

Rothamsted Repository Download

A - Papers appearing in refereed journals

Mutasa-Göttgens, E. S., Chwarszczynska, D. M. and Asher, M. J. C. 1996. Single-tube, nested PCR for the diagnosis of *Polymyxa betae* infection in sugar beet roots and colorimetric analysis of amplified products. *Phytopathology*. 86 (5), pp. 493-497.

The publisher's version can be accessed at:

- <https://dx.doi.org/10.1094/Phyto-86-493>

The output can be accessed at: <https://repository.rothamsted.ac.uk/item/876xw>.

© 1 May 1996, American Phytopathological Society (APS).

Single-Tube, Nested PCR for the Diagnosis of *Polymyxa betae* Infection in Sugar Beet Roots and Colorimetric Analysis of Amplified Products

E. S. Mutasa, D. M. Chwarszczynska, and M. J. C. Asher

IACR Broom's Barn, Higham, Bury St. Edmunds, Suffolk IP28 6NP, U.K.

The work was financed by the U.K. Sugar Beet Industry through the Sugar Beet Research and Education Committee.

We thank G. Williams and M. Le Bloa for technical assistance; M. Acaster for supplying sterile sugar beet seedlings; M. Adams of IACR-Rothamsted for *P. graminis*, *O. brassicae*, *P. brassicae*, and *Ligniera* sp. isolates; R & D Systems Europe Ltd. for providing Captagene-GCN4 kits; and R. Rowling for invaluable technical advice.

Accepted for publication 22 January 1996.

ABSTRACT

Mutasa, E. S., Chwarszczynska, D. M., and Asher, M. J. C. 1996. Single-tube, nested PCR for the diagnosis of *Polymyxa betae* infection in sugar beet roots and colorimetric analysis of amplified products. *Phytopathology* 86:493-497.

Nested primers for the specific amplification of DNA sequences from the obligate parasitic root-infecting fungus *Polymyxa betae* in a single-tube reaction are described. The choice of primers, DNA purity, and relative concentration of outer to inner primers were critical to the success of single-tube reactions. The polymerase chain reaction (PCR) test discriminated against background DNA from the host plant and contam-

inating microorganisms and detected *P. betae* in as little as 1 pg of total genomic DNA from infected roots. For rapid analysis of amplified products, primers were modified to generate products that could be detected in a colorimetric assay with the commercially available Captagene-GCN4 kit. It was essential to design a PCR protocol that reduced primer dimerization to levels that did not lead to high background absorbance readings. Results from the Captagene-GCN4 test were compared to those obtained by agarose gel analysis of PCR products.

Additional keywords: beet necrotic yellow vein virus, obligate parasite, plant breeding, rhizomania.

The fungus *Polymyxa betae* Keskin is an obligate parasite of sugar beet roots and the vector of beet necrotic yellow vein virus (BNYVV) that causes rhizomania (16), a very damaging (13) and highly infectious disease. The virus is transmitted in small amounts of soil in which it survives for many years protected by the fungal resting spores (14). *P. betae* is widespread in sugar beet-growing areas and, in the absence of BNYVV, is relatively harmless, especially in temperate, cool climates (3). For some time there has been a growing interest in developing sugar beet cultivars that are resistant to the virus and, more recently, to *P. betae* (1), as the only effective way of controlling the disease.

Antiserum to BNYVV has been developed (5,17) and is widely available for screening plant material. However, because of its obligate parasitic habit and the difficulty of purifying *P. betae*, there has not been an equivalent diagnostic tool for the fungus until very recently. A specific DNA probe (pPbKES-1) has been cloned (12) and used for the detection of *P. betae* in sugar beet roots by Southern and dot blot analyses. Subsequently, polymerase chain reaction (PCR) primers were developed for improved sensitivity and faster detection of the fungus and have been used successfully in a field trial (10). The availability of specific PCR primers for *P. betae* provides the opportunity to develop fast, accurate diagnostic tests for the fungus, with the levels of sensitivity required for reliable selection of resistant plants in breeding programs.

In this paper, we describe a new set of improved PCR primers that allows amplification of *P. betae* DNA sequences in a single-tube nested reaction. The previous set of nested primers (10) only allowed nested amplification in two separate reactions, requiring the transfer of amplified products from the first round into a sec-

ond reaction tube for amplification with the nested primers. Single-tube nested reactions that do not require this additional step have the advantage of being less liable to carry-over contamination. We also demonstrate the specificity and sensitivity of the new PCR test and discuss some of the technical problems experienced in its development. Finally, detection of products by the Captagene-GCN4 (supplied by R & D Systems Europe Ltd., Abingdon, England) colorimetric assay kit was evaluated and compared with agarose gel electrophoresis.

MATERIALS AND METHODS

Fungi. The main *P. betae* isolate (BB-1) used was obtained from naturally infested field soil (New Piece field, IACR Broom's Barn, Higham, Bury St. Edmunds, Suffolk, England), using bait plants. Other isolates were from field soils collected from various locations in the United Kingdom: Humberside (BB-5), Suffolk (BB-6), Norfolk (BB-8), Bedfordshire (BB-10), and South Yorkshire (BB-13). Isolates of *Polymyxa graminis*, *Olpidium brassicae*, *Plasmodiophora brassicae*, and *Ligniera* sp. were supplied from the IACR-Rothamsted collection by M. Adams. *Pythium ultimum* and *Aphanomyces cochlioides* were isolated from sugar beet roots as previously described (10).

Plant infections. Artificial infection of sugar beet seedlings with infested soil and fungal zoospores was carried out as described by Barr and Asher (2). Naturally infected seedlings were collected from fields in East Anglia.

DNA extraction. Purified DNA from infected sugar beet roots, fungal resting spores, zoospores, and mycelia was extracted for 1 h at 65°C in cetyltrimethylammoniumbromide high-salt buffer (19). After phenol/chloroform extraction, the DNA was ethanol precipitated in 0.3 M sodium acetate and redissolved in 1× Tris-EDTA buffer, treated with RNase A, reextracted with phenol/chloroform, precipitated with ethanol, washed in 70% ethanol, air-

Corresponding author: E. S. Mutasa; E-mail address: mutasa@bbsrc.ac.uk

dried, and dissolved in sterile distilled water and was ready for use. The full and detailed protocol has been described elsewhere (11). Crude DNA was extracted from crushed roots by boiling in 1 M Tris, pH 8.0, for 10 min as described by Mutasa et al. (10).

PCR primers. The PCR primers were based on the sequence of cloned fragment pPbKES-1 (EMBL accession number X83745) and were designed by the computer programs Primer (9) and Amplify (4). The outer primer pair was designated Pb-5ab (where Pb-5a = 5'-CAGGGGCAGACGGATCGCAG and Pb-5b = 5'-CGTCGAGCGCAGTTCTTGGC), and the nested primers were Pb-6a4b (where Pb-6a = 5'-AGATGAGGATGTCAGTCAGG and Pb-6b = 5'-CTATGTGGCAAACCCAAG). For the Captagene-GCN4 test, primer Pb-6a was modified by the addition of a GCN4-recognition site (5'-GGATGACTCATT) at the 5' end, and Pb-4b was modified by 5' biotinylation. Optimum annealing temperatures for each of the primers were 70.7°C for Pb-5a, 68.8°C for Pb-5b, 55.0°C for Pb-6a, and 54.9°C for Pb-4b. The outer primers were designed to amplify a product of 1.14 kb from which a further product of 0.8 kb could be amplified by the nested primers.

DNA amplification conditions. Unless otherwise specified, standard 50- μ l reactions contained 10 nM of each outer primer (Pb-5a and Pb-5b), 100 nM of each nested primer (Pb-6a and Pb-4b), 125 μ M deoxynucleotide triphosphates (Pharmacia polymerization mix [Pharmacia Biotech, St. Albans, England]), 1 \times PCR buffer (Boehringer Mannheim, Lewes, E. Sussex, England), and 1.0 units of *Taq* DNA polymerase (Boehringer). Varying amounts of DNA were added depending on the test being done; 100 ng was the level above which PCR was inhibited. The two rounds of amplification were separated by limiting the amount of outer primers. The reactions were incubated in a HYBAID OmniGene thermal cycler (HYBAID, Teddington, England) for 35 cycles: stage 1: 4 min at 95°C, 30 s at 65°C, and 30 s at 72°C for 1 cycle; stage 2: 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C for 14 cycles; and stage 3: 30 s at 94°C, 30 s at 52°C, and 30 s at 72°C for 20 cycles.

For quality control, all reagents were preassembled in a bulk mix from which 49- μ l aliquots were dispensed into each tube before the DNA was added in 1- μ l volumes. This was carried out on ice to reduce primer dimerization. Positive (artificially infected sugar beet previously examined under a microscope) and negative (healthy sugar beet grown under sterile conditions) controls also were included with each run. To reduce carry-over contamination, aerosol resistant tips were used for all operations, and different pipette sets were reserved for preparing PCR reagents and manipulating the products. All reactions were repeated at least once by two researchers.

Primer titration. To determine optimum primer concentrations for single-tube nested reactions, inner primers were kept at 100 nM, whereas the outer primers were titrated to cover a range between 2 and 10 nM. Reactions were carried out with pure and crude DNA from artificially infected plants and plants naturally infected in the field.

Analysis of PCR products. Products (10 μ l) were separated by electrophoresis in 1.2% agarose gels with Tris-borate buffer, stained with ethidium bromide, and visualized on a UV transilluminator. Gels were documented on Polaroid 665 film (Polaroid Corp., Cambridge, MA). Alternatively, products were captured on Captagene-GCN4 plates and detected as specified by the manufacturer (Amrad Corp., Kew, Victoria, Australia). Absorbance values were determined at 450 nm with a reference filter at 620 nm and a plate reader (Molecular Devices, EMAX colorimeter, Sunnyvale, CA). Threshold values were determined for each PCR run from five negative controls according to the relationship $[CN1 + 3 \times SD(CN1)]$, where *CN1* is the mean absorbance of negative controls and *SD(CN1)* is the standard deviation from the mean.

Primer specificity. PCRs were carried out in reaction mixes containing 10 ng of purified DNA from five *P. betae* isolates (resting spores in dried sugar beet roots), five isolates of *P. graminis* (resting spores in dried barley roots), *O. brassicae* (zoospores), *Plas-*

modiophora brassicae (resting spores), *Pythium ultimum* (mycelium), *A. cochlioides* (mycelium), and *Ligniera* sp. (zoosporangia in sweet sugar pumpkin roots). Products were analyzed on agarose gels and Captagene-GCN4 plates.

Sensitivity of single-tube nested PCR. PCRs were carried out with 10-fold serial dilutions of pure DNA from artificially infected roots. After agarose gel electrophoresis, products were transferred onto Hybond N nylon membrane (Amersham International, Little Chalfont, England) by Southern blotting and were probed with digoxigenin-dUTP-labeled pPbKES-1 as previously described (10). Hybridizations were carried out under stringent conditions at 68°C with a final wash in 0.1 \times SSC (1 \times SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0). Products were simultaneously detected by the Captagene-GCN4 colorimetric system.

Field application of nested PCR as a diagnostic test for *P. betae*. Field application of nested PCR as a diagnostic test was assessed by carrying out PCRs with pure DNA extracted from roots of young sugar beet seedlings (44 to 54 days old) randomly collected from fields in East Anglia. Products were simultaneously analyzed on agarose gels and Captagene-GCN4 plates.

RESULTS

The combined effects of outer primer concentration and target DNA source and purity on PCR product yield are shown in Figure 1. With pure DNA from inoculated roots (Fig. 1, panel A), products were clearly visible across the entire range of primer concentrations tested (2 to 10 nM); the best product yield was obtained with 10 nM. With crude DNA from the same source, products were clearly visible only at 8 and 10 nM and were only just visible at 6 nM (Fig. 1, panel B). When the same test was carried out with crude DNA from naturally infected roots (Fig. 1, panel C) primer concentrations leading to successful amplification again were different. Here, products were clearly visible between 4 and 10 nM, with the best product yields at 6 and 8 nM.

When primer specificity was assessed with pure DNA, PCR products were amplified only in reactions known to contain *P. betae* target sequences (Fig. 2A, lanes 3 through 7). There was no amplification from nonsterile sugar beet root DNA (i.e., DNA that included sequences from root microorganisms other than *P. betae*), sterile sugar beet root DNA (Fig. 2A, lanes 1 and 2), other

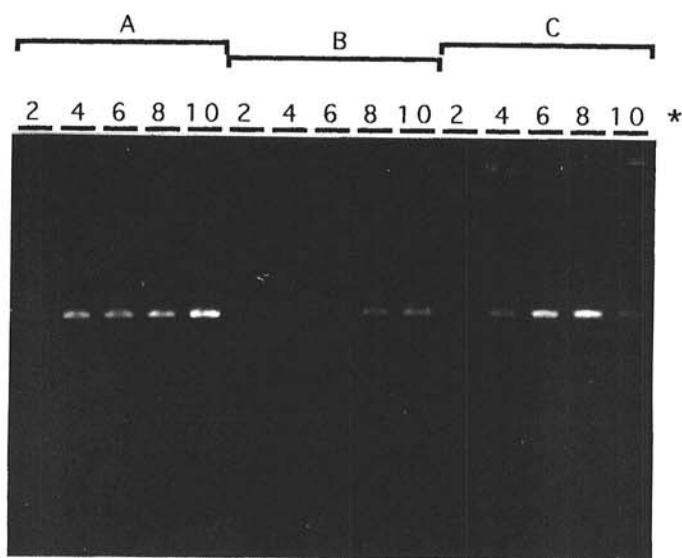


Fig. 1. The effect of changing outer primer concentration, DNA extraction method, and source material on the amplification of *Polymyxa betae* target sequences in single-tube nested polymerase chain reactions. A is purified DNA from inoculated sugar beet roots; B is crude DNA from inoculated sugar beet roots; and C is crude DNA from sugar beet roots naturally infected in the field. * indicates the outer primer concentration (nanomolar). NB inner (nested) primers were kept at 100 nM for each reaction.

root pathogens (Fig. 2A, lanes 8 through 12), or isolates of *P. graminis* (Fig. 2A, lanes 13 through 17). Results were confirmed by the Captagene-GCN4 test (Fig. 2B), in which only PCRs corresponding to lanes with visible bands gave a positive absorbance reading. However, the absorbance values were not correlated with visual assessment of product yield. For example, a comparison of products in lanes 5 and 6 (Fig. 2A) shows that although there appeared to be more product in lane 5 it had an absorbance value of only 0.098 (only just above the threshold of 0.090), whereas lane 6 had an absorbance value of 0.189. Similarly, products in lanes 3 and 7 appeared to have approximately the same amount of product as that amplified from the control reaction in lane 18, yet had absorbance values of only 0.243 and 0.185, respectively, compared with 0.541 for the control.

The nested PCR test was sensitive enough to detect *P. betae* in as little as 1 pg of total DNA from infected roots, as determined

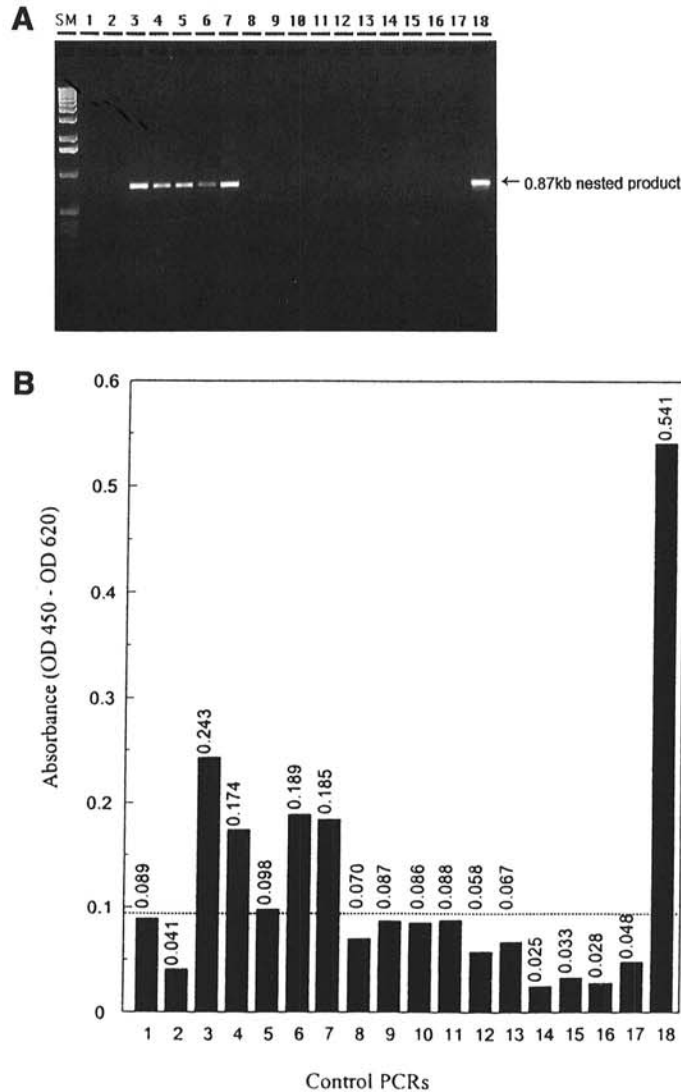


Fig. 2. A, Agarose gel showing the results of nested polymerase chain reactions carried out with known positive and negative DNA samples, and **B**, a bar chart of associated Captagene-GCN4 colorimetric assay results. The dotted line on the bar chart indicates the threshold value (OD = 0.90) above which results were recorded as positive. The DNA samples used for PCR were extracted from: 1 = nonsterile *Polymyxa betae*-free sugar beet roots; 2 = sterile roots from aseptically grown sugar beet; 3 through 7 = *Polymyxa betae* isolates BB6, BB8, BB10, BB13, and BB5, respectively; 8 = *Plasmodiophora brassicae*; 9 = *Olpidium brassicae*; 10 = *Ligniera* sp.; 11 = *Pythium ultimum*; 12 = *Aphanomyces cochlioides*; 13 through 17 = *Polymyxa graminis* isolates RES F1 (zoospores), RES F51, RES F60, RES (resting spores), and RES F24, respectively; and 18 = 1 ng of control plasmid pPbKES-1 containing target sequence.

by agarose gel electrophoresis (Fig. 3A) and Southern blot analyses (Fig. 3B). Southern blotting confirmed the presence of a product in the 1-pg lane and also that there was no amplification below this level. When the same products were tested on Captagene-GCN4 plates, all template concentrations gave positive absorbance values, including reactions below the detection limit (Fig. 3C). Closer examination of the agarose gel clearly shows that this was due to primer dimers, which accumulated as the DNA concentration was reduced. Once again, there was no direct correlation between absorbance values and product yield; indeed, there was very little difference in absorbance over the range of 0.1 ng to 1 pg of DNA, even though the Southern blot data showed that there was a marked difference in product yield.

When the PCR test was applied to DNA extracted from randomly selected plants grown in naturally infested fields, 8 of 12 samples contained detectable levels of *P. betae* DNA (Fig. 4A).

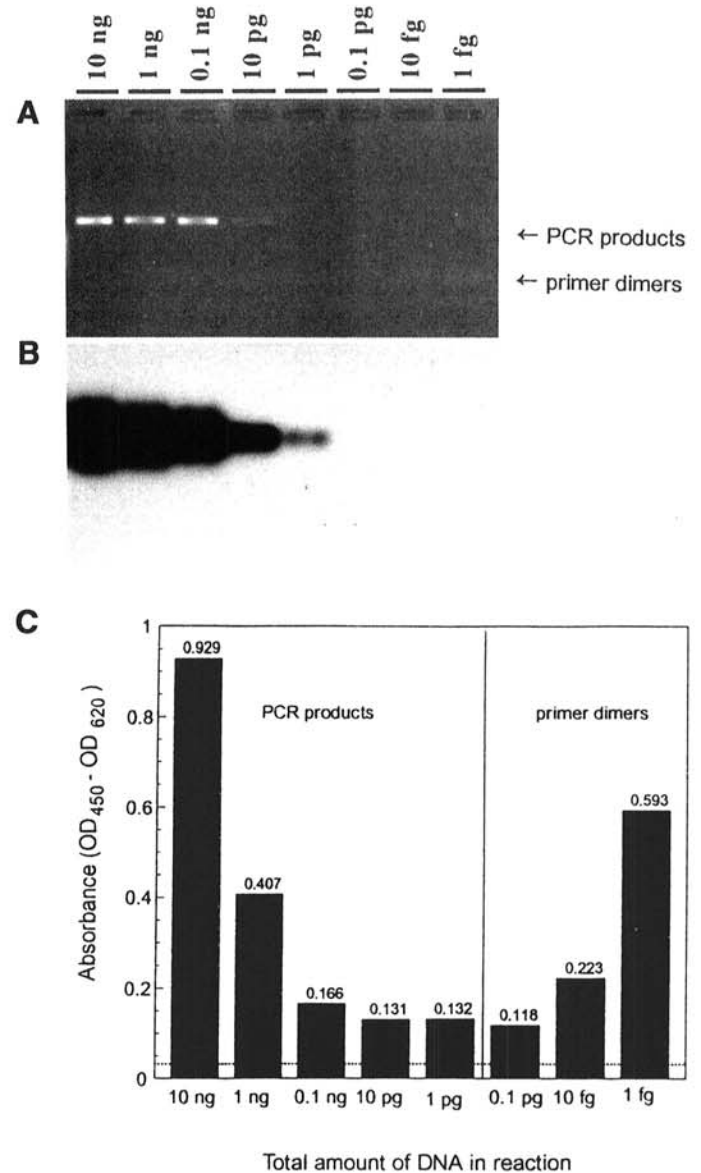


Fig. 3. A, Ethidium bromide-stained gel of nested polymerase chain reaction products amplified from 10-fold serial dilutions of DNA from sugar beet roots heavily infected with *Polymyxa betae*, showing an increase in primer dimers as DNA concentration is reduced. **B**, A Southern blot of the same gel probed with DIG-11-UTP-labeled target sequence (pPbKES-1) confirmed amplification down to 1 pg of total DNA. **C**, Associated Captagene-GCN4 colorimetric assay results illustrated in a bar chart clearly showing false positives due to primer dimerization. The dotted line indicates the threshold value (OD = 0.03) above which results were read as positive.

Results were confirmed with a different set of primers (data not shown). Captagene-GCN4 test results (Fig. 4B) agreed with the data from the gel, showing the same positive and negative samples.

DISCUSSION

The development of a PCR detection method specific for *P. betae* provides a sensitive and accurate test for diagnosing infection in sugar beet roots. However, for widespread practical application, protocols must guard against cross-contamination and employ uncomplicated techniques for detecting amplified products. This has been addressed here by the successful development of single-tube nested PCR that not only reduces the risk of contamination but allows interpretation of results from agarose gels without the need for further manipulation and also by the use of a commercially available kit for colorimetric detection of products.

Our results show that the ratio of outer to inner primers was critical for successful amplification of nested products and that the exact concentration was dependent on the source and purity of

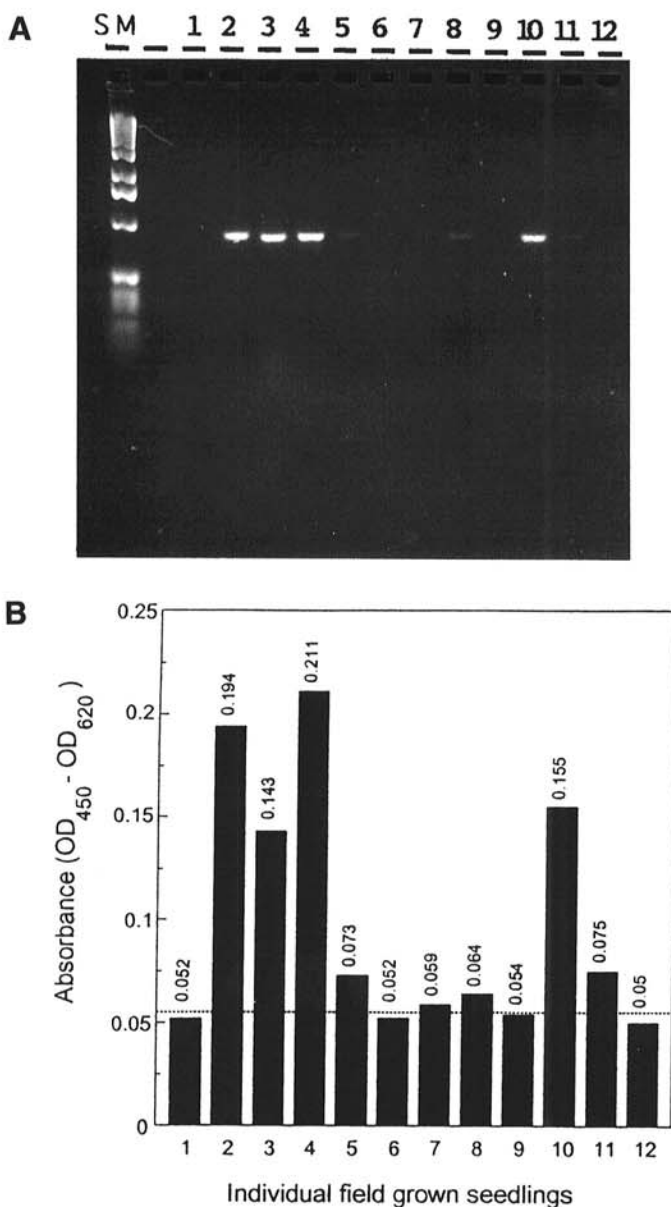


Fig. 4. A, Agarose gel showing the results of nested polymerase chain reactions carried out on DNA from a random selection of sugar beet seedlings (1 through 12) grown in naturally infested fields, and B, a bar chart of associated Captagene-GCN4 colorimetric assay results. The dotted line indicates the threshold value (OD = 0.058) above which results were read as positive.

target DNA. For example, with purified DNA our primers were efficient over a wider range of concentrations, whereas with crude DNA the range was narrower, and there was a marked difference in efficiency depending on whether the original plants were inoculated or naturally infected in the field. Therefore, optimization of primer concentrations for each batch of source material and DNA extraction procedure is recommended. Similarly, Kemp (6) also stressed the need for careful primer titration for single-tube nested PCR. The possibility of interference from inhibitory substances coextracted from plant and soil material should not be overlooked and may be a particular problem with crude DNA samples. However, for the purpose of large-scale screening of breeding material, it is encouraging that our primers could be optimized for use with crude DNA preparations, otherwise the need for DNA purification would become a serious rate-limiting step. Our experience also shows that the actual choice of primers influences the success or failure of the single-tube nested reaction. For example, we were unable to optimize conditions for single-tube nested PCR with our original set of *P. betae* primers (10), even though they were designed from the same DNA fragment as those described here. Indeed, we found that even for simple PCR primers from different regions of fragment pPbKES-1 did not always give the same level of amplification efficiency, and it was necessary to test several primer sets.

Because the nested PCR test is designed for screening very young sugar beet seedling roots (4 to 12 weeks old), from which we regularly obtain low DNA yields, amplification conditions were optimized on 10 ng of total DNA per 50- μ l reaction. Subsequently, we found that these conditions were susceptible to inhibition when the total DNA exceeded 100 ng. However, it was still possible to detect *P. betae* sequences in only 1 pg of total DNA per reaction. This is equivalent to a 10,000-fold increase in sensitivity compared to detection by Southern hybridization as previously reported (11). We have shown previously that detection of PCR products by molecular hybridization is as sensitive as ethidium bromide staining of nested products in agarose gels (10). This was confirmed by data obtained with serially diluted templates, in which hybridization signals on the Southern blot were obtained only in lanes containing visible bands on the stained gel.

Colorimetric detection of amplified products was considered not only because its microtiter-plate format allows for the mass screening required in a plant breeding program, but also because it utilizes equipment already available in many diagnostic laboratories. Color detection of PCR products is not new and is widely applied in medical research (15,18); however, most protocols depend on the use of DNA capture probes attached to a solid support (12). The Captagene-GCN4 system was selected not only because it is well characterized (6,7,8) and available in kit form, but also because PCR products are captured specifically by the DNA-binding GCN-4 protein. This protein has a higher affinity for double-stranded DNA and, therefore, binds PCR products in preference to single-stranded oligonucleotides, and unlike systems using DNA capture probes, there is no need for further manipulation of PCR products (e.g., denaturation) before they are captured.

Our data show that if great care is taken to guard against the formation of primer dimers, the Captagene-GCN4 system can be relied on to differentiate between infected and uninfected samples. From the combined electrophoresis and Southern data, we recommend that the total amount of DNA per reaction be kept above 1 ng to keep primer dimers below levels that interfere with absorbance readings. Hence, it was important to ensure that all the negative controls contained *P. betae*-free sugar beet DNA. However, because the data show that some of our positive samples were precariously close to the threshold values, cautious interpretation of data is required, because the kit may occasionally lead to selection of false negatives. Although use of the Captagene-GCN4 system for quantitative analysis has been proposed (Captagene Bulletin No. 2), in our study there was no direct correlation between

the amount of PCR product visible on agarose gels and associated absorbance readings.

LITERATURE CITED

1. Asher, M. J. C. 1993. Rhizomania. Pages 311-346 in: *The Sugar Beet Crop: Science into Practice*. D. A. Cooke and R. K. Scott, eds. Chapman and Hall, London.
2. Barr, K. J., and Asher, M. J. C. 1992. The host range of *Polymyxa betae* in Britain. *Plant Pathol.* 41:64-68.
3. Blunt, S. J., Asher, M. J. C., and Gilligan, C. A. 1991. Infection of sugar beet by *Polymyxa betae* in relation to soil temperature. *Plant Pathol.* 40: 257-267.
4. Engels, W. R. 1992. *Amplify*. University of Wisconsin, Genetics, Madison.
5. Heberle-Bors, E., Merten, O. W., Himmler, G., Reiter, S., Messner, P., and Katinger, H. 1984. Monoclonal antibodies for ELISA of BNYYV-(rhizomania) virus in sugar beet (*Beta vulgaris*). Pages 111-123 in: *Proc. 1st Int. Congr. Rhizomania*. INRA, Colmar, France.
6. Kemp, D. J. 1992. Colorimetric detection of polymerase chain reaction-amplified DNA segments using DNA-binding proteins. *Methods Enzymol.* 216:116-126.
7. Kemp, D. J., Smith, D. B., Foote, S. J., Samaras, N., and Peterson, M. G. 1989. Colorimetric detection of specific DNA segments amplified by polymerase chain reactions. *Proc. Natl. Acad. Sci. USA* 86:2423-2427.
8. Lew, A. M., Marshall, V. M., and Kemp, D. J. 1993. Affinity selection of polymerase chain reaction products by DNA-binding proteins. *Methods Enzymol.* 218:526-534.
9. Lincoln, S. E., Daly, M. J., and Lander, E. S. 1991. *Primer*. MIT Center for Genome Research and Whitehead Institute for Biomedical Research, Cambridge, MA.
10. Mutasa, E. S., Chwarszczynska, D. M., Adams, M. J., Ward, E., and Asher, M. J. C. 1995. Development of PCR for the detection of *Polymyxa betae* in sugar beet and its application in field studies. *Physiol. Mol. Plant Pathol.* 47:303-313.
11. Mutasa, E. S., Ward, E., Adams, M. J., Collier, C. R., Chwarszczynska, D. M., and Asher, M. J. C. 1993. A sensitive DNA probe for the detection of *Polymyxa betae* in sugar beet roots. *Physiol. Mol. Plant Pathol.* 43:379-390.
12. Nagata, Y., Yokota, H., Kosuda, O., Yokoo, K., Takemura, K., and Kikuchi, T. 1985. Quantification of picogram levels of specific DNA immobilized in microtiter wells. *FEBS* 183:382.
13. Richard-Molard, M. S. 1985. Rhizomania: A world-wide danger to sugar beet. *Span* 28:92-94.
14. Schlösser, E. 1988. Epidemiology and management of *Polymyxa betae* and beet necrotic yellow vein virus. Pages 281-292 in: *Viruses with Fungal Vectors. Developments in Applied Biology*. Vol. 2. J. I. Cooper and M. J. C. Asher, eds. Association of Applied Biologists, Wellesbourne, England.
15. Shindo, M., Di Bisceglie, A. M., Silver, J., Limjoco, T., Hoofnagle, J. H., and Feinstone, S. M. 1994. Detection and quantitation of hepatitis C virus RNA in serum using polymerase chain reaction and a colorimetric enzymatic detection system. *J. Virol. Methods* 48:65-72.
16. Tamada, T., and Baba, T. 1973. Beet necrotic yellow vein virus from rhizomania-affected sugar beet in Japan. *Ann. Phytopathol. Soc. Jpn.* 39:325-332.
17. Torrance, L., Pead, M. T., and Buxton, G. 1988. Production and some characteristics of monoclonal antibodies against beet necrotic yellow vein virus. *Ann. Appl. Biol.* 113:519-530.
18. Van Der Vliet, G. M. E., Hermans, C. J., and Klaster, P. R. 1993. Simple colorimetric microtiter hybridization assay for detection of amplified *Mycobacterium leprae* DNA. *J. Clin. Microbiol.* 31:665-670.
19. Zolan, M. E., and Pukkila, P. J. 1986. Inheritance of DNA methylation in *Coprinus cinereus*. *Mol. Cell. Biol.* 6:195-200.