Bacteriology

Integrated Approach for Detection of Nonculturable Cells of *Ralstonia solanacearum* in Asymptomatic *Pelargonium* spp. Cuttings

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ABSTRACT

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Ralstonia solanacearum (biovar 2, race 3) is a soil and water-borne pathogen that causes serious diseases in several solanaceous hosts. It can also infect geranium plants, posing an important threat to their culture when latently infected cuttings are imported from countries where the pathogen is endemic. *R. solanacearum* can be present in very low numbers in asymptomatic geranium cuttings, and/or in a particular stressed physiological state that escapes direct isolation on the solid media usually employed. Consequently, an integrated protocol has been developed to analyze asymptomatic geranium cuttings routinely. The first screening tests include isolation and co-operational-polymerase chain reaction (Co-

PCR), based on the simultaneous and co-operational action of three primers from 16S rRNA of *R. solanacearum*. This method was selected as the most sensitive one, able to detect only 1 cell/ml including non-culturable cells. When isolation is negative but Co-PCR is positive, the bioassay in tomato plants is proposed, since stressed bacterial cells or those present in low numbers that do not grow on solid media can be recovered from inoculated tomato plants and retain pathogenicity. This methodology has been demonstrated to be useful and has allowed us to assess the relevance of the physiological status of bacterial cells and its implications in detection. It also reveals the risk of introducing *R. solanacearum* through asymptomatic geranium material when relying only on bacterial isolation.

Additional keywords: latent infection, survival.

Ralstonia solanacearum (E. F. Smith) (67) is the causal agent of bacterial wilt and is widespread in tropical, subtropical, and warm temperate regions, leading to serious losses in crops of economic importance (8,24). *R. solanacearum* race 3 (biovar 2) is considered a quarantine organism in the European Union (EU) (2) and was among 10 plant pathogens listed in the Agricultural Bioterrorism Protection Act of 2002 in the United States (32). The incidence of potato brown rot, the disease specifically caused by *R. solanacearum* biovar 2, race 3 (23,28), has increased in the EU after the first case reported in Sweden in 1976 (47). Since then, outbreaks have been more frequent in the last decade (15,25, 28,49,58,63,65), with most foci being related to crops irrigated with water contaminated by the pathogen.

The host range of *R. solanacearum* (biovar 2, race 3) is not limited to solanaceous plants since it can potentially infect many ornamental and weed species (29,52,59,63). In geranium, *R. solanacearum* was detected in the United States for the first time in 1979 (55). Since 1999 up to the present, *R. solanacearum* biovar 2, race 3 has been isolated from geraniums growing in different greenhouses in both the United States and Canada (3–5,22,26,64). The imported plants came from Guatemala, Costa Rica, Mexico, or Kenya. In Europe, geranium samples infected with *R. solanacearum* biovar 2, race 3 were detected in 1999 and 2000 in the United Kingdom, Belgium, Germany, and The Netherlands, and the origin of infections was traced to cuttings from Kenya (29). Because latent infections in other host plants are considered to play a role in the spread of bacterial wilt within and between countries (23), the latent or symptomatic presence of

R. solanacearum in geranium raises concern that the bacterium could move from ornamental plants into other hosts, soil, or irrigation water. Since there is an overwhelming volume of imported geranium cuttings, the interest in developing sensitive, reliable, and accurate detection and diagnostics methods of *R. solanacearum* from this plant material is increasing, but the fact this pathogen can latently infect geraniums (57) makes it difficult to detect.

Although isolation of this quarantine organism is usually required in detection protocols, as stated in the EU Directive 98/57/EC (2), culture-independent molecular techniques can be used as a screening method to circumvent the problems associated with the possible presence of stressed, injured, or viable but nonculturable (VBNC) cells. Cells in such states are those that progressively lose the ability to form colonies on solid media yet remaining viable, as a possible survival strategy under adverse environmental conditions. Related to the VBNC state, the induction factors can be variable depending on the bacterial species (43), and low temperatures have been demonstrated as responsible for the entry of *R. solanacearum* cells in the VBNC state in water (6,11,60).

Among culture-independent methods, several polymerase chain reaction (PCR) protocols and oligonucleotides have been described for *R. solanacearum* (7,14,17,48,50,51,53,62). Furthermore, an innovative and sensitive co-operational-PCR (Co-PCR) has recently been described (9), which has proven useful for detecting the target from environmental water samples. Co-PCR detection levels were as sensitive as the nested-PCR technique, with the advantage that this method does not require two different annealing temperatures (46). Furthermore, there is increasing interest in combining different techniques in integrated approaches for detection in order to avoid false negatives as well as false positives results (1,36). In this context, we have developed and evaluated an integrated protocol, combining several techniques,

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for specific and sensitive detection of *R. solanacearum* in asymptomatic geranium cuttings.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *R. solanacearum* biovar 2 strains PD 2762, IVIA 2833-6.2, and UW551 were used in this study. Strain PD 2762 was isolated from potato (*Solanum tuberosum*) in The Netherlands and strain IVIA 2833-6.2 from geranium cuttings imported to Spain in 2003. Strain UW551 was also isolated in 2003 from a wilted geranium plant (*Pelargonium hortorum*) growing in a Wisconsin greenhouse, but from cuttings produced in Kenya. Strains were routinely grown on modified yeast extract-peptone-glucose agar (YPGA) medium (34) with filter sterilized glucose, at 29°C for 72 h and maintained at –80°C with 30% glycerol.

Plant material. Tomato plants (*Lycopersicon esculentum* 'Roma') at the third true-leaf stage were used for bioassays and pathogenicity tests as previously described (2). Two-month-old geranium plants (*Pelargonium × hortorum* and *P. peltatum*) from a nursery in Valencia (Spain), with no previous history of *R. solanacearum* infection, were used for the protocol standardization. For protocol evaluation, samples of asymptomatic geranium cuttings of cultivars imported to Spain from Kenya in 2003 and 2004 were analyzed.

Spiked geranium samples preparation. In order to simulate latent *R. solanacearum* infections, basal stem parts from healthy geraniums were incubated in 4.5 ml of phosphate-buffered saline (PBS) for 2 h with shaking, as described later for sampling. Then, extracts were inoculated with a bacterial suspension of the strain IVIA 2833-6.2 at serial decimal decreasing doses (from 10^8 to 1 colony forming units [CFU]/ml) prepared from a culture growing in YPGA for 72 h. The protocols for testing these spiked samples were the same as for naturally infected ones, described later.

Analysis of imported asymptomatic geranium cuttings. Portions of vascular tissue from geranium cuttings of about 0.5 × 0.5 cm from the lowest part of the stems were aseptically comminuted in 4.5 ml of PBS and incubated for about 2 h at room temperature with shaking. The extracts were individually processed by isolation on modified YPGA (34) and modified semiselective agar (SMSA) medium (14), indirect immunofluorescence (IIF), double-antibody-sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA) and Co-PCR. For isolation, 0.1 ml of the extracts were used by spreading; 0.1 ml from the enrichment culture were used for DASI-ELISA; 30 µl for immunofluorescence; 0.2 ml for DNA extraction, and 5 µl of the undiluted or 10-fold diluted after DNA extraction were used as sample for PCR amplifications. In these two last cases, because of the small sample sizes, several aliquots were processed. All the analyses were performed, at least twice, in separate experiments. Aliquots of 0.5 ml of the extracts were conserved at –80°C with 30% glycerol for further inoculation in tomato plants.

Isolation, enrichment, and identification of *R. solanacearum*. *R. solanacearum*-like colonies were observed and counted, after incubation on SMSA for 48 h to 5 days at 29°C. Presumptive *R. solanacearum* colonies were purified on YPGA and confirmed by IIF, DASI-ELISA, and Co-PCR before biovar determination (19). During the direct isolation process, part of the sample (0.5 ml) was used for enrichment by mixing with 4.5-ml of semiselective modified-Wilbrink broth (MWB) according to Caruso et al. (10) in order to improve *R. solanacearum* detection. Geranium extracts in MWB were incubated at 29°C for 48 to 72 h with gentle shaking (125 rpm) in a Lab-line orbital shaker model 4628. Enriched samples were plated on modified YPGA and modified SMSA agar and also processed by DASI-ELISA and Co-PCR. All analyses were performed at least twice, in separate experiments.

IIF. Antiserum 1546-H IVIA obtained against a Spanish *R. solanacearum* biovar 2 strain was used for IIF according to Caruso et al. (10), to confirm that isolated colonies were *R. solanacearum* and to detect the pathogen in geranium extracts directly. Only a staining similar to that exhibited by the homologous strain (positive control) was scored as positive. Filter sterilized PBS was used as negative control.

DASI-ELISA. DASI-ELISA was basically performed as described by Caruso et al. (10) using the detection kit supplied by Plant Print Diagnostics (Valencia, Spain), which includes the specific monoclonal antibody 8B-IVIA. This technique was used to analyze spiked and nonspiked geranium samples at least in duplicate, in separate assays. Strain PD 2762, used to raise the MAb, was employed as a positive control, whereas strain P 27 of *Chryseobacterium indologenes* was the negative one. Values of optical density (OD) at 450 nm that were over twice those of the negative control were considered positive.

DNA extraction. DNA was isolated from geranium plant extracts (200 µl) using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol, eluted with 50 µl of DNase-free water, and stored at –20°C. DNA extractions were used directly or 1/10 diluted in sterile water to dilute potential polymerase inhibitors.

Conventional PCR and Co-PCR. Conventional PCR was performed according to Caruso et al. (9) using OLI1 (14,53) and Z (7) primers, for amplification of a 403-bp fragment. The reaction was performed in a final volume of 25 µl with the following reagents: 10× *Taq* buffer, 2.5 mM MgCl2, 0.1 mM concentrations of each deoxynucleoside triphosphate, 0.1 µM of each primer, 1 U of *Taq* DNA polymerase and 5 µl of sample. Amplification conditions included a denaturation step at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min and then one cycle of 72°C for 10 min in a Master-Cycler Gradient (Eppendorf) thermal cycler. Co-PCR was carried out following the methodology also described by Caruso et al. (9), with OLI1, OLI2 (14), and JE-2 (14). The reaction was performed in a final volume of 25 µl with 10 × *Taq* buffer, 4 mM MgCl2, 0.2 mM concentrations of each deoxynucleoside triphosphate, 5% dimethyl sulfoxide (DMSO), 3% formamide, 0.15 µM OLI1 and OLI2 primers, 0.075 µM JE-2 primer, 1 U of *Taq* DNA polymerase (GIBCO-BRL), and 5 µl of sample. The amplification was performed by a denaturation phase at 95°C for 3 min, followed by 55 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 45 s, and then one cycle of 72°C for 10 min in a MasterCycler Gradient (Eppendorf) thermal cycler. Both conventional and Co-PCR were used to detect *R. solanacearum* in geranium extracts, both directly and after enrichment, and to confirm the identification of suspected colonies grown on solid media. Positive and negative controls were the strain PD 2762 and sterile PBS, respectively. Amplified products were separated by electrophoresis (100 V) on 2% agarose gels.

Biochemical tests. The biochemical tests used for the presumptive *R. solanacearum* colonies were: oxidative metabolism of glucose through O/F medium, oxidase activity, use of Simmons citrate, and hydrolysis of esculin. Biovar determination was also performed, based on using cellobiose, lactose and maltose, and oxidation of the hexose alcohols dulcitol, mannitol, and sorbitol (19), and the methodology used was that described in the EU Directive as official protocol (2). Positive and negative controls were the same as for the Co-PCR.

Bioassays and pathogenicity tests. Bioassays were performed according to the EU Directive (2). Briefly, 10 µl of the geranium extracts was inoculated into tomato stems between the cotyledons (27). Plants were maintained at 28°C 16 h light/8 h dark and high relative humidity for 28 days, assessed daily for wilt symptoms, and sampled to recover the pathogen by isolation. Stem pieces approximately 0.5 cm³ from the inoculation site and 5 cm above and below that site were comminuted in saline solution and the

extract spread on LPGA and SMSA media. Furthermore, to confirm the pathogenicity of strains identified as *R. solanacearum*, virulence assays were performed both in tomato and geranium by stem inoculation, as described previously, with suspensions of suspected colonies at 10^6 to 10^8 CFU/ml. Ten plants were inoculated for each strain. The negative control was the same as for the Co-PCR, and strain UW551 was used as positive control for comparative purposes.

RESULTS

Sensitivity assessment of the different detection techniques. Spiked geranium extracts, inoculated with serial 10-fold dilutions of *R. solanacearum* suspensions, were analyzed by isolation (on general and semiselective media), serological (IIF and DASI-ELISA), and molecular techniques (conventional and Co-PCR), with or without previous enrichment in liquid medium. Moreover, a tomato bioassay was included to potentially multiply bacteria from samples with very low numbers of the pathogen. Results from all these analyses are shown in Table 1. The detection limit was about 10^3 CFU/ml for both YPGA and SMSA plate counts and 10^2 CFU/ml after enrichment; 10^4 cells/ml for conventional PCR, and 1 cell/ml for Co-PCR. PCR amplifications were sometimes inhibited in geranium extracts as observed by processing at the same time the DNA extracts and their dilutions, but they always worked when samples were serially (10-fold) diluted (Fig. 1). DASI-ELISA and IIF were positive for all dilutions, with strong background fluorescence observed in all samples stained by IIF, regardless of the dilution considered and also after the enrichment step. In DASI-ELISA most of the wells in the plates also provided very high OD values, even in some negative controls with only healthy geranium extracts. Regarding pathogenicity, the dilutions of *R. solanacearum* in spiked geranium extracts with 10^4 CFU/ml or higher concentrations caused the typical wilting symptoms in inoculated tomatoes after 7 days, leading to the death of nearly 100% of the tomatoes after 2 to 3 weeks of inoculation; however, extracts with bacterial cell counts ranging from 10^2 to 10^0 CFU/ml did not produce visible symptoms in all the inoculated plants, and the bacterium was not always re-isolated from them.

Protocol for detecting *R. solanacearum* in asymptomatic geranium cuttings. Based on the results described previously, a protocol was developed to detect *R. solanacearum* in asymptomatic geranium cuttings (Fig. 2), for which isolation and Co-PCR were proposed as the two basic tools to analyze asymptomatic cuttings. According to this protocol, bioassays would be performed when isolation was negative and Co-PCR was positive; then, *R. solanacearum*-like colonies recovered from inoculated tomatoes would be subjected to identification and pathogenicity tests to confirm pathogen detection.

Evaluation of protocol for detection of *R. solanacearum*. A total of 107 samples of asymptomatic geranium cuttings (*Pelargonium* spp.), imported from Kenya in 2003 and 2004, were analyzed according to the previously designed protocol. The direct isolations were all negative because the colonies on general or semiselective media, before and after enrichment, did not exhibit the typical characteristics of *R. solanacearum* or were not identified as this pathogen. However, 23 samples tested positive by DASI-ELISA after enrichment. Results from IIF were confusing because high background fluorescence was observed in all samples, which masked detection of target cells. With respect to the Co-PCR, though no amplification was obtained from the direct geranium DNA extractions, the specific band of 408 bp was clearly amplified in 30 samples when these extractions were diluted 10-fold. Ten samples that were negative for isolation and positive for Co-PCR were randomly selected and extracts inoculated in tomato stems. Plants did not show evident symptoms of bacterial wilt, but typical *R. solanacearum* fluid colonies were re-isolated after 7 days and their identity as *R. solanacearum* biovar 2 confirmed by biochemical, serological, and molecular tests. Results for positive and negative controls were as expected.

R. solanacearum inoculated in tomato and geranium plants. All tomatoes inoculated with the strains recovered from bioassayed tomatoes showed the typical wilt symptoms after 7 days.

TABLE 1. Sensitivity of different techniques for *Ralstonia solanacearum* detection in spiked geranium extract

Techniques	Sensitivity	
	Before enrichment	After enrichment[a]
Plate count on YPGA[b]	3×10^3 CFU/ml	10^2 CFU/ml
Plate count on SMSA[b]	2.5×10^3 CFU/ml	10^2 CFU/ml
Co-PCR[c]	1 cell/ml	1 cell/ml
PCR[d]	10^4 cells/ml	10^3 cells/ml
DASI-ELISA[e]	ND	1 CFU/ml
IIF[f]	ND	ND
Pathogenicity[g]	10^4 CFU/ml	ND

[a] Enrichment performed in modified-Wilbrink broth, according to Caruso et al. (10).

[b] Plate counts were determined, directly from the plant extract or after enrichment, on the nonselective medium yeast extract-peptone-glucose agar (YPGA) (34) and the semiselective agar SMSA (14).

[c] Co-operational-polymerase chain reaction (Co-PCR) carried out after DNA extraction, according to Caruso et al. (9), using OLI1 (14,53), OLI2 (14), and JE-2 (14) primers.

[d] Conventional PCR performed with OLI1 and Z (7) primers, according to Caruso et al. (9).

[e] Double-antibody-sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA) basically performed as described by Caruso et al. (10) using the detection kit supplied by Plant Print Diagnostics (Valencia, Spain), which includes the specific monoclonal antibody 8B-IVIA. (Performed only after enrichment because the sensitivity of this technique before enrichment is very low.)

[f] Indirect immunofluorescence (IIF), with the antiserum 1546-H IVIA obtained against a Spanish *R. solanacearum* biovar 2 strain according to Caruso et al. (10): results not included because of the high background fluorescence in all samples.

[g] Pathogenicity assays made in tomato plants by inoculation of 10 µl of geranium extract into stem between the cotyledons (27).
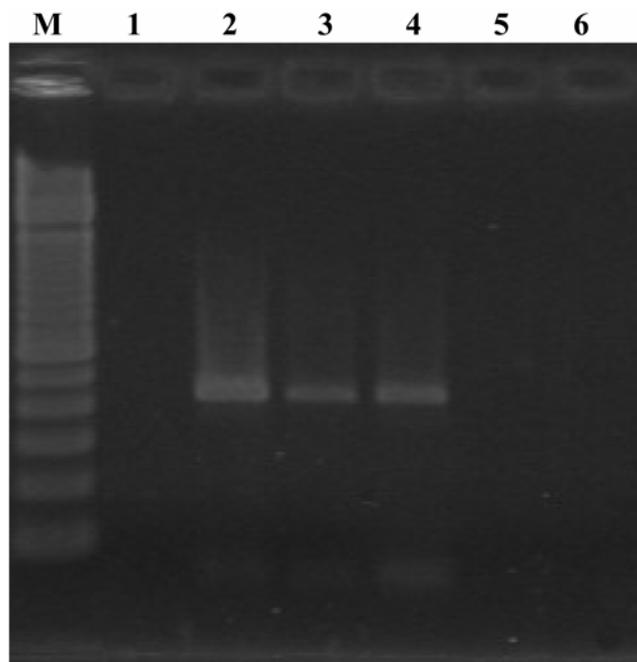

Fig. 1. Amplification by co-operational polymerase chain reaction using DNA extracted from geranium cutting samples. M, Molecular marker; 1, undiluted *R. solanacearum* spiked geranium extract sample; 2 to 5, 10-fold serial diluted samples (10^2, 10^1, 10^0, and 10^{-1} CFU/ml); and 6, negative control.

However, most geraniums inoculated with the same strains showed only minor symptoms, such as some wilted leaves and other leaves with V-shaped spots (Fig. 3), at 20 days postinoculation. Nevertheless, *R. solanacearum* was recovered from both wilted and asymptomatic plants. At the same postinoculation time, strain UW551, used as positive control, caused wilting in almost 40% of the plants, and the pathogen was re-isolated. All colonies were confirmed as *R. solanacearum* by IIF and Co-PCR.

DISCUSSION

The ability of *R. solanacearum* biovar 2, race 3, to produce latent infections in geranium plants is well known (57) and can lead to false negative results when analyzing asymptomatic cuttings only by isolation. Therefore, the threat of potential introduction of this pathogen in asymptomatic geranium cuttings is real and should not be underestimated. In an effort to minimize the risk of introducing this bacterium through asymptomatic plant material, we have developed a rapid and sensitive protocol to detect *R. solanacearum* in geranium cuttings. Moreover, this is the first report to deal with the detection of this quarantine organism in imported geraniums in Spain.

The analyses of geranium cuttings for *R. solanacearum* according to the EU Directive 98/57/EC (2) by cultural, serological, and

Fig. 2. Flow chart diagram of the proposed protocol to detect *Ralstonia solanacearum* in asymptomatic geranium plants. Co-PCR indicates co-operational-polymerase chain reaction according to Caruso et al. (9). IF indicates indirect immunofluorescence with *R. solanacearum*-specific antibodies. ELISA indicates ELISA with *R. solanacearum*-specific monoclonal antibodies.

molecular methods require the isolation of the bacterium on solid media to confirm its detection. In our samples, a discrepancy between cultural and molecular methods was revealed, since no colonies of *R. solanacearum* were recovered, whereas positive detection was obtained by Co-PCR. To assess the sensitivity of the different detection techniques employed, we inoculated geranium extracts with decreasing dilutions of the pathogen, and then compared the detection efficiency of cultural and molecular techniques in these spiked samples. The Co-PCR technique displayed the highest sensitivity, being able to detect 1 cell/ml of plant extract. The high sensitivity of this PCR protocol in comparison to other methods was previously reported in the detection of the target bacteria in environmental waters (9). Sensitivity by isolation was around 10^3 CFU/ml, and it improved detection to 10^2 CFU/ml when an enrichment step was performed prior to isolation. However, these CFU numbers may overestimate the efficiency of isolation under natural conditions, since in nature the pathogen must compete with other microbes which can overgrow target organism on the culture media, thus masking detection of *R. solanacearum*.

Furthermore, target bacterial cells in the sample could be present at very low levels or in a particular physiological state, such as injured (38) or VBNC (42), thus escaping detection by cultural methods. Injured cells are not detected on selective solid media but they can grow on general ones; cells in the VBNC state are even unable to grow on general solid media but remain viable as measured by culture independent methods. Both injured and VBNC cells can be induced by several environmental factors. In fact, the VBNC state has been demonstrated in *R. solanacearum* at low temperatures (6,11,60,61). Therefore, detection of *R. solanacearum* based mainly on culturability could lead to an underestimation of its presence. In contrast, Co-PCR, which is a more sensitive technique, detects injured and VBNC cells, and is not affected by the growth of competitors as it does not need an enrichment step, something that also contributes to the speed of analysis. The only caution to consider in PCR analysis is to dilute the samples to minimize the effect of inhibitors, a common problem in PCR detection of plant pathogens in crude plant extracts (31,37,39,56), which has already been reported in geranium tissue (21,51). Dilutions in the range of 1/100 to 1/500, which involve a drastic reduction in sensitivity, have been reported as successful for *Pelargonium asperum* (51) with no DNA extraction; by contrast, in present work, 10-fold dilutions of DNA extractions were enough to give a strong target signal by Co-PCR, as previously described for certain water samples with the same protocol (9), allowing positive results with the pathogen at low levels even in the presence of high numbers of indigenous microbiota (up to 10^5 CFU/ml). Thereafter, it has been successfully applied to potato plant material (*unpublished data*) and in this study to geranium. In spiked geranium extracts, Co-PCR was much more sensitive than conventional PCR, and therefore the former was chosen as a screening method. In fact, it has been stated that the detection threshold of single round PCR is not sensitive enough to detect early or latent infections of *R. solanacearum* (51). However, Co-PCR has several advantages over other developed PCR protocols, such as high sensitivity and reduced risk of cross-contamination, because all reactions occur in just one tube. Indeed, such a technique has been considered as appropriate for routine use, due to its good specificity and sensitivity (9).

Serological methods, such as IIF and ELISA, were of limited interest because the assayed geranium extracts caused a strong background fluorescence that hampered the visualization of target cells by IIF or gave positive result in most wells of ELISA plates. Furthermore, the background was observed in geranium extracts even after dilution in water (IIF) or dilution in modified Wilbrink broth used for enrichment (DASI-ELISA), and true positive results obtained by IIF and ELISA could not be distinguished from false positive ones.
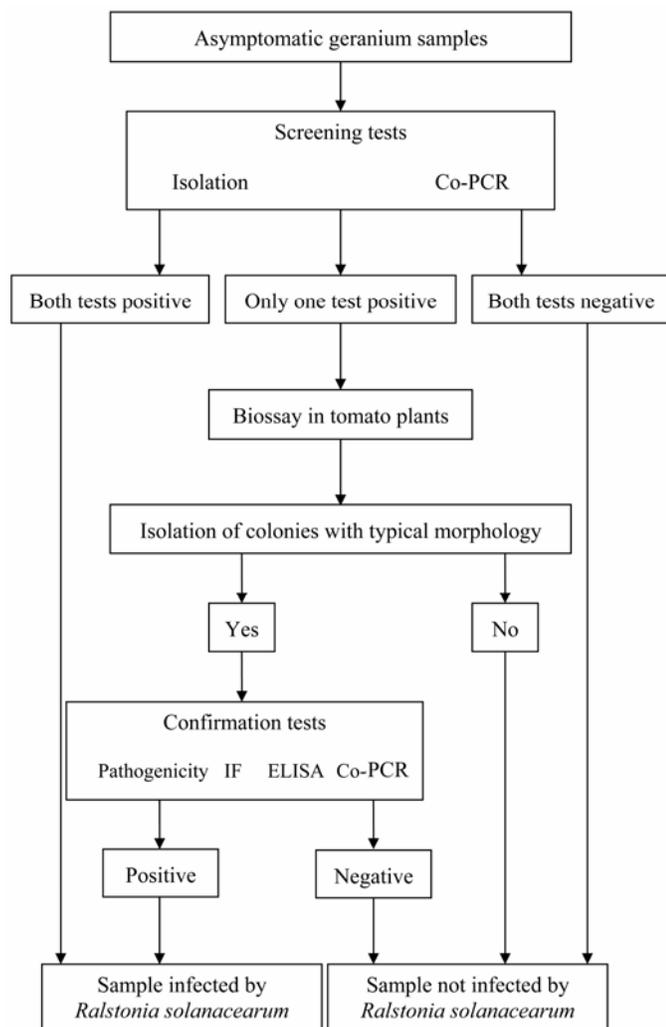
Regarding bioassays, these were performed with geranium extracts containing *R. solanacearum* cells in the range of 10^8 to 10^0 CFU/ml. The analysis of the inoculated plants provided positive results by Co-PCR in all of them, but only positive by isolation at concentrations higher than 10^3 CFU/ml. The inoculation of extracts with 10^2 CFU/ml in tomatoes produced the typical bacterial wilt symptoms in only some plants, and *R. solanacearum* was reisolated from them. In this respect, Swanson et al. (57), assaying soil-soak inoculations, observed that 20% of geranium plants were latently infected, with the proportion of plants developing active infections varying greatly between experiments. Obviously, the inclusion of a larger number of bioassayed plantlets (25 to 50 per extract instead of 10 plantlets) could increase the reliability of the protocol. A large proportion of sites in latently infected plants has even been reported to contain zero or no detectable *R. solanacearum* cells because of the uneven distribution of bacterial cells (57). This fact indicates that random stem sampling can be an unreliable testing method. *R. solanacearum* evaluated strains seem not to reveal a particularly aggressive pathogen for some geranium cultivars (41,57), and their ability to cause latent infections can generate plants with inoculum that led to the disease spreading only after variable periods of time under favourable conditions (57).

We have demonstrated that the lack of accordance between isolation and molecular results could mask the presence of this pathogen and subsequently play down the real danger of introducing the bacterium on asymptomatic plants. Based on the results of spiked geranium extracts, we have designed a protocol to analyze asymptomatic geraniums for the presence of *R. solanacearum* when this pathogen is present in low numbers, and/or in particular physiological states and/or in latent infections.

The protocol was evaluated with geranium cuttings imported from Kenya, where *R. solanacearum* biovar 2 had been detected in watercourses as reported by Janse et al. (29). In our study, from a total of 107 samples, no *R. solanacearum* cell was isolated directly from the cuttings, even after enrichment. However, in 30 samples the pathogen was detected by Co-PCR. According to our protocol, some of the samples testing positive by Co-PCR and negative by isolation were analyzed by bioassay, and *R. solanacearum* was reisolated from tomatoes developing typical bacterial wilt symptoms. When these strains were tested in geranium plants, they developed latent infections producing only minor symptoms, and again *R. solanacearum* was re-isolated from most challenged plants. Variability in susceptibility of geranium cultivars to *R. solanacearum* and in the pathogen variation to produce symptoms has been reported (41). Our results demonstrated the usefulness of the protocol, because target cells (alive, injured, VBNC, or even in low numbers) can be detected by Co-PCR. Moreover, these cells, although not recovered by direct isolation, can be recovered in a second isolation step from tomato plants inoculated with the geranium extracts.

R. solanacearum cells can be released from the roots of infected geraniums during cultivation, and the ebb-and-flow sub-irrigation systems are very effective means of disseminating the pathogen (57). A common practice for routine treatment of contaminated irrigation water involves clarification by filtration, then UV radiation, and addition of chlorine dioxide. The presence of particles in water, even the very small particle sizes, has been reported to influence the efficacy of disinfection with UV radiation and chlorine dioxide (12,30,35,40,54,66). This is because pathogens associated with the particles can escape from full exposure to chlorine (20) or UV radiation (12,16,30); moreover, they can form biofilms which can be more resistant to disinfection than unattached cells (18). Furthermore, it has been demonstrated that both UV radiation and treatment with chlorine can induce the VBNC state in several bacteria (42,43,44,45,68) or reversible cell injury (38). Following such water treatments, three populations of the target cells could probably be distinguished: a majority of dead cells, a few culturable cells, and a large population of injured or VBNC cells, as reported for *E. coli* after chlorination
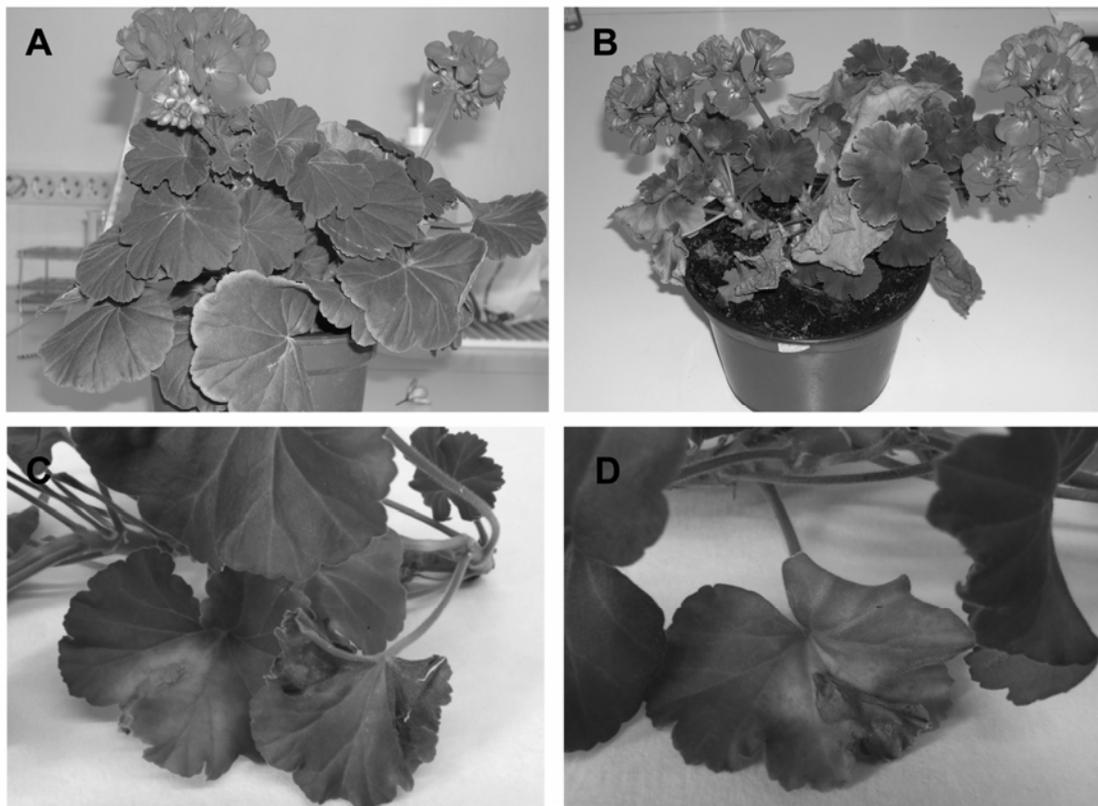

Fig. 3. *Pelargonium* plants challenged by stem inoculation. **A,** Geranium inoculated with phosphate-buffered saline as a negative control; **B, C, and D,** geraniums inoculated with strain IVIA 2833-6.2; in **B,** typical symptoms accompanied by some wilted leaves; in **C and D,** characteristic V-shaped lesions on leaves.

(13,33). Thus, the particular physiological states that *R. solanacearum* can adopt under these conditions (that have not been studied yet) necessitates the use of detection techniques complementary to isolation, able to detect nonculturable or injured bacterial cells. In our survey, *R. solanacearum* cells that were not recovered by isolation but detected by Co-PCR were probably in a stressed state, potentially reversible by passage through a host plant.

In the developed protocol, both isolation and Co-PCR are proposed as basic tools for testing plant material. Isolation on SMSA medium, directly from extracts or after bioassay in tomato plants, was selected because it provides information about the active physiological state of the bacterium, which could divide and proliferate in the plant and, also, because it is the main test outlined by the EU Directive for detection of *R. solanacearum* in potato. The Co-PCR technique was chosen because of its sensitivity, specificity, speed, and ease of use (9) as explained above.

In conclusion, improved detection methods are needed given the fact that *R. solanacearum* could possibly spread from ornamental plants (originally grown in tropical or subtropical areas) to susceptible hosts in European or other countries. This improved detection can be achieved through a combination of different techniques, such as those outlined in the protocol developed as result of this study. Therefore, such protocols should be considered for the analysis of asymptomatic cuttings in the implementation of preventive measures to reduce the likelihood for introduction of geranium bacterial wilt to new areas.

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