LABORATORY-SCALE EVALUATION OF COMPOSITE SPIKED SAMPLES FOR ERWINIA AMYLOVORA DETECTION IN ASYMPTOMATIC PLANTS

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SUMMARY

Erwinia amylovora, the fire blight pathogen, can survive in plants and produce latent infections that can lead to false negative results in routine diagnostic procedures, since the pathogen can be present at very low numbers under the detection limit of available techniques. A protocol combining different techniques included in the EPPO diagnostic standard for E. amylovora (2013) was designed in order to determine the minimum amount of plants necessary for detecting the pathogen in asymptomatic plant material. To this aim, single samples of flowers and shoots from pear, apple, and loquat were spiked with an E. amylovora strain at different concentrations, and then analyzed by isolation, real-time PCR and ELISA-DASI. Thereafter, using the single spiked samples that were positive for any of the techniques used, composite spiked samples were prepared by adding to single spiked samples increasing amounts of plant material. Interestingly, whole results show that the most robust technique, less affected by the amount of plant material, was the real-time PCR after enrichment, since 10^1-10^2 cfu ml^-1 of E. amylovora could be detected in most cases, both in flowers and in shoots, regardless of the amount of simulated plants analyzed, 3, 5, 10 or 20. The enrichment was confirmed as a necessary step in the analysis of bulked samples to achieve a greater sensitivity. This study is a relevant contribution for programs of extensive screening surveys for prevention of fire blight disease, since it shows that samples can be processed as a pool, improving the detection of E. amylovora in asymptomatic samples.

Keywords: fire blight, latent infection, survey, EPPO, real-time PCR, ELISA-DASI, enrichment.

INTRODUCTION

E. amylovora causes fire blight, the most devastating bacterial disease of rosaceous plants. The disease has great economic importance, especially in apple (Malus domestica) and pear (P. communis) (van der Zwet et al., 2012), but also in other fruit trees as loquat (Eriobotrya japonica) or quince (Cydonia oblonga) with relevant production in the Mediterranean basin. In total, around 200 plant species are considered hosts for E. amylovora, also including ornamentals such as cotoneaster (Cotoneaster sp.), hawthorn (Crataegus sp.), firethorn (Pyracantha sp.) or rowan tree (Sorbus sp.) (Momol and Aldwinckle, 2000).

The fire blight pathogen can survive in plants in an epiphytic or endophytic phase, and the colonization, even with high cell numbers, is possible without subsequent symptom development (Crepel et al., 1996; Ge and van der Zwet, 1996; Momol et al., 1998; López et al., 1999; Crepel and Maes, 2000; Hinze et al., 2015). In fact, the occurrence of asymptomatic fire blight infection has been observed in nature and reported in several studies (McManus and Jones, 1994; van der Zwet, 1996; Smith, 2002; Billing, 2011; Tancos et al., 2016). The ability of E. amylovora to survive in plants and produce latent infections can lead to false negative results in routine diagnostic protocols, especially because the asymptomatic samples can contain a low number of bacterial cells. The European Plant Protection Organization (EPPO) diagnostic protocol for E. amylovora (EPPO, 2013) includes a section for detection in asymptomatic samples, warning that this task has been shown to be difficult. The development of a protocol for analysing large amounts of plants is essential for certification and sanitary programs for healthy plant production as well as for the analysis of imported plants in order to avoid the risk of introduction of E. amylovora into fire blight free areas.

The aim of this study was to evaluate at the laboratory-scale the E. amylovora detection limit of different techniques of the diagnostic protocol EPPO (2013) in the analysis of single and composite spiked samples from different hosts, with the objective of determining the appropriate number of plants to be analyzed for a sensitive detection of this pathogen.
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\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strain conditions and plant material.} \textit{E. amylovora} CFBP 1430, isolated from \textit{Crataegus} sp. in France in 1972, was routinely grown on King’s medium B (KB) (King \textit{et al.}, 1954) at 26ºC for 48-72 h. Flowers and shoots from healthy pear (cv. ‘Conference’), apple (cv. ‘Gala’) and loquat (cv. ‘Algerie’) trees, from greenhouses at Instituto Valenciano de Investigaciones Agrarias (Spain) or State Plant Service (Lithuania) were used for both single and composite spiked samples.

\textbf{Detection of \textit{E. amylovora} in single spiked samples.} The sensitivity of the different techniques was evaluated first in single spiked samples and then in composite spiked samples. Healthy plant material was crushed with a hammer in sterile antioxidant maceration buffer described by Gorris \textit{et al.} (1996a) as indicated in the EPPO diagnostic protocol, in a proportion of 1:50 (w/v). Ten-fold dilution series ranging from 10^8 to 10^2 cfu ml^{-1} were prepared from a suspension of strain CFBP 1430 in phosphate buffered saline (PBS) 10 mM (pH 7.2) at OD_{600nm} = 0.2. The concentration of the initial suspension was determined by plating dilutions on KB medium. Then, 3 ml aliquots of the serial dilutions were inoculated in 27 ml of the pear (flowers and shoots), apple (flowers and shoots) and loquat (flowers and shoots) extracts to obtain spiked samples containing final \textit{E. amylovora} concentrations ranging from 10^7 to 10 cfu ml^{-1}. In all assays, appropriate negative controls containing no template DNA were subjected to the same procedure. Four aliquots containing 5 ml of each dilution of single samples plus 1.5 ml of glycerol 30% were kept at -20ºC for further analyses, and the rest was analyzed according to the EPPO diagnostic protocol, by using isolation, real-time PCR and ELISA-double-antibody sandwich indirect (ELISA-DASI).

Isolation was evaluated by plating 50 µl of each dilution on KB medium with 250 mg l^{-1} cycloheximide (Sigma-Aldrich, USA), sucrose nutrient agar (SNA) medium (Billing \textit{et al.}, 1961) with 250 mg l^{-1} cycloheximide (Sigma-Aldrich, USA) and CCT solid media (Ishimaru and Klos, 1984). For molecular detection, DNA was extracted by the method described by Llop\textit{et al.} (1999) and 2 or 5 µl of each DNA extract were used for real-time PCR according to Gottsberger (2010), with the primers hpEaF/hpEaR and the probe hpEaP. Pear and apple shoots were evaluated by real-time PCR following Pirc \textit{et al.} (2009), by using the primers Ams116F/Ams189R and the probe Ams141T. A LightCycler 480 (Roche, Germany) thermocycler and its software for data acquisition and analyses were employed. An analysis was considered positive when an exponential amplification from the DNA curve was generated with cycle threshold (CT) value < 40, whereas an analysis was considered negative if it did not produce an amplification curve. An enrichment step was performed by mixing the dilutions with KB or CCT liquid media in 1:1 proportion and incubating at 26ºC for 72 h. Then, serological detection was assayed by the ELISA-DASI commercial kit (Plant Print Diagnòstics S.L., Spain) based on specific monoclonal antibodies (Gorris \textit{et al.}, 1996b), following the manufacturer’s instructions. The absorbance was measured with an automatic reader (Titertek Multiskan Ascent, Labsystems, United Kingdom) at OD_{405nm} and, after 1 h, values greater than twice the value of the negative controls were considered to be positive. After enrichment, real-time PCR according to Gottsberger (2010) was also performed.

\textbf{Detection of \textit{E. amylovora} in composite spiked samples.} Once single spiked samples had been analyzed, the positive samples by any of the assayed techniques were conserved at -20ºC and utilized for preparing composite spiked samples. Simultaneously, macerated plant material was obtained, maintaining the ratio 1:50 (w/v), as previously described, to prepare four composite samples, as shown in Fig. 1: sample simulating 3 plants, prepared with the content of one thawed single sample plus 10 ml of macerated plant material; sample simulating 5 plants, with the content of one thawed single sample plus 20 ml of macerated plant material; sample simulating 10 plants, with the content of one thawed single sample plus 45 ml of macerated plant material; and sample simulating 20 plants,
with the content of one thawed single sample plus 95 ml of macerated plant material.

After an enrichment step, made as described above, composite spiked samples were also analyzed by isolation on CCT media, real-time PCR according to Gottsberger (2010) and Pirc et al. (2009) and ELISA-DASI. All the experiments with both single and composite spiked samples were repeated at least twice.

RESULTS AND DISCUSSION

The use of accurate procedures to obtain a reliable result in the detection of pathogenic bacteria when analyzing asymptomatic carrier plants is a major challenge in the diagnostic protocols. The choice between individual or bulked samples is a question of great interest that has obvious benefits but can decrease the sensitivity of the diagnostic protocols. In this study we have evaluated at a laboratory-scale single and composite spiked samples with two types of plant material and three hosts to optimize the E. amylovora detection in asymptomatic plants.

The first approach by using single spiked samples showed that the detection limit for each technique assayed ranged between $10^1$ and $10^4$ cfu ml$^{-1}$ of plant extract (Fig.2). The isolation allowed detecting up to $10^6$ cfu ml$^{-1}$ in almost all the combinations of plant organ and host evaluated, whereas enrichment-ELISA-DASI (E-ELISA-DASI) and real-time PCR showed a greater variability. The robustness and sensitivity of isolation in the conditions of the assay was not surprising, since E. amylovora cells inoculated in plant extracts came from a fresh culture in a rich medium, so probably they were able to grow on solid medium faster than the native microbiota present in plant samples, which is adapted to more restrictive conditions (Bulgarelli et al., 2013). In fact, when an additional enrichment step in liquid culture medium was included before isolation for improving the recovery of the target pathogen, the detection limit of isolation became worse than that of direct isolation (data not shown). This could be due to the masking of E. amylovora colonies by plant microbiota with a faster and more efficient growth, which probably competes with the fire blight pathogen, both in culture medium and also in the host, for nutrients and space (Stockwell et al., 1999; Mikiciński et al., 2016).

The detection limit reached by E-ELISA-DASI, $10^1$, $10^4$ cfu ml$^{-1}$ (Fig.2), is in accordance with Gorris et al. (1996b) and the EPPO diagnostic protocol (2013). As this serological technique is dependent on the type of microbiota present that allows the multiplication of the target in the enrichment, it would seem advisable to use several culture media to promote the selective growth of E. amylovora over competitor bacteria. However, in our assays any of the two media used for enrichment showed a higher efficiency than the other in recovering E. amylovora cells. This is indicative of a certain grade of variability in the composition of microbiota in plant samples, depending on host characteristics and environmental conditions and other factors, suggesting that the simultaneous use of the two media advised in the EPPO diagnostic protocol can increase the probability of finding E. amylovora. Moreover, the composition of growth media affects the biotic relationships among bacteria co-cultivated on it (Mikiciński et al., 2016).

With respect to the real-time PCR, the detection limit was also ranging from $10^1$ to $10^4$ cfu ml$^{-1}$ (Fig.2). In shoots of any of the hosts tested, real-time PCR did not allow detecting E. amylovora below $10^2$-$10^3$ cfu ml$^{-1}$, maybe due to the presence of inhibitors in this type of samples (Rezadost et al., 2016). Although many inhibitors are removed during the extraction process, some of them co-elute with the DNA, maybe leading to PCR inhibition (Rezadost et al., 2016). In all cases, the extraction method by Llop et al.
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(1999) was employed because it is a simple method, not using toxic reagents, with a good price/quality ratio and showed good results in preliminary ring tests (López *et al.*, 2010). In any case, an enrichment step in liquid medium, which also contributes to dilute the possible inhibitors present in a sample, was very useful and allowed consistently to detect up to 10^1 cfu ml^{-1} (Fig. 2), regardless of the primers and protocol assayed.

Secondly, the analysis of composite samples simulating increased amounts of 3, 5, 10 or 20 plant specimens revealed some interesting insights and allowed developing a more useful protocol to detect *E. amylovora* in asymptomatic plants. The first remarkable observation was the divergence in results obtained among organs and hosts. In general, a worse sensitivity was reached in flowers than in shoots. Flowers provide ephemeral but nutrient-rich and protective habitats for microorganisms (Shade *et al.*, 2013) and they support a relatively large microbial load compared to that of other plant parts (Stockwell *et al.*, 1999; Pusey *et al.*, 2009). Moreover, not only the plant organ but also the weather conditions have a great influence on the composition of plant microbiota, and the composition markedly determines the efficiency of the enrichment step in the diagnostic protocol and so the success of detecting the target pathogen. Microbial communities change over time and there is a successional pattern among the prevalent bacteria (Shade *et al.*, 2013; Aleklett *et al.*, 2014). The selective advantages or disadvantages of *E. amylovora* with respect to native microbiota depend mostly on the composition of the native microbiota. Nevertheless,
enrichment-real-time PCR was again the most sensitive technique in most cases, even when the plant material corresponding to 20 plants was simulated. Therefore, this molecular technique was the less influenced by the abundance and diversity of native microbiota.

In the flowers, the comparison among hosts showed variability depending on the technique considered (Fig. 3A, C, E). Thus, the sensitivity of enrichment-isolation (E-isolation) ranged between $10^1$-$10^2$ cfu ml$^{-1}$ in pear (Fig. 3A), $10^1$-$10^3$ cfu ml$^{-1}$ in apple (Fig. 3C) and $10^1$-$10^4$ cfu ml$^{-1}$ in loquat (Fig. 3E). In pear and loquat flowers the E-ELISA-DASI and between $10^1$-$10^3$ cfu ml$^{-1}$ by E-real-time PCR (Fig. 3A, E) was not suitable for detection in composite asymptomatic organs of these two hosts. However, in apple flowers the detection limit of this technique was between $10^2$-$10^4$ cfu ml$^{-1}$ (Fig. 3C), in agreement to the usual limit of this technique. The detection limit of enrichment-real-time PCR (E-real-time PCR) was around $10^1$ cfu ml$^{-1}$, regardless of the host evaluated (Fig. 3A, C, E). The different composition of microbiota among the three hosts could explain the differences observed in detection, since the enrichment can promote or not the growth of certain bacteria with some selective advantage over *E. amylovora*.

In shoots, the differences among techniques decreased because the sensitivity by E-isolation in the three hosts was around $10^1$-$10^2$ cfu ml$^{-1}$, between $10^1$-$10^3$ cfu ml$^{-1}$ by E-ELISA-DASI and between $10^1$-$10^3$ cfu ml$^{-1}$ by E-real-time PCR (Fig. 3B, D, F), depending on the number of plants analyzed in the composite samples. The best detection limit was reached by the PCR in loquat, *ca.* 10 cfu ml$^{-1}$, regardless of the amount of plants simulated (Fig. 3F). The primers used for the PCR are also another factor to take into account. The use of those designed by Pirc *et al.* (2009) reduced slightly the sensitivity of the technique, as shown in Fig. 3D, where the detection limit was even higher than that of E-isolation.

Several main conclusions can be drawn from this study. Asymptomatic samples from *E. amylovora* hosts can be bulked for analysis with no loss of reliability in detection results, as revealed in the analysis of plant material simulating an increasing number of plants. The most robust technique, less affected by the amount of plant material, was the E-real-time PCR, since $10^1$-$10^2$ cfu ml$^{-1}$ of *E. amylovora* could be detected in most cases, both in flowers and in shoots, regardless of the amount of simulated plants analyzed, 3, 5, 10 or 20. Finally, the enrichment is confirmed as a very advisable step for improving the probability of detection of *E. amylovora* (López *et al.*, 2004) even by the most sensitive technique, the real-time PCR. Of course, the main drawback for processing bulk samples can be the irregular distribution of the bacteria in the plants but this aspect requires further investigations.

In summary, this laboratory-scale study has provided interesting data that will be assayed with *E. amylovora* naturally infected asymptomatic plant material, and it will contribute to the implementation of analysis focused on reducing the risk of introduction of infected plants in areas free of fire blight.

**ACKNOWLEDGEMENTS**

This work was funded by Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) through the ERA-Net EUPHRESCO project “Phytosanitary diagnostic, on-site detection and epidemiology tools for *Erwinia amylovora* (Phytfire)” and through project “IVIA 51407”. J.F. Catalá-Senent acknowledges support from IVIA and the European Social Fund for his scholarship. The authors also thank Clara Morente, Adela Monterde, and Encarnación Martínez for the technical assistance.

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Accepted April 10, 2017
Received June 21, 2017