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SHORT COMMUNICATION / *COMUNICAÇÃO*

Detection of phytoplasma in desiccated tissue of *Momordica charantia*, *Catharanthus roseus* and *Sechium edule*

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ABSTRACT

Phytoplasmas can cause yield losses in numerous crops worldwide. In Brazil, phytoplasmal diseases have been reported in plant species, distributed among 29 botanical families. Chayote witches'-broom (ChWB) is reported as one of the diseases of economic importance, associated with phytoplasma in the country, and bitter melon is a natural alternative host of the phytoplasma associated with the disease. Periwinkle is an ornamental plant that can naturally host phytoplasmas. An attempt to detect phytoplasma in desiccated leaf tissues collected from naturally diseased chayote, bitter melon and periwinkle plants was made. On the basis of phytoplasma-specific DNA amplification in PCR, phytoplasma was detected in desiccated chayote and bitter melon samples affected by witches'-broom, as well as in desiccated periwinkle exhibiting chlorosis and small leaves. DNA extraction was achieved using 0.25 g and 0.5 g of bitter melon and periwinkle dessicated tissue. These results encourage trials in utilizing dried plant tissues for the detection of phytoplasmas in other plant species, since dried samples are easy to preserve and transport, and yield good amounts of DNA.
Key words: bitter melon, periwinkle, chayote, DNA, witches'-broom.

RESUMO

Detecção de fitoplasma em tecido dessecado de *Momordica charantia*, *Catharanthus roseus* e *Sechium edule*

Fitoplasmas causam severas perdas na produção em diversos cultivos de importância econômica, no mundo. No Brasil, há relatos de doenças associadas a fitoplasmas em espécies vegetais distribuídas em 29 famílias botânicas. O superbrotamento do chuchuzeiro, também conhecido como "irizado" é considerado uma das principais enfermidades de natureza fitoplasmática no Brasil e o melão-de-São-Caetano é um hospedeiro alternativo natural do fitoplasma associado à doença. Vinca é uma planta ornamental, hospedeira natural de fitoplasmas. O presente trabalho objetivou detectar fitoplasma em tecido foliar dessecado, a partir de plantas de chuchuzeiro, melão-de-São-Caetano e vinca, naturalmente infectadas. Através de amplificação, por PCR, do 16S rDNA detectou-se a presença de fitoplasma em amostras dessecadas de chuchuzeiro e de melão-de-São-Caetano com superbrotamento, bem como de vinca apresentando clorose e folhas de tamanho reduzido. Para a obtenção do DNA dos tecidos dessecados foram utilizados 0,25g e 0,5 g de amostras de melão-de-São-Caetano e vinca. Os resultados obtidos demonstram o potencial da utilização de tecido vegetal dessecado na detecção de fitoplasmas associados a outras espécies botânicas, pois amostras de tecido seco apresentam facilidade de preservação e de transporte, além de fornecerem boa quantidade de DNA.
Palavras-chave: melão-de-São-Caetano, vinca, chuchu, DNA, superbrotamento.

Phytoplasmas are plant pathogens that cause yield losses in numerous crops worldwide (Bertaccini, 2007). In Brazil, the diseases associated with phytoplasmas have been reported in diverse hosts, comprising wild, weed and cultivated species (Montano et al., 2007). *Momordica charantia* L., known as bitter melon or bitter gourd, is easily found growing over fences, near commercial crops. In Brazil, bitter melon is a natural alternative host of the phytoplasma associated with chayote witches'-broom disease (ChWB). Diseased plants exhibt yellowing, reduction in size of leaves and witches' broom (Montano et al., 2000).

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Catharanthus roseus (L.) G. Don is an herbaceous plant with ornamental and medicinal importance. In Brazil, there are reports of periwinkle naturally infected with phytoplasma (Barros et al., 1998; Bedendo et al., 1999; Montano et al., 2001a, b). Chayote (*Sechium edule* (Jacq.) Sw.) is an economically important vegetable crop grown in several states of Brazil (Makishima, 1991). The productivity and quality of chayote can be markedly reduced by ChWB. Besides witches'-broom growths, diseased plants can exhibit generalized stunting and yellowing, leaf malformation, flower and fruit drop, as well as fruit malformation (Kitajima et al., 1981; Montano et al., 2000). In Brazil, the diseases associated with phytoplasmas have been reported in a wide range of families and there is the likelihood of new disease records, determined by the diversity of wild and cultivated

plant species, and by climatic conditions (Kitajima, 1994; Davis, 1995).

Since Brazil is a country of continental dimension, and new diseases outbreaks may occur in remote locations, preserved tissue material could be useful to eventual detection of associated phytoplasma diseases, and the establishment of control measures. Several methods have been reported for preservation of phytoplasmas, including maintenance in plant tissue cultures, in living host plants, in leafhopper vectors under –70ºC, and freeze drying as traditional methods for preservation. These methods, sometimes, are not suitable for exchanging phytoplasma-infected tissues with other research centers (Wang & Hiruki, 1998). However, the information concerning the use of preserved infected tissue for the isolation of phytoplasma DNA is very scarce. Therefore, a simple, reliable, and practical method needs to be developed for maintenance of phytoplasmas for future research. Thus an attempt to detect phytoplasma in desiccated leaf tissues collected from naturally diseased chayote, bitter melon and periwinkle plants was carried out.

Leaves of bitter melon were collected from naturally diseased plants exhibiting symptoms of witches'-broom, in Seropédica, State of Rio de Janeiro. Symptomatic leaves of periwinkle were sampled from plants exhibiting chlorosis, grown in the *Campus* of the Universidade Federal Rural do Rio de Janeiro. Leaves of chayote exhibiting symptoms of ChWB were collected in the location of Mendanha, State of Rio de Janeiro. Leaf midribs or whole small leaves were excised from diseased plants of bitter melon (10 plants/10 samples), periwinkle (five plants/five samples) and chayote (10 plants/10 samples). Each sample was disinfected in 1% sodium hypochlorite solution, 1 g of leaf tissues was cut, with sterilized scissors, into pieces of approximately 2 cm^2, placed in sterile Petri dishes and submitted to 60 mm^2 Hg vacuum, for two minutes, in a flask desiccator, containing 5 cm silica gel layer.

After seven days, dehydrated tissues were reduced to a fine powder, collected into 1.5 mL tubes, and stored at –20ºC in a flask with a layer of silica gel for 31 days. Fresh leaves of asymptomatic chayote plants were collected and submitted to the same procedure. Fresh leaves of naturally diseased bitter melon, periwinkle and chayote were used as controls. In another dehydration process tested in this work - a herborization-like procedure -plant material collected in the field was spread flat on sheets of newsprint, and dried at room temperature. Instead of a plant press, newsprint sheets filled with the samples were topped with a lightweight to ensure water transfer, from plant tissue to the sheets. Leaf samples of symptomatic bitter melon and periwinkle collected from the same locations above mentioned were kept for eight days, under dehydration process. In order to compare the DNA efficiency yield from desiccated tissues, fresh samples of naturally diseased bitter melon, periwinkle and chayote were used in the present work, after disinfestations.

Total DNA was extracted following Montano et al. (2000) and phytoplasma 16S rDNA was further amplified in PCR primed by universal primer pairs P1/P7 (Deng & Hiruki, 1991; Schneider et al. 1995) and R16F2n/R2 (Gundersen & Lee, 1996) in nested PCR assays. DNA fragment size standard was 1-kb-ladder (Invitrogen). Negative controls consisted of reaction mixtures devoided of templates and desiccated leaf tissues of healthy chayote plants. PCR products were analyzed by electrophoresis through 1% agarose gel, staining with ethidium bromide, and visualization of DNA bands using a UV transilluminator.

On the basis of phytoplasma-specific DNA amplification in PCR, phytoplasmas were detected in all of the desiccated tested samples, exhibiting symptoms of phytoplasma infection. Samples from diseased bitter melon, periwinkle and chayote submitted to desiccation with silica gel and herborization, as well as fresh samples collected from bitter melon and periwinkle contained phytoplasma DNA, which was amplified with universal primer pairs, yielding products of approximately 1.2 kb. Samples from desiccated asymptomatic chayote did not yield amplicons. Nested PCR products, amplified with universal primer pairs P1/P7 and F2n/R2, from bitter melon and periwinkle tested samples and controls is shown in Figure 1. Bitter melon samples desiccated with silica gel (MD1 and MD2) were positive for the presence of phytoplasma, the same result observed for bitter melon fresh sample (MF). All periwinkle samples dessicated with silica gel (CS) or herborized (CN), and fresh tissue samples (CF) gave positive results for the presence of phytoplasma. Bitter melon samples herborized during eight days were positive for the presence of phytoplasma (data not shown). Figure 2 shows nested PCR products, amplified with universal primer pairs P1/P7 and F2n/R2, from chayote tested samples. Symptomatic samples dessicated with silica gel (SE) resulted positive for phytoplasma, as well as the positive control obtained from fresh chayote sample (P).

In this work, the use of dehydrated leaf tissue with silica gel and a herborization-like procedure provided the isolation of good-quality 16S rDNA of phytoplasma, for PCR assays. Dehydration of bitter melon, periwinkle and chayote infected with phytoplasma showed no detectable effect on the stability of phytoplasma DNA. As for bitter melon and periwinkle samples submitted to dehydration for eight days, using the procedure similar to herborization, the phytoplasma DNA kept its integrity, what was confirmed after the PCR assay. However, bitter melon samples submitted to the same kind of desiccation, for 21 days, did not yield amplicons. This could be attributed to the longer period of desiccation that the samples were submitted or it was probably due to the changing in DNA molecular structure.

This finding is coincident with the reported by Wang & Hiruki (1998), in paulownia tissues infected with the witches' broom phytoplasma, submitted to air-drying during four weeks. These authors suggest that the genomic

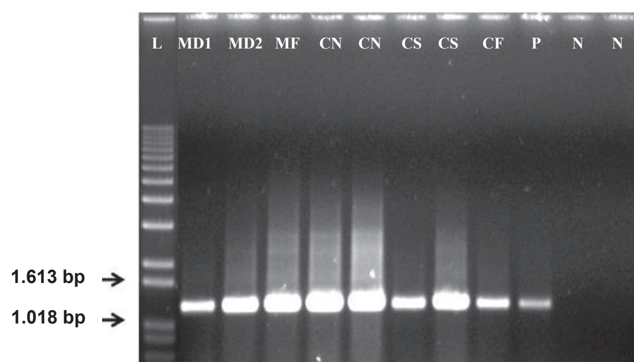

FIGURE 1 - Nested PCR detection of phytoplasmas in desiccated and fresh tissues, primed by universal primer pairs P1/P7 and R16F2n/R2. **L:** DNA size standard 1 kb-DNA-ladder (Invitrogen). ***Momordica charantia*: MD1** – 0.5 g tissue dessicated with silica gel; **MD2** – 0.25 g tissue dessicated with silica gel; **MF** – 1 g fresh tissue. ***Catharanthus roseus*: CN** – 0.5 g tissue dessicated with herborization-like procedure; **CS** – 0.25 g tissue dessicated with silica gel; **CF** – 1 g fresh tissue. **P:** positive control, *Dimorphandra gardneriana* phytoplasma. **N:** negative control, reaction control devoid of DNA template.

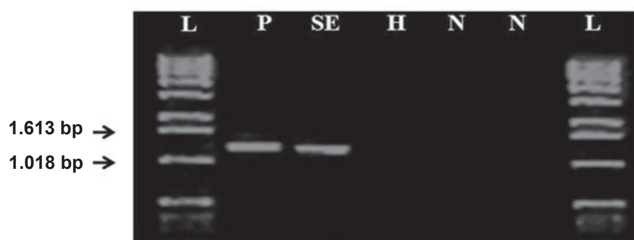

FIGURE 2 - Nested PCR detection of phytoplasmas in desiccated tissue of chayote (*Sechium edule*) primed by universal primer pairs P1/P7 and R16F2n/R2. **L:** DNA size standard 1 kb- DNA-ladder (Invitrogen). **H:** Desiccated asymptomatic chayote sample. **SE:** Desiccated samples from diseased chayote. **N:** reaction control devoid of DNA template, negative control. **P:** ChWBIII phytoplasma from chayote fresh tissue, positive control.

DNAs were damaged during the air-drying of samples or, alternatively, bound to host cellular components or both. In the case of bitter melon and periwinkle dehydrated with silica gel, the amplicons were identical to those formed with DNA from fresh samples, which were used as controls. Interestingly, it was observed that for bitter melon samples desiccated with silica gel, amplicons were obtained from 0.25 g, as well as from 0.5 g of dried tissues. The amplified band from 0.25 g tissue (MD2) is stronger than the band seen from 0.5 g sample (MD1). This finding could be explained by the fact that pelleted phytoplasma DNA of sample MD2, was diluted with the same volume of TE buffer than sample MD1, thus provinding a more diluted DNA suspension and, consequently, less amount of inhibitors of *Taq* polymerase enzyme, in PCR assays. Dehydration with silica gel provided good phytoplasma DNA from chayote infected samples, what was demonstrated after nested PCR with universal primer pairs. The advantage of using dried infected material relies on the fact that dehydrated suspicious samples could be easily handled and preserved, especially in the case of remote sampling locations, for the detection of associated phytoplasmas. The use of preserved samples infected with phytoplasma for molecular diagnosis and analysis was reported by Khadair et al. (1995) and by Wang & Hiruki (1998), who adopted thermal treatment for infected tissue dehydration.

The results obtained in the present work encourage trials in utilizing plant tissues dried with silica gel or with a herborization-like procedure, for the detection of phytoplasmas in other plant species, since dried samples are easy to preserve and transport, and yield good amounts of DNA, as also reported in other works (Bult et al., 1992; Thomson & Henry, 1993; Khadair et al., 1995; Wang et al., 1996; Wang & Hiruki, 1998). In the case of molecular diagnosis and analysis of phytoplasmas, the use of fresh plant material is largely employed, and the reports regarding the adoption of dried plant samples seem to be scarce. The use of dehydrated plant samples will offer a suitable method for preservation of phytoplasma DNA, which would be useful for molecular diagnosis of associated diseases in remote areas, and also for safe international exchange of experimental materials without violating quarantine regulations.

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