Attenuation of PCR inhibition in the presence of plant compounds by addition of BLOTTO

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Amplification of DNA extracted from complex milieus such as soil and plants by the polymerase chain reaction (PCR) is often limited by the presence of compounds inhibitory to the reaction (4). Inhibition is attributed to humic substances in soil which adsorb to various organic substances and form complexes with metal ions (1). Simple dilution of sample attenuates inhibition but also decreases test sensitivity when DNA template is a limiting factor (5). We encountered inhibition of PCR in experiments to detect low levels of plant pathogenic bacteria and fungi in plant tissue. The inhibition was probably due to plant polyphenolic molecules in the extracts. We found that the addition of non-fat milk eliminated the inhibitory effect in all of our samples.

Non-fat milk cocktails, termed BLOTTO (Bovine Lacto Transfer Technique Optimizer; 3), have been widely used to prevent non-specific binding of proteins and nucleic acids to nitrocellulose in Western and Southern blotting procedures. BLOTTO is also used to inhibit non-specific binding in enzyme-linked immunosorbent assays (2).

In our experiments we extracted DNA from potato tuber tissue to detect Erwinia carotovora subsp. atroseptica and Clavibacter michiganensis subsp. sepedonicus the causal agents of the blackleg and ring rot diseases of potato, and cucumber roots for the presence of the damping-off pathogen, Pythium spp. Portions of potato tissue (0.5–2.0 g) were crushed and shaken with 1 ml of sterile distilled water in zip-lock plastic bags. One ml of fluid was removed and centrifuged in an Eppendorf tube at 17 300 g for 20 min and the pellet resuspended in 100 μl of Tris–EDTA buffer (pH 8.0) containing 1% (w/v) SDS. The EDTA concentration was increased to 50 mM and the samples treated with Proteinase K at 10 μg/ml at 50°C for 3 h. Then one half volume of 7.5 M ammonium acetate was used to precipitate protein debris in the sample. Subsequently, DNA was precipitated from the supernatant fraction with 1 vol of isopropanol and then washed with 70% ethanol. Cucumber root samples were processed in a similar manner but after Proteinase K digestion they were treated with RNase and extracted with phenol/chloroform rather than being clarified by ammonium acetate treatment. In each case the DNA fraction was dried at 58°C for 10 min, resuspended in 50 μl purified water and heated to 50–55°C prior to PCR.

PCR for each pathogen was done in 20 μl vol in a mix consisting of 0.5 μM of each of two primers specific for the target pathogen, 100 μM of each dNTP, 2.5 or 5 mM Mg2+ and 0.5 U Taq polymerase. One μl of template, usually consisting of a crude DNA extract, was added to the mix. The effect of adding 2% (v/v) BLOTTO (10% skim milk powder and 0.2% NaCl3) to the PCR mix of DNA extracts from potato for detecting E. carotovora subsp. atroseptica was shown in Figure 1. Amplification products were only detected in those reactions to which BLOTTO had been added. Similar results were obtained with potato tissue extracts for detection of C.m.sepedonicus to which 2.5% (v/v) BLOTTO had been added (Fig. 2) and for Pythium spp. in cucumber root extracts to which 1% (v/v) BLOTTO had been added (data not shown). The optimum concentration of BLOTTO was in the range of 1–5% and varied with the batch of Taq polymerase being used. In all cases yield of PCR products was reduced with BLOTTO concentrations >5%.

To determine whether the presence of any sacrifice protein would attenuate PCR inhibition, bovine serum albumin and acid hydrolyzed casein were also tried instead of BLOTTO at comparable concentrations but they did not eliminate the inhibitory effect. Since polyphenols could inhibit PCR by sequestering Mg2+,

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Figure 1. Gel electrophoresis of PCR products obtained with primers specific to E.carotovora subsp. atroseptica and using potato tuber extract as template. After an initial 5 min treatment at 95°C, PCR conditions for 40 cycles were: denaturation at 94°C for 30 s, annealing at 62°C for 45 s and extension at 72°C for 45 s. An 8 min extension period was given in the final cycle. Lanes 2, 4, 9, 11 and 13 were loaded with PCR products from five different potato samples in which PCR amplification was apparently inhibited since these samples were all positive in an enzyme-linked immunosorbent assay using a monoclonal antibody specific for the bacterial pathogen. Lanes 3, 5, 8, 10 and 12 were loaded with PCR products from the same five potato sample extracts but to which 2% (v/v) BLOTTO had been added to the PCR mix. Lane one has a 100 base pair ladder (Bethesda Research Laboratories, Burlington, Ont.). Lane 6 is a negative control to which no template DNA was added, and lane 7 is the same negative control but with 2% (v/v) BLOTTO added to the PCR mix.
increasing the concentration of Mg\(^{2+}\) was also tested but this did not attenuate inhibition.

Addition of BLOTTO to the PCR mix for amplifying DNA in environmental samples may be a generally useful means of eliminating the effect of inhibitory compounds that are co-extracted with the DNA fraction. The mechanism of attenuating the inhibition, however, is not understood.

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**REFERENCES**