Detection of Begomoviruses in Clarified Plant Extracts: A Comparison of Standard, Direct-Binding, and Immunocapture Polymerase Chain Reaction Techniques

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Accepted for publication 14 April 2003.

ABSTRACT

Rampersad, S. N., and Umaharan, P. 2003. Detection of begomoviruses in clarified plant extracts: A comparison of standard, direct-binding, and immunocapture polymerase chain reaction techniques. *Phytopathology* 93:1153-1157.

Three polymerase chain reaction (PCR) techniques—standard PCR (Std-PCR), direct-binding PCR (DB-PCR), and immunocapture PCR (IC-PCR)—using degenerate primers were optimized and evaluated for the detection of begomoviruses. Tomato leaf samples were ground in three different extraction buffers and subjected to Std-PCR. The effect of the buffers on the detection limits of amplification of the virus (detection of the initial and end points of dilution) was determined. With the optimal extraction buffer determined by the first experiment, the antibody concentration and incubation conditions for IC-PCR were evaluated to determine the requirements for maximum capture of antigens during the capture phase of the technique. The incubation conditions of DB-PCR

were also investigated to determine the most favorable conditions for adsorption of the viral template. The reproducibility of all assays was evaluated. With the results of the optimization experiments, the applicability of the three techniques to different plant species was assessed. Extracts of plant species belonging to three families were subjected to the optimized Std-, DB-, and IC-PCR protocols. Std- and IC-PCR both achieved reproducible detection of begomoviruses, but the detection limits and amplified band intensity for all plant species tested were superior for the latter. DB-PCR was an unreliable method of detection, because of poor reproducibility and low intensity of amplified bands. These results indicate that the optimized IC-PCR detection system using degenerate primers is the most effective for the detection of begomoviruses in clarified plant extracts.

Additional keywords: antigen-capture polymerase chain reaction, diagnosis, tube-capture polymerase chain reaction.

With the recent epidemic status of whitefly-transmitted geminivirus diseases in the Western Hemisphere, the family *Geminiviridae* has become one of the major groups of phytopathogens in many vegetable agroecosystems throughout the region (1,19). Whitefly-transmitted geminiviruses belong to the genus *Begomovirus*, members of which possess a bipartite genome and are transmitted by an insect vector, the whitefly *Bemisia tabaci*, to many dicotyledonous host plants. During the past decade numerous begomoviruses have been reported in the region (2,6,9,16,19–21,23,24,27,30). In Trinidad and Tobago, *Potato yellow mosaic virus* Trinidad isolate (PYMV-TT) has been identified as the causal agent of viral epidemics observed in tomato since the late 1980s (30). PYMV-TT infection of tomato is manifested by interveinal chlorosis; yellow mottling or mosaic of leaves; leaf malformations, including severe epinasty and crinkling; leaf size reduction; and, in severe infection, stunting of the whole plant.

Historically, begomoviruses have been characterized by symptomatology and host range tests. A reliance on symptom development as the sole indicator of infection may impede diagnosis, as symptoms are affected by several variables, such as the virus strain, the growth stage of the plant at the time of infection, the plant cultivar, and environmental conditions (1,19). However, fast, accurate, highly sensitive, and reproducible diagnostic assays for detecting begomoviruses have been developed. Among several diagnostic tools available, the polymerase chain reaction (PCR) using degenerate primers has been the most useful for the detection of geminiviruses (8,25,28,33). PCR is especially useful for detect-

ing begomoviruses, because of its sensitivity to the viral template in low titers. In addition, taxonomically informative domains may be amplified by PCR and used in identification and phylogenetic studies (11,18,25,27,30).

Conventional PCR protocols require the preparation of total nucleic acids from infected plants for successful amplification of viral DNA templates. Methods of DNA isolation and purification are not only laborious but also may not be universally applicable to different plant species, especially those with high levels of polysaccharides or phenolics (7,33). As a consequence, template preparation can be a limitation on PCR detection of begomoviruses. Direct-binding PCR (DB-PCR), based on the adsorption of viral particles to the surface of a polypropylene microcentrifuge tube, has been assessed for the detection of viruses (26,33). Similarly, immunocapture PCR (IC-PCR), or antigen-capture PCR, has been used to detect plant viruses, such as *Plum pox virus*, *Apple chlorotic leaf spot virus*, *Prunus necrotic ringspot virus*, and *Banana streak virus* (3–5,10,14,22,26,31,32).

The objectives of this study were to optimize and evaluate three PCR techniques using degenerate primers—standard PCR (Std-PCR), DB-PCR, and IC-PCR—for efficient detection of begomoviruses in clarified leaf extracts of different plant species.

MATERIALS AND METHODS

Collection of plant material. The plants selected for study were *Lycopersicon esculentum* Mill. (Solanaceae); *Sida acuta* J. Burm. and *S. rhombifolia* L. (Malvaceae); and *Calopogonium mucunoides* Desv. and *Rhynchosia minima* (L.) DC. (Fabaceae), each a known host of geminiviruses. Young, newly emerged leaves from symptomatic plants of each species were placed in labeled bags and transported to the laboratory on ice. *L. esculentum* was col-

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Publication no. P-2003-0616-03R

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lected from Aranguez and Pasea, large commercial tomato-growing areas in north Trinidad. *S. acuta* and *S. rhombifolia* were collected as wayside weeds from Toco Main Road, in northeastern Trinidad. *C. mucunoides* and *R. minima* were collected as wayside weeds at the University of the West Indies Field Station in Mt. Hope, in north Trinidad. Healthy leaf samples of *L. esculentum* (used as negative controls) were collected from virus-free greenhouse-maintained plants at the University of the West Indies. Uninfected leaves of *Sida* spp. were collected from Santa Cruz, in northwestern Trinidad, and uninfected leaves of *C. mucunoides* and *R. minima* were collected at Mt. Hope.

Preparation of clarified extracts. One gram of fresh foliar tissue was weighed, washed briefly in distilled water, and cut into strips with a sterile scalpel blade. Samples were ground separately in a sterile mortar and pestle in each of the three buffers (A, B, and C) at a ratio of 1:5 (wt/vol) at room temperature. The extracts were subsequently filtered through a rayon polyester filter (Miracloth, Calbiochem-Novabiochem, La Jolla, CA) and serially diluted (2^0 to 2^{-10}) in the same buffers used for grinding. Buffer A contained 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0); buffer B, 500 mM Tris-HCl (pH 8.0), 2% polyvinylpyrrolidone (PVP-40), 1% polyethylene glycol (PEG-8000), 140 mM NaCl, and 0.05% Tween 20 (pH 8.3); and buffer C, 20 mM Tris-HCl (pH 8.0), 138 mM NaCl, 1 mM PVP, 0.05% Tween 20, 3 mM KCl, and 3 mM NaN_3 per liter (pH 7.4).

Oligonucleotide primers and PCR amplification. For a single reaction of 50 μl , the PCR included 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl_2 , 0.2 mM deoxynucleoside triphosphate (dNTP), 1% Tween 20, 2.5 U *Taq* DNA Polymerase (Gibco BRL, Gaithersburg, MD), and 50 pmol of each primer. The reaction mix was overlaid with PCR-grade sterile mineral oil and subjected to amplification in a Touchgene Gradient thermal cycler (Techne, Cambridge, U.K.) programmed for 5 min at 94°C , followed by 40 cycles of 1 min at 94°C , 1 min at 55°C , and 2 min at 72°C , with a final extension of 5 min at 72°C (30).

The degenerate primers MP16 (5'-CCTCTAGATAATATTA-CCRKWKGRCC-3') and MP82 (5'-CGGAATTCYTGNACY-TTRCANGNCCYTCRCA-3') were designed to anneal to the invariable sequence of a nonanucleotide (TAATATTAC) located in the conserved stem-loop region and the eight invariable codons that are present in the 5' terminal domain of the begomovirus capsid protein gene, respectively. These primers were predicted to direct the amplification of a fragment of PYMV DNA-A consisting of approximately 400 bp (nucleotides 167 to 506) (30).

Optimization of Std-PCR. The effects of several additives to the general PCR mix were evaluated in an attempt to maximize amplification. Three additives—glycerol (20%), Tween 20 (1%), and dimethyl sulfoxide (5%)—and varying concentrations of MgCl_2 (2.5, 2.0, 1.5, and 1.0 mM) were tested. The combination of Tween 20 (1%) and 1.5 mM MgCl_2 in the PCR mix resulted in amplification of geminivirus fragments from highly diluted tomato extracts and was used in all subsequent PCR assays. Serial dilutions (2^0 to 2^{-10}) were prepared from extracts of PYMV-TT-infected tomato in each buffer, and 1 μl of each dilution was dispensed in 50 μl of PCR mix for amplification. The extracts were subjected to Std-PCR in at least two replications. The differences in detection limits (detection of the initial and end points of dilution) and amplified band intensity were recorded. Negative controls consisted of healthy tomato extracts diluted to 2^{-5} in buffer C.

Optimization of DB-PCR. With the optimum buffer identified in the previous experiment, the requirements for maximum adsorption of the viral template to microcentrifuge tube walls were determined by varying the incubation conditions in the adsorption phase of this method. Serial dilutions (2^0 to 2^{-10}) of extracts of infected tomato tissue were prepared in buffer C, and 50- μl aliquots of each dilution were dispensed in sterile 0.5-ml polypropylene microcentrifuge tubes, which were either incubated at 37°C for 2 h and then washed twice with enzyme-linked immunosorbent assay

(ELISA) wash buffer (Bioreba, Reinach, Switzerland), containing 138 mM NaCl, 1.5 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 3 mM KCl, 0.05% Tween 20, and 3 mM NaN_3 per liter (pH 7.4), or incubated at 4°C for 16 h and then washed three times. The tubes were then air-dried for approximately 15 min and subjected to PCR amplification to determine detection limits (initial and end points of dilution). Two replications were tested, to assess reproducibility. Negative controls consisted of extracts of healthy tomato leaves diluted to 2^{-5} in buffer C.

Optimization of IC-PCR. The effect of antibody concentration (1:500 and 1:1,000) and incubation conditions (37°C for 2 h versus 4°C for 16 h) in the precoat and capture phases were evaluated in factorial combinations (2×2) to determine the detection limits of PCR reactions using serial dilutions of tomato extracts in buffer C. The antibodies (Bioreba) used in the coating step are polyclonal, produced in rabbits and raised against a recombinant coat protein of cabbage leaf curl virus (CaLCV). In ELISA, the antibodies reacted with CaLCV, *Bean golden mosaic virus* (BGMV), and *Tomato mottle virus* (ToMoV). In Western blots, CaLCV, BGMV, ToMoV, *Tomato yellow leaf curl virus-Israel*, and *Sida golden mosaic virus* Florida isolate were recognized. For this optimization study, the antibodies were diluted to 1:500 and 1:1000 in precoat carbonate buffer (Bioreba), containing 15 mM Na_2CO_3 , 35 mM NaHCO_3 , and 3 mM NaN_3 (pH 9.6). Aliquots (50 μl) of the diluted antibody were dispensed in sterile 0.5-ml microcentrifuge tubes. The tubes were incubated at 37°C for 2 h and then washed twice with ELISA wash buffer or incubated at 4°C for 16 h and then washed three times. Infected tomato leaf tissue was ground in extraction buffer C in a ratio of 1:5 (wt/vol) and filtered through Miracloth, and serial dilutions of 2^0 to 2^{-20} were prepared. Aliquots (50 μl) of each dilution were dispensed in the antibody-coated tubes. The tubes were incubated for another 2 h at 37°C and then washed two times or incubated for 16 h at 4°C and then washed three times. The tubes were then dried by inversion for 15 min. The PCR mix (50 μl) was then added to each tube, and PCR amplification was performed. Two replications were carried out, to assess reproducibility. Negative controls consisted of healthy tomato extracts prepared and diluted to 2^{-5} in buffer C.

Comparison of Std-, DB-, and IC-PCR in assaying different plant species. With the optimized PCR protocols developed, a comparative evaluation was carried out with *L. esculentum*, *R. minima*, *C. mucunoides*, and *Sida* spp. infected with geminiviruses. Infected leaves of each plant species were ground in extraction buffer C (pH 7.4), and serial dilutions (2^0 to 2^{-20}) were prepared. The detection limits for each species were determined by subjecting the diluted extracts to the three PCR techniques. Negative controls consisted of extracts of healthy leaves of each species, ground and diluted to 2^{-5} in buffer C (this dilution was tested for the presence of nonspecific amplified band formation).

Analysis of PCR products. The PCR amplification products were analyzed in 1.4% agarose gels stained with ethidium bromide and photo-documented with the Imagestore 7500 Gel Documentation System (UVP, Cambridge, U.K.).

RESULTS

Optimization of Std-, DB-, and IC-PCR. Among the three extraction buffers tested with Std-PCR, amplification from the greatest number of dilutions (2^0 to 2^{-9}) occurred in buffer C, followed by buffer B (2^{-1} to 2^{-5}) and buffer A (2^{-2} to 2^{-5}). As a result, only buffer C was used with the other two methods.

In DB-PCR, there was a small improvement in detection from the lower dilution end point of tomato extracts (2^{-8} to 2^{-10}) when the incubation conditions were changed from 37°C for 2 h with two washes to 4°C for 16 h with three washes (the initial detection point remained unchanged, at 2^0). There was also some increase in band intensity over the first three dilutions under the latter incubation

conditions. However, the reproducibility was poor with respect to both detection limits and intensity of the amplified band, even for comparisons with the same dilutions (Table 1). Because of these results, DB-PCR was not used for the other test plants, except for *Sida* spp. Extracts of *Sida* spp. are very mucilaginous, and the high level of polysaccharides may affect the amplification reaction in Std-PCR (7). DB-PCR is intended to isolate the viral template from the rest of the extract through adsorption of viral DNA to the microcentrifuge tube wall, thereby improving amplification. However, results were again inconsistent for *Sida* spp., both for amplified band intensity and detection of the initial and end points of dilution.

Optimal conditions for IC-PCR assay of all plant species were an antibody concentration of 1:500 and overnight incubation (16 h at 4°C) followed by three washes with ELISA wash buffer. After incubation at 4°C for 16 h, the antibody dilution of 1:500 allowed detection at a considerably lower dilution end point (2^{-13}) and extended band intensity, compared to the 1:1,000 antibody dilution (lower dilution end point of 2^{-7}), while the initial detection point was 2^0 for both protocols. Preliminary studies using clarified leaf extracts of *Sida* spp. showed that an antibody dilution of 1:250 did not provide additional improvement in the detection limit (with dilutions of 2^{-10} to 2^{-20}), and hence this antibody dilution was not tested with extracts of other plant species. Incubation for 16 h at 4°C improved both the detection limit and the intensity of the amplified band with the 1:500 antibody dilution, indicating a more efficient capture of antigens by antibodies, compared with 2 h of incubation at 37°C. The bands obtained after the 16-h incubation were very distinct and of high intensity. Figure 1 shows the relative amplified band intensities obtained when the same tomato extract dilutions were subjected to PCR amplification with all three assays. Amplification products were not obtained for the negative controls.

Comparison of Std-, DB-, and IC-PCR in assaying different plant species. Table 2 lists the detection limits for *Sida* spp., *R. minima*, *C. mucunoides*, and *L. esculentum* when serial dilutions of each were subjected to the three optimized PCR-based assays. The lowest dilution in which detection was achieved by Std-PCR was for *L. esculentum* (2^0 , undiluted extract), and the greatest dilution was for *C. mucunoides* (2^{-10}). Viral templates in undiluted extracts of both *L. esculentum* and *C. mucunoides* were detected with the optimized IC-PCR protocol. IC-PCR detected viral templates at greater dilutions of extracts of all plant species under investigation (*L. esculentum* at a dilution of 2^{-10} , *Sida* spp. at 2^{-10} , and *R. minima* and *C. mucunoides* at 2^{-15}) than Std-PCR (*L. esculentum* and *R. minima* at 2^{-8} , *Sida* spp. at 2^{-9} , and *C. mucunoides* at 2^{-10}) and DB-PCR (*L. esculentum* at 2^{-8} and *Sida* spp. at 2^{-5} ; *R. minima* and *C. mucunoides* were not tested). Apart from differ-

ences in minimum and maximum detectable limits, which varied in a species-specific manner and according to the assay used for detection, the amplified band intensity also varied among the three assay techniques. IC-PCR consistently resulted in amplified band intensity superior to that obtained with the other two assays, irrespective of the plant species.

DISCUSSION

The use of clarified plant extracts in detecting viral DNA through PCR amplification provides several advantages. It elimi-

Fig. 1. Comparison of three methods for detection of begomoviruses by polymerase chain reaction (PCR). **A**, Direct-binding PCR. Lane 1: 1-kb DNA ladder; lanes 2 to 12: extract of infected tomato leaves diluted to 2^0 to 2^{-10} in buffer C (20 mM Tris-HCl, pH 8.0; 138 mM NaCl; 1 mM polyvinylpyrrolidone; 0.05% Tween 20; 3 mM KCl; and 3 mM NaN_3 per liter, pH 7.4); lane 13: extract of healthy tomato leaves diluted to 2^{-5} in buffer C. **B**, Standard PCR. Lane 1: 1-kb DNA ladder; lanes 2 to 12: extract of infected tomato leaves diluted to 2^0 to 2^{-10} in buffer C; lane 13: extract of healthy tomato leaves diluted to 2^{-5} in buffer C. **C**, Immunocapture PCR. Lane 1: 1-kb DNA ladder; lanes 2 to 12: extract of infected tomato leaves diluted to 2^0 to 2^{-10} in buffer C; lane 13: 1-kb DNA ladder; lane 14: extract of healthy tomato leaves diluted to 2^{-5} in buffer C. Amplified DNA fragments are approximately 400 bp.

TABLE 1. Detection of initial and end points of dilution and respective amplified band intensity obtained by standard, direct-binding, and immunocapture polymerase chain reaction (PCR) assays of tomato extract diluted 2^0 to 2^{-10} in buffer C^{a,b}

	Dilution											NC ^c
	2 ⁰	2 ⁻¹	2 ⁻²	2 ⁻³	2 ⁻⁴	2 ⁻⁵	2 ⁻⁶	2 ⁻⁷	2 ⁻⁸	2 ⁻⁹	2 ⁻¹⁰	
Standard PCR												
Replicate 1	+	++	+++	+++	+++	++++	++++	++	++	—	—	—
Replicate 2	+	++	+++	+++	+++	++++	++++	++	++	—	—	—
Direct-binding PCR ^d												
Replicate 1	+	+	+	—	—	—	—	—	—	—	—	—
Replicate 2	—	—	+	++	++	+	—	—	—	—	—	—
Immunocapture PCR ^{d,e}												
Replicate 1	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	—
Replicate 2	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	—

^a Band intensity: — = no amplified band; + = barely visible amplified band; ++ = visible amplified band; +++ = intense amplified band; ++++ = highly intense amplified band.

^b Buffer C contained 20 mM Tris-HCl (pH 8.0), 138 mM NaCl, 1 mM polyvinylpyrrolidone, 0.05% Tween 20, 3 mM KCl, and 3 mM NaN_3 per liter (pH 7.4).

^c Negative controls: extracts of healthy tomato leaves were prepared, diluted to 2^{-5} in buffer C, and amplified.

^d Diluted extracts were incubated at 4°C for 16 h.

^e Antibody concentration of 1:500.

nates the time-consuming steps of total nucleic acid isolation and purification, avoids possible inhibitory effects of co-isolated impurities on PCR amplification, and allows detection of viruses that occur in low titers (32). Std-PCR using clarified plant extracts is the least time-consuming, but it may be affected by inhibitory substances in the extracts.

We first optimized the PCR amplification in order to maximize sensitivity and reproducibility of detection of virus templates in crude extracts using degenerate primers (8,30). Annealing temperature, type and concentration of PCR additives, and MgCl₂ concentration were first adjusted to improve sensitivity and reproducibility. Sensitivity was assessed by the detection limits (detection of the initial and end points of dilution) and the intensity of amplified bands.

The type of buffer and capture conditions were varied to improve the sensitivity and reliability of detection by IC-PCR and DB-PCR. Extraction buffer C provided the greatest sensitivity and reproducibility. The extraction buffer may affect the stability of the viral particle, which usually exists over a narrow pH range (12,13). In instances in which immunoaffinity is the basis of detection, capsid integrity becomes especially important (13). The components of a successful buffer may also assist in releasing the virus from cell constituents, while protecting it from endogenous nucleases (29) and polyphenoloxidases present in plant extracts (7). Buffer components may also act as competitors to exclude inhibitors, such as tannins, in the extraction medium (12).

The efficacy of the optimized detection system was determined by subjecting serial dilutions of crude extracts of geminivirus-infected plants of different species to the optimized PCR-based protocol. IC-PCR had the lowest detection limits for all species and was superior to Std-PCR by one to seven orders of dilution. Depending on the species and the tissue specificity of certain begomoviruses (e.g., phloem limitations), detection may reflect the variation in the begomovirus titer in different species. This study indicates that IC-PCR may be the most sensitive tool for the detection of begomoviruses, and it may be particularly useful for those that are present in low or variable titers and for plant species that contain various levels of PCR inhibitors.

The efficiency of IC-PCR may be explained by its ability to concentrate the viral template in the extract by the selective affinity of the epitope-antibody complex during the capture phase. Wetzel et al. (32) and Rowhani et al. (26) found IC-PCR to be effective with low-titer viruses, such as *Plum pox virus*, and viruses of some woody plants. This efficiency may also stem from an ability to selectively eliminate PCR inhibitors during washing.

However, detection with IC-PCR was not achieved with undiluted plant extracts, with the exception of tomato and *C. mucunoides* (and with Std-PCR using tomato extracts only). These results may show that while excess PCR inhibitors may be eliminated during the washing step of the IC-PCR process, such compounds may hinder the capture phase of IC-PCR. For instance, it is plausible that the high mucilage content of extracts of *Sida* spp. may retard the interaction between antibody and antigen during the capture phase. Detection is then achieved only as the level of inhibitors is reduced (by dilution), so that a sufficient amount of antigen can be captured and subsequently amplified. Further dilution of extracts decreases the level of inhibitors, but it also decreases the concentration of the viral template. Std-PCR is more sensitive to these effects than IC-PCR (i.e., 1 µl of extract is added to the PCR mix for Std-PCR, compared with 50 µl of extract incubated for IC-PCR) in its detection limits and amplified band intensity, as confirmed by the results of this study.

DB-PCR gave inconsistent results for amplified band intensity and detection limits in extracts of infected tomato. Optimizing this assay would be difficult, because the precise mechanism by which the viral DNA is adsorbed to or immobilized on the solid support of the microcentrifuge tube wall is not well understood (26,33) and may vary according to the individual properties of each tube. Hence, it is understandable that we were unable to obtain consistent results with this method, despite varying the incubation conditions.

Diagnostic PCR should fulfill several qualitative characteristics, of which the most important are specificity, sensitivity, efficiency, and reproducibility (8,10,15,17). Deng et al. (8) designed degenerate primers to flank the 5' terminal region of the coat protein gene to specifically detect begomoviruses. Umaharan et al. (30) showed that amplification followed by sequencing of this region can be used to identify new begomoviruses. Here we show that specificity, selectivity, efficiency, and reproducibility in detecting begomovirus infection can be achieved by an optimized IC-PCR protocol using degenerate primers flanking the 5' terminus of the coat protein gene.

In summary, our results show that IC-PCR is the most efficient among the systems tested in detecting begomoviruses in different species. IC-PCR can be used for detecting and identifying begomoviruses in clarified plant extracts with degenerate primers, without prior isolation of total nucleic acids. This technique takes advantage of commercially available polyclonal geminivirus antisera and may lead to the development of sensitive and rapid single-tube assays.

TABLE 2. Amplified band intensity obtained by standard, direct-binding, and immunocapture polymerase chain reaction (PCR) assays of diluted clarified leaf extracts of different plant species^a

	Dilution															NC ^b	
	2 ⁰	2 ⁻¹	2 ⁻²	2 ⁻³	2 ⁻⁴	2 ⁻⁵	2 ⁻⁶	2 ⁻⁷	2 ⁻⁸	2 ⁻⁹	2 ⁻¹⁰	2 ⁻¹¹	2 ⁻¹²	2 ⁻¹³	2 ⁻¹⁴		2 ⁻¹⁵
<i>Sida</i> spp.																	
Standard PCR	—	—	—	—	+	+	+	+	+	+	—	—	—	—	—	—	—
Immunocapture PCR	—	—	++	+	+	++	+++	+++	++	++	++	—	—	—	—	—	—
Direct-binding PCR	—	—	—	—	++	+	—	—	—	—	—	—	—	—	—	—	—
<i>Rhynchosia minima</i>																	
Standard PCR	—	—	+	+	++	+	+	+	+	—	—	—	—	—	—	—	—
Immunocapture PCR	—	—	+	++	+++	++++	++++	++++	++++	+++	+++	++	++	++	++	++	—
<i>Calopogonium mucu-</i> <i>noides</i>																	
Standard PCR	—	—	—	+++	++++	++++	++++	++++	++++	++++	+++	—	—	—	—	—	—
Immunocapture PCR	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	++	++	++	—
<i>Lycopersicon esculentum</i>																	
Standard PCR	+	+++	++++	++++	++++	++++	++++	+	+	—	—	—	—	—	—	—	—
Immunocapture PCR	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	—	—	—	—	—	—
Direct-binding PCR	+++	+++	++	++	+	+	+	+	+	—	—	—	—	—	—	—	—

^a Band intensity: — = no amplified band; + = barely visible amplified band; ++ = visible amplified band; +++ = intense amplified band; ++++ = highly intense amplified band.

^b Negative controls: extracts of healthy tomato leaves were prepared, diluted to 2⁻⁵ in buffer C (20 mM Tris-HCl, pH 8.0; 138 mM NaCl; 1 mM polyvinylpyrrolidone; 0.05% Tween 20; 3 mM KCl; and 3 mM NaN₃ per liter, pH 7.4), and amplified.

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