence analysis-

based genotypic relatedness among pathogenic variants of *Xanthomonas citri* pv. *citri* and *Xanthomonas campestris* pv. *bilvae*. International Journal of Systematic and Evolutionary Microbiology 60, 515–525.

Cubero, J., Graham, J.H., 2002. Genetic Relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. Appl Environ Microbiol 68, 1257–1264.

Cubero, J., Graham, J.H., 2005. Quantitative real-time polymerase chain reaction for bacterial enumeration and allelic discrimination to differentiate *Xanthomonas* strains on citrus. Phytopathology 95, 1333–1340.

European and Mediterranean Plant Protection Organization, EPPO Standards, Diagnostic PM 7/44.

Mavrodieva, V., Levy, L., Gabriel, D.W., 2004. Improved sampling methods for real-time polymerase chain reaction diagnosis of citrus canker from field samples. Phytopathology 94, 61–68.

Rigano, L.A., Marano, M.R., Castagnaro, A.P., Do Amaral, A.M., Vojnov, A.A., 2010. Rapid and sensitive detection of citrus bacterial canker by loop-mediated isothermal amplification combined with simple visual evaluation methods. BMC Microbiol 10, 176.

Robène, I., Maillot-Lebon, V., Chabirand, A., Moreau, A., Becker, N., Moumène, A., Rieux, A., Campos, P., Gagnevin, L., Gaudeul, M., Baider, C., Chiroleu, F., Pruvost, O., 2020. Development and comparative validation of genomic-driven PCR-based assays to detect *Xanthomonas citri* pv. *citri* in citrus plants. BMC Microbiol 20, 296.

Test performance study on *Xanthomonas* on *Citrus*, Final report, Valitest (Horizon 2020 GA 773139).