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ADVANTAGES OF AN INTEGRATED APPROACH FOR DIAGNOSIS OF QUARANTINE PATHOGENIC BACTERIA IN PLANT MATERIAL

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Abstract

The accurate and reliable diagnosis of quarantine bacteria and their detection in asymptomatic material requires the use of an integrated approach based on the use of several techniques. This strategy is more expensive and time consuming but it combines conventional, serological and molecular tests in order to get better results. Bacterial isolation is required for having pure cultures necessary for target identification and to perform pathogenicity tests. However, its results can be negative, especially for slow growing pathogens, or when the bacteria in the samples are in the viable but non culturable state. Serological tests, especially indirect immunofluorescence (IIF) and ELISA are very useful for routine testing. Nevertheless, both can have specificity problems due to the poor quality of some available antibodies. The first one has usually an acceptable sensitivity and can be employed as a screening test. On the opposite, ELISA sensitivity is, in general, poor and a previous enrichment step is recommended. The use of PCR has improved the sensitivity and specificity of the diagnosis and in many different variants, is a very efficient method for rapid screening of samples. However, there are many cases where the presence of inhibitors gives false negative results, or for which the positive ones by PCR cannot be confirmed with any other technique. Bioassays have also proved their usefulness, especially for recovering stressed or low populations of bacteria. They require few weeks for being performed when doing the biological enrichment *in planta*, but other possibilities such as *in vitro* growing plants or detached leaves, can also be used. The experience in applying this integrated methodology for diagnosis (isolation, serological tests, PCR and bioassays) has allowed to detect more accurately *Erwinia amylovora*, *Ralstonia solanacearum* and *Xanthomonas axonopodis* pv. *citri* in plant material.

Key words: *Erwinia amylovora, Ralstonia solanacearum, Xanthomonas axonopodis* pv. *citri,* detection, identification, PCR, ELISA, IIF

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Introduction

The available techniques for detection of pathogenic bacteria in plant material are numerous, but the most widely used can be classified in four groups: isolation, serological, molecular techniques, the combination of all of them and bioassays. Isolation techniques include, among others, plating on standard and/or selective media, plating after a preliminary enrichment step in liquid media (standard and/or selective), and the isolation after bioassays or inoculation of plants or detached organs, with the sample extracts. The more commonly used serological techniques for detection of plant pathogenic bacteria include indirect immunofluorescence (IIF) and several ELISA formats, both using polyclonal or specific monoclonal antibodies, with different specificity characteristics. The low sensitivity observed for ELISA, especially when using specific monoclonal antibodies, can be overcome by the use of an enrichment step in liquid media. The available molecular techniques reported for detection of plant pathogenic bacteria are very abundant, but in the last 10 years only PCR in different variants is widely used because of its high sensitivity and specificity using the appropriate primers.

The main goal of this paper is to describe the advantages of a combination of several methods for the most accurate detection of quarantine pathogenic bacteria. When analysing samples for these bacteria, is strictly necessary to avoid the risk of false positives and false negatives results. Consequently, we should use the techniques and probes with maximum specificity, but also those with maximum sensitivity and many times it is difficult to combine them in only one technique and protocol. Then, the use of two or more techniques in an integrated approach is necessary for obtaining the better diagnostic results. This approach was selected for the European Union (EU) official protocols for *Clavibacter michiganensis* subsp. *sepedonicus* (Spieckermann & Kotthoff) Davies et al. (Council Directive 93/85/EEC… 1993) and *Ralstonia solanacearum* (Smith) Yabuuchi et al. (Council Directive 98/57/EC… 1998) diagnosis. It has also been adviced more recently for Detection of *Erwinia amylovora* (Burrill) Winslow et al. and *Xanthomonas fragariae* Kennedy & King detection, after a ring test evaluation of several techniques, in the context of an EU granted project (www.csl.gov.uk/science/organ/ph/diagpro).

To show the interest of this polyphasic analysis, we have selected, as example, three models of EU quarantine bacteria, in which the use of an integrated approach has provided new information and accurate diagnosis.

Advantages and disadvantages of techniques used for routine analysis of plant samples

Bacterial isolation, IIF, ELISA, PCR and bioassays techniques have many advantages for routine detection of plant quarantine bacteria, but also some limitations. The advantages of bacterial isolation are very well known because of using the appropriate media, the sensitivity and specificity can be very good reaching a detec-

tion level of up to 1–10 cfu/ml, as it has been shown in several models in comparative evaluations (Elphinstone et al. 2000, López et al. in press). However, when analysing plant samples in natural conditions, the isolation will not detect stressed or injured bacteria or those in the viable but non culturable state (VBNC), which it has been recently described for *R. solanacearum* and *E. amylovora*. In this physiological state the bacterial cells are unable to divide sufficiently on non-selective solid medium to yield visible growth as a colony. The factors that induce this state can be variable in different bacterial species as low temperatures, or copper in *R. solanacearum* (Van Elsas et al. 2001, Grey and Steck 2001) or copper and lack of nutrients in *E. amylovora* (Ordax et al. in press, Biosca et al. in press). Furthermore, colonies with the same morphological features in the plates can belong to different bacterial species and their serological, molecular and pathogenic characteristics must be analysed consuming a lot of time.

The IIF is very useful for routine analysis and it shows an acceptable sensitivity (10^3–10^4 cfu/ml) but requires specific antibodies of good antigenic affinity and avidity, that are not commercially available for all quarantine bacteria, while more specific monoclonal antibodies, generally are not appropriate for IIF. In addition to that, it is considered a tedious technique that requires subjective and time-consuming microscopical reading. ELISA is a very convenient technique processing large number of samples because it is simple to perform and all the steps can be automated. The most commonly used types of ELISA are indirect ELISA, DASI-ELISA and there are commercially available kits for many models. The problems of ELISA are also mainly related to the need of specific antibodies, which cannot always be commercially available. The sensitivity generally observed (10^5–10^6 cfu/ml) can be improved by the use of an enrichment step previous to the ELISA technique (López et al. 1997).

The PCR technique has, in its high sensitivity (up to 1 cfu/ml or less) and specificity, the main advantage over other methods. There are many PCR formats as conventional PCR, nested-PCR, nested-PCR in a single tube, Cooperative-PCR, multiplex PCR, etc. (López et al. 2003). The new developments of real-time PCR overcome the risk of contamination when handling the amplicons and the quantitative approach expands the potential uses of PCR, not only for diagnosis but also for many research purposes. The main disadvantage of PCR is due to the lack of a universal DNA extraction protocol that could avoid unexpected inhibition problems, commonly observed when working with different types of plant material. Consequently, the most appropriate DNA extraction protocol must be used for each type of analysed material (López et al. 2003) and in many cases, enrichment step in solid or liquid medium (Schaad et al. 1995, López et al. 1997) can improve the amplification results. In addition to that, the specificity and sensitivity of the reported PCR primers and protocols are not always as appropriate as initially reported for some quarantine bacteria (Ruiz-Arahal et al. 2004).

Bioassays have been developed in many models, especially for the analysis of quarantine pathogens in asymptomatic potato tubers (Council Directive 93/85/EEC… 1993, Council Directive 98/57/EC… 1998). They are very useful when the target populations are very low or for recovering stressed, injured or

VBNC bacteria. The main disadvantage is that bioassays usually require several weeks when doing the bioassay *in planta* and must be performed under biosafety conditions, to avoid dissemination of the pathogens. Then, they are expensive and time consuming, but for several models cheaper and faster protocols have been developed, as the use of inoculation of plant extracts on *in vitro* growing plants or on detached leaves or other plant organs, as for *X. fragariae* (www.csl.gov.uk/science/organ/ph/diagpro/Xanthomonas.pdf).

Integrated approach for diagnosis of *Erwinia amylovora* in rosaceous hosts

Erwinia amylovora, causal agent of fire blight is a quarantine bacterium, according to EU legislation (Council Directive 2000/29/EC… 2000) although it is present in many European countries. The use of isolation, immunofluorescence (IF), enrichment-ELISA and PCR techniques for its accurate detection in plant material is adviced (EPPO… 2004) and the details of the protocol are also available at www.csl.gov.uk/science/organ/ph/diagpro/Erwinia.pdf. This polyphasic approach for routine analysis of the suspected samples has been used for several years in Spain, a protected zone for *E. amylovora*. Surveys are carried out yearly in all the Spanish regions, including laboratory analysis of plants with suspected symptoms and also of asymptomatic hosts in nurseries. During these surveys, some previously undescribed *Erwinia* isolates have been found and their characteristics are reported in Table 1. One isolate is a fully virulent *E. amylovora* strain without pEA29, the plasmid previously considered ubiquitous in all *E. amylovora* isolates. The integrated approach for diagnosis allowed us to identify such as a true *E. amylovora* strain. The use of other techniques, as shown in Table 1, was very useful for correct identification, although the sample was PCR negative when using the primers described by Bereswill et al. (1992) and Llop et al. (2000), previously considered as universal for detection of *E. amylovora* strains. The evaluation of the virulence confirmed that it was *E. amylovora*.

Another isolates were a new *Erwinia* sp., causal agent of necrotic pear blossoms in Valencia, Spain, that share several morphological, serological and molecular characteristics with *E. amylovora*, but not all and is not pathogenic in all *E. amylovora* hosts (Table 1). We did not identify it as the fire blight pathogen after its negative reaction in ELISA (with specific monoclonal antibodies) PCR with primers based on pEA29 sequences and lack of pathogenicity on pear and apple fruits. However, the colony morphology on standard and selective media, biochemical tests, IIF and PCR with primers from Maes et al. (1996) were as expected for *E. amylovora*.

All these results show the risk of false negatives when using only pEA29 based PCR or false positives, when relying only on bacterial isolation. Although *E. amylovora* PCR by using pEA29 based primers was considered universal, and isolation followed by only biochemical or serological characterization is widely used, both approaches can miss atypical strains or new *Erwinia* species.

Table 1

Characteristics of an *Erwinia amylovora* strain without pEA29 and a new *Erwinia* species, causing necrotic pear blossoms

Tests (according to EPPO… 2004)	*Erwinia amylovora* without pEA29	*Erwinia* sp.
Colonies on King's B medium	+	+
Colonies on NSA medium	+	+
Colonies on CCT medium	+	+
Immunofluorescence (polyclonal antibodies)	+	+
DASI-ELISA (polyclonal and monoclonal antibodies)	+	–
PCR (Bereswill et al. 1992)	–	–
PCR (Llop et al. 2000)	–	–
PCR (Maes et al. 1996)	+	+
PCR (Guilford et al. 1996)	+	–
Pathogenicity on pear fruits	+	–
Pathogenicity on pear blossoms	+	+
Pathogenicity on apple fruits	+	–

Integrated approach for diagnosis of *Ralstonia solanacearum* in geranium cuttings

Ralstonia solanacearum is another quarantine bacterium, according to EU legislation (Council Directive 2000/29/EC… 2000) and it is able to cause bacterial wilt in many *Solanaceae* and other hosts. A specific protocol was published in an EU Directive for its detection in different types of plant material (Council Directive 98/57/EC… 1998) and a new one was recently designed (Optimised… 2001). It included isolation on SMSA medium, IIF and PCR as screening techniques, and a tomato bioassay for confirmation of the diagnosis. However, the VBNC state has been recently described for *R. solanacearum,* being low temperatures, and copper treatments the induction factors (Van Elsas et al. 2001, Grey and Steck 2001). This makes the integrated approach strictly necessary because the target cells could not appear on the SMSA plates.

A polyphasic analysis was used in the analysis of geranium cuttings produced in Kenya that were imported to Spain. The standard plating on SMSA medium (Council Directive 98/57/EC… 1998) and the Co-PCR described by Caruso et al. (2003) were used but not IIF because of autofluorescence of the plant tissue. As shown in Table 2, the results were always negative for isolation, but some samples were Co-PCR positive. This Co-PCR protocol combined with an efficient DNA extraction using the DNA-easy kit (Quiagen) was very sensitive when using with this type of plant material for *R. solanacearum* detection (unpublished data) and the PCR positive samples were refused. Unfortunately, as it was a perishable material, it

Table 2

Detection of *Ralstonia solanacearum* in imported geranium cuttings

Tests (according to Council Directive 98/57/EC… 1998 and Optimised… 2001)	Positive samples/analysed samples
Isolation on SMSA	0/97
Co-PCR (Caruso et al. 2003)	20/97
Tomato bioassay	1/10
Isolation after bioassay	1/10

was not possible to perform more analysis on the cuttings. However, when analysing 10 cuttings received later, one of them was Co-PCR positive, and the tomato bioassay performed with the macerate of the cutting detected living and pathogenic cells of *R. solanacearum* in it. The lack of detection of the bacterium by direct plating and its isolation after the bioassay, strongly suggested that the cells were stressed or in the VBNC state before being inoculated on tomato and confirmed the interest of using Co-PCR as a screening test.

Integrated approach for diagnosis of *Xanthomonas axonopodis* pv. *citri* on imported citrus fruits

Xanthomonas axonopodis pv. *citri* is a quarantine bacterium, causal agent of citrus canker, affecting only citrus and related plants. It is not present in Europe or Mediterranean countries, but it is widespread in many South American areas from which the EU imports citrus fruits. Then, inspection of the imports and analysis of citrus with suspicious symptoms is compulsory, because the EU legislation does not allow the introduction of fruits with symptoms of canker. Such analysis is performed in Spain by an integrated approach that includes bacterial isolation, ELISA, PCR and a detached leaf bioassay. Some results of the analysis of imported citrus fruits (sweet oranges, mandarines and limes) are shown in Table 3. The direct isolation on YPGA medium succeed in detecting living cells of the bacterium in only 20 out of 139 analysed samples. However, the PCR with primers designed by Hartung et al. (1993) were able to confirm the bacterial etiology of the lessions in 107 of them. ELISA using polyclonal antibodies confirmed some of the PCR positive results.

Table 3

Detection of *Xanthomonas axonopodis* pv. *citri* in imported citrus fruits

Technique	Positive samples/analysed samples
Isolation on YPGA medium	20/139
PCR (Hartung et al. 1993)	107/319
ELISA	7/109

When some samples were inoculated in detached grapefruit leaves it was possible to reisolate *X. axonopodis* pv. *citri* from two more samples and to confirm its pathogenicity. All these data show, again, the interest of using several techniques for diagnosis of this quarantine bacterium in plant material and the PCR protocol selected confirmed the higher efficiency of PCR as screening test.

Conclusion

In conclusion, an integrated approach for diagnosis should combine isolation, serological and molecular techniques and bioassays. This combination of complementary tests, improves the accuracy of the detection of quarantine bacteria decreasing the number of false positives and false negatives results. In many cases, the analysis of samples requires the use of several specific monoclonal and polyclonal antibodies and primers based on sequences of different origins, for maximum accuracy of the diagnosis. Furthermore, the bioassays are very useful for detecting low populations of stressed or VBNC cells.

The integrated approach has allowed detecting strains with differential characteristics or new bacterial species. This would had been not possible using only one or few techniques in a more rapid analysis. This polyphasic methodology for diagnosis is time consuming and expensive, but the risk of introducing quarantine bacterial pathogens in new areas because of false negative samples, or of taking eradication measures based on false positive results, justifies its use when the maximum accuracy in the diagnosis is required.

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