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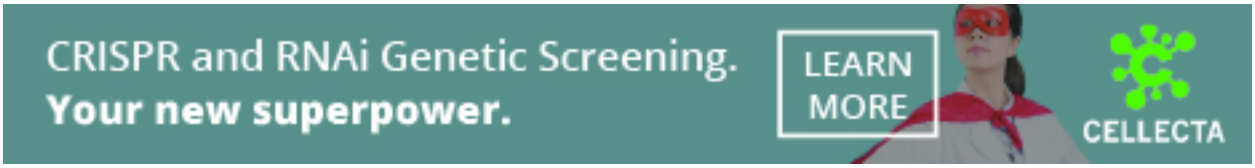

# A DNA Extraction Method that Allows Reliable PCR Amplification of MLO DNA from "Difficult" Plant Host Species

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Molecular techniques have facilitated the study of plant pathogenic mycoplasma-like organisms (MLOs) in the last few years, and PCR has been used to detect MLOs in infected plant and insect tissue.[1–3] Where very little is known about MLOs and wide-scale screening of plant host species is required, or quarantine restrictions prevent the propagation of infected plant material, it is essential that a "universal DNA extraction method" be developed that can be applied to a range of plant host species to give good quality DNA suitable for molecular applications such as PCR. Some of these species may contain high levels of polyphenolics, making it difficult to obtain good quality DNA.[4] To study MLOs in Australia, a screening program was initiated in which a range of plant host species with suspected MLO-associated diseases were collected. A DNA extraction method was needed to obtain DNA suitable for use in PCR. A number of techniques have been published for extraction of MLO DNA.[1–3,5,6] One of the problems we encountered was that DNA extracted by some of these methods deteriorated with time when stored at −20°C. Furthermore, DNA extracted from some plants with classical MLO-associated disease symptoms did not give an amplified product using PCR. This posed significant problems for long-term genetic studies using stockpiled DNA as well as severe limitations for a screening program in which a wide range of plant host species have to be accommodated. The method described here gave DNA that produced consistent results in PCR after storage at −20°C. It also allowed the detection of MLO DNA by PCR from plant host species that had symptoms but were consistently negative using DNA extracted by other methods. Our method is based on modified protocols and combines MLO enrichment and maintenance in an osmotically supplemented buffer, with polyvinylpyrrolidone (PVP) to complex polyphenolic compounds[7] followed by selective precipitation of nucleic acids using cetyltri-methylammonium bromide (CTAB) and sodium chloride.[3]

## MATERIALS AND METHODS

Leaf midribs, petioles, and young stems (5 grams) were sliced, using a single-edge blade, into 0.2–0.5-cm lengths, placed in a prechilled mortar and pestle, and ground in 20 ml of ice-cold isolation medium (0.1 M $Na_2HPO_4$, 10% sucrose, 2% PVP-40 at pH 7.6 to which 0.15% bovine serum albumin and 1 mM ascorbic acid were added just before use). The extract was filtered through cheesecloth and centrifuged (1500*g* for 5 min at 4°C) to help remove plant nuclei, chloroplasts, and other plant debris. The supernatant fluid was centrifuged (18000*g* for 25 min at 4°C) to pellet MLOs and plant organelles of similar size, such as mitochondria, and the pellet was resuspended in 20 ml of Tris–sucrose (TS) buffer (20 mM Tris-HCl at pH 8.0, 10% sucrose) by gentle pipetting. The resuspension was centrifuged again (1500*g* for 5 min at 4°C) to remove residual plant debris, and the supernatant fluid was transferred to a clean tube and centrifuged (18000*g* for 25 min at 4°C) to pellet MLOs and similar sized plant organelles. The pellet was resuspended in 800 µl of extraction buffer (100 mM Tris-HCl at pH 8.0, 100 mM EDTA at pH 8.0, 250 mM NaCl) containing proteinase K at 100 µg/ml and Sarkosyl (10% solution) to a final concentration of 1%. The extract was incubated for 1–2 hr at 55°C with occasional mixing. Ice-cold isopropanol (0.6 volume) was added to the extract, mixed gently, and left either for 1 hr or overnight at −20°C. The extract was centrifuged (7500*g* for 15 min at 4°C), and the pellet, which at this stage was still green and required further processing to remove plant compounds, was resuspended in 9 ml of TE buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA at pH 8.0) to which was added proteinase K (to give 100 µg/ml) and SDS (to give a final concentration of 0.5%). The extract was mixed thoroughly and incubated for 1 hr at 37°C, after which 1575 µl of 5 M NaCl and 1260 µl of CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) were added, mixed thoroughly, and the extract incubated for 10 min at 65°C. The sample was then extracted with an equal volume of chloroform/isoamyl alcohol (24:1) followed by extraction with an equal volume of TE-saturated phenol/chloroform/isoamyl alcohol (25:24:1). The nucleic acid was precipitated from the supernatant fluid by the addition of 0.6 volume isopropanol at −20°C overnight and pelleted by centrifugation (7700*g* for 10 min at 4°C). The pellet was washed with 2 ml of ice-cold 70% ethanol and resuspended in TE buffer. Average yield was 30–40 µg of DNA/100 mg fresh weight.



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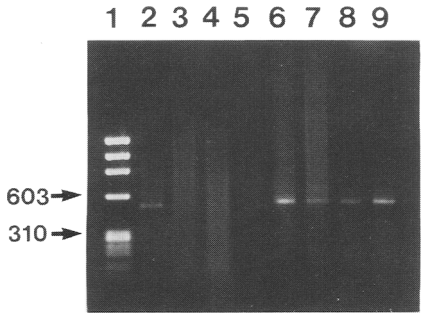


**FIGURE 1** The modified method for DNA extraction allowed the detection of MLO DNA from some host species that gave negative results when the DNA was extracted by published methods 1 and 2 as described in the text. MLO DNA was detected by amplification of a 500-bp region of the 16S rRNA gene using PCR and visualization of products (5 μl) by agarose gel electrophoresis (80 V, 1 hr, 1.5% agarose) followed by staining with ethidium bromide. (Lane *1*) *Hae*III-digested ϕX174 DNA; (lanes *2–4*) DNA extracted by method 2 from sweet potato, *E. sonchifolia*, and *Stylosanthes* sp., respectively; (lane *5*) DNA extracted by method 1 from *Evolvulus* sp; (lanes *6–9*) DNA extracted by the modified method from the same plant host species as above. In all tests, a healthy sweet potato DNA extract was included and no product was amplified in PCR.

## RESULTS AND DISCUSSION

This method has been used successfully for different plant species, including sweet potato, lettuce, tomato, soybean, chick-pea, bean, passionfruit, papaya, and a range of weed species. An ornamental *Evolvulus* sp. and two weeds, *Emilia sonchifolia* and *Stylosanthes* sp., showed typical little-leaf symptoms but were negative by PCR screening when the DNA was extracted by other published methods hereafter referred to as method 1[(2)] and method 2.[(3)] When these same host species were extracted by the modified method described here, they were positive in PCR (Fig. 1). The modified method, which can be used with "difficult hosts" such as these, is very useful in enhancing the reliability and range of a screening program. The quality of DNA extracted from infected sweet potato plants, using the three different methods and stored at −20°C, was tested using PCR. DNA extracted from sweet potato plants with little-leaf symptoms by methods 1 and 2 and the modified method was used immediately in the PCR with primers that amplified a 500-bp region of the 16S rRNA gene.[(8)] All three preparations gave 500-bp products when analyzed by gel electrophoresis. The DNA was stored for 2 months and subjected to PCR. This time, no PCR product was observed using DNA extracted by method 1. PCR products were, however, observed using DNA extracted by method 2 and the modified method (Fig. 2). The ability to store DNA successfully and to amplify PCR products from difficult hosts may be affected by inhibitors copurified with host plant DNA. An experiment was done to study the role of inhibitors in the amplification of MLO DNA that had been stored at −20°C. DNA that had been extracted from a healthy sweet potato by each of the three methods and stored for >2 months was spiked with 20 ng of DNA freshly extracted from an infected sweet potato, before being subjected to PCR. Regardless of the method used to extract the DNA, a 500-bp PCR product was observed when this "healthy DNA" template was spiked with DNA from an infected sweet potato (Fig. 3). No 500-bp PCR product was observed when the template was healthy DNA only (Fig. 3).

Both method 2 and the modified method described here allowed the preparation of DNA that stored well for PCR amplification and genetic studies. The modified method also allowed the detection of MLO DNA from a wider range of plant host species than was possible using other DNA extraction methods. DNA extracted from healthy sweet potato by any of the three methods and stored for >2 months did not inhibit PCR when this healthy DNA was spiked with DNA freshly extracted from an infected sweet potato. This indicates that under these experimental conditions, compounds copurified with the DNA were probably not responsible for the inability to amplify MLO DNA extracted by method 1 after it was stored for 2 months at −20°C. This does not exclude the possibility that inhibitors were copurifed with other plant host species, because only sweet potato was tested here. It is often not clear whether unsuccessful amplification in the PCR is the result of poor quality template, the presence of inhibitors, or a combination of both factors. Regardless of the reason, however, any method that overcomes the problem is invaluable in a screening program.

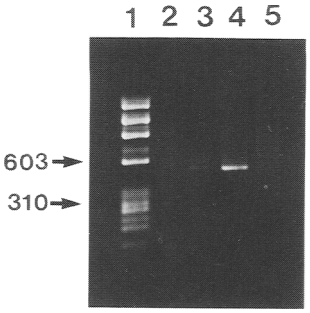


**FIGURE 2** The quality of DNA extracted from sweet potato showing "little-leaf" symptoms by the three methods was tested after it was stored at −20°C for 2 months. The DNA used was prepared by method 1 (lane *2*), method 2 (lane *3*), or the modified method described in this paper (lane *4*). Lane *5* is a healthy sweet potato control using DNA extracted by the modified method. MLO DNA was detected by amplification of a 500-bp region of the 16S rRNA gene using PCR and visualization of products by agarose gel electrophoresis (conditions as for Fig. 1). (Lane *1*) *Hae*III-digested ϕX174 DNA.

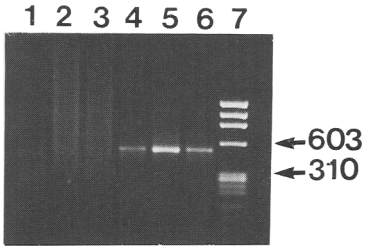


**FIGURE 3** The presence of inhibitors of PCR in DNA extracted by each of the three methods was tested by spiking healthy DNA template with DNA extracted from an infected plant. DNA (20 ng) extracted from a healthy sweet potato by methods 1, 2, or the modified method and stored for >2 months was subjected to PCR in the absence (lanes *1–3*, respectively) and the presence (lanes *4–6*, respectively) of 20 ng of DNA freshly extracted from an infected sweet potato by the modified method. No PCR product was observed when healthy DNA alone was the template; however, regardless of the extraction method used, healthy DNA spiked with DNA extracted from an infected plant gave a 500-bp PCR product that was visualized by agarose gel electrophoresis (conditions as for Fig. 1). (Lane *1*)) *Hae*III-digested ϕX174 DNA.

## ACKNOWLEDGMENTS

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