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著者	斎藤, 勝晴 西脇, 亜也 菅原, 和夫
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Short Report

DNA Extraction from Arbuscular Mycorrhizal Roots of *Miscanthus sinensis* Anderss. Collected in the Native Grassland

Katsuharu SAITO, Aya NISHIWAKI* and Kazuo SUGAWARA

Laboratory of Land Ecology, Graduate School of Agricultural Science, Tohoku University, Kawatabi, Narugo, Miyagi, 989-6711 Japan

*Faculty of Agriculture, Miyazaki University, Gakuen Kibanadai, Miyazaki, 889-2192 Japan

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野草地から採取したススキのアーバスキュラー菌根からの DNA 抽出

斎藤勝晴・西脇亜也*・菅原和夫

東北大学大学院農学研究科陸圏修復生態学（989-6711 宮城県玉造郡鳴子町川渡），

*宮崎大学農学部（889-2192 宮崎市学園木花台西 1-1）

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Introduction

Arbuscular mycorrhizal (AM) fungi are symbiotic microorganisms that colonize the roots of most terrestrial plant species[11]. AM fungi play an important role in plant nutrient acquisition (especially phosphorus) by the spread of their hyphae into the rhizosphere[7], and hence affect plant community structure and plant diversity[12,13]. The diversity of AM fungi has been investigated based on the morphological characteristics of their spores in the rhizosphere. On the other hand, study of AM fungal diversity in mycorrhizal roots is lacking because of the difficulty of distinguishing AM fungal species based on hyphal morphology in root. DNA markers have recently been developed to identify AM fungi[8]. Specifically, a polymerase chain reaction (PCR) method using AM fungi-specific and its taxon-specific primers has been attempted to detect AM fungal DNA in roots. High quality template DNA must be prepared from AM roots for PCR. DI BONITO *et al.*[4] showed that the boiling method is a useful and easy way to extract DNA from laboratory-grown mycorrhizal roots inoculated with AM fungi. In contrast to laboratory-grown mycorrhizal roots, field-collected mycorrhizal roots contain various amounts of AM fungal tissue and many kinds of PCR inhibitors such as phenolic compounds, polysaccharides and proteins, whose compositions and quantities are always influenced by environmental factors. For field-collected mycorrhizal roots, selection of the proper DNA extraction method is therefore necessary. However, there are few studies on DNA extraction from field-collected mycorrhizal roots, although PCR amplification method has been modified [*e.g.* SEAD method[3] and nested PCR (SAITO *et al.* unpublished)]. In this study, we tested several methods for consistent and simple extraction of PCR-quality AM fungal DNA from field-collected mycorrhizal roots.

Materials and Methods

Miscanthus sinensis Anderss. roots were collected from a native grassland at the Experimental Farm of Tohoku University, Narugo, Miyagi, from June to November 1997 (Table 1). The roots were washed in running water and cut into portions approximately 1 cm long. About thirty root segments were cleaned three times with 10 s sonication pulses followed by rinses with distilled water. The cleaned root segments were crushed in liquid nitrogen with a pestle. The DNA was extracted by the following five methods : 1) CTAB method. The crushed root was added to 300 μl of 2×CTAB solution (100 mM Tris-

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HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB and 0.2% 2-mercaptoethanol). The solution was incubated at 56℃ for 90 minutes. After two extractions with chloroform/isoamyl alcohol (24 : 1, v/v), DNA in the aqueous phase was precipitated by the addition of an equal volume of isopropanol and a tenth volume of 3 M sodium acetate and incubation at room temperature for 30 minutes. The DNA pellet was washed with 70% ethanol and resuspended in 50 μl sterilized water. 2) Boiling method. The crushed root was added to 400 μl of cold 0.75 M Tris-HCl pH 8.0 and 5% Chelex 100 resin (Sigma), which reduces metal ions in the solution. The samples were boiled for 40 seconds and the resin was then removed by centrifugation at 12,000 rpm for 2 minutes. The supernatant was used as the DNA extract. 3) Boiling method combined with isopropanol precipitation (boiling method+IP). DNA obtained by the boiling method was precipitated by the addition of an equal volume of isopropanol and a tenth volume of 3 M sodium acetate and incubation at room temperature for 30 minutes. The DNA pellet was washed with 70% ethanol and resuspended in 50 μl of sterilized water. 4) Freezing-thawing method. The crushed root was added to 400 μl of cold 0.75 M Tris-HCl pH 8.0 and 5% Chelex 100 resin. The solution was frozen at −20℃ and then thawed at room temperature. The cycle was repeated three times. The resin in the solution was removed by centrifugation at 12,000 rpm for 2 minutes. The supernatant was used as the DNA extract. 5) Freezing-thawing method combined with isopropanol precipitation (freezing-thawing method+IP). DNA obtained by the freezing-thawing method was precipitated with isopropanol and a tenth volume of 3 M sodium acetate, washed, and resuspended in sterilized water. DNA solution obtained by each method was diluted 100 to 1,000-fold and used as template for PCR amplification.

PCR was performed using a Program Temp Control System PC-800 (ASTEC) in a 50 μl reaction mixture containing 10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.025 U μl^{-1} *Taq* DNA polymerase (TaKaRa), 0.5 μM of each primer, and 5 μl of template DNA. PCR was carried out with primer pair VANS 1-NS 21 (direct PCR) or SS 38-NS 21 followed by VANS 1-NS 21 (nested PCR). VANS 1[9] is an AM fungi-specific primer, SS 38[1] and NS 21[9] are universal primers. The temperature profile was programmed to repeat 40 cycles of denaturation (60 s at 94℃), annealing (45 s at 50℃), and polymerization (60 s at 72℃), followed by a final extension step of 10 min at 72℃[10]. The amplified products were separated by electrophoresis on 2% agarose gels and visualized on a UV transilluminator after staining with ethidium bromide.

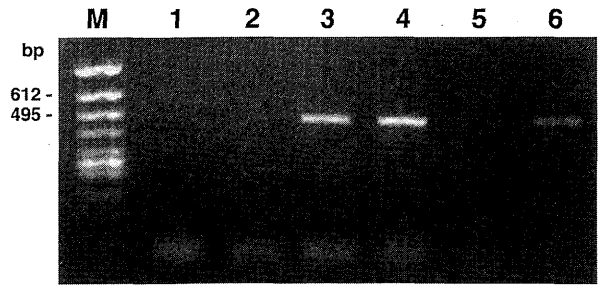

Fig. 1. PCR products obtained using template DNA prepared from *M. sinensis* mycorrhizal roots collected from a native grassland on 6 August 1997. Template DNA was prepared by the CTAB method (lane 2), boiling method (lane 3), boiling method combined with isopropanol precipitation (lane 4), freezing-thawing method (lane 5) and freezing-thawing method combined with isopropanol precipitation (lane 6). PCR was carried out with primer pairs VANSI-NS 21 (direct PCR). Negative control (lane 1) contained water instead of template DNA. The size marker (M) is φX174-*Hin*cII.

Results and Discussion

We initially investigated five DNA extraction methods using *M. sinensis* mycorrhizal roots collected on 6 August. Figure 1 shows the results of the electrophoretic analysis of the PCR products. Using the boiling method and the boiling method+IP for extracting AM fungal DNA, PCR products about 550 bp in length were obtained. On the other hand, no PCR product was obtained with either the CTAB or freezing-thawing methods. However, a small amount of PCR product was obtained by the freezing-thawing method+IP. Hence, the boiling method and the boiling method+IP were more reliable than the other methods. In subsequent experiments, we used the boiling method+IP because this can prepare higher quality DNA than the boiling method.

We next examined PCR amplification with template DNA extracted from June to November. Using direct PCR, PCR product was obtained from samples taken up to 20 August, while no PCR product was obtained from samples taken after 28 August (Table 1). With nested PCR, however, PCR product could be obtained even after 28 August (Table 1). Nested PCR has been shown to be a powerful method for amplifying target DNA[2,5,6,14]. The boiling method+IP is therefore a useful method for extracting PCR-quality AM fungal DNA from field-collected *M. sinensis* mycorrhizal roots throughout its growing season, provided that the appropriate PCR method (nested PCR) is selected. Furthermore, the boiling method+IP is simple procedure that is suitable for processing

Table 1. Detection (+) of PCR products from of *M. sinensis* mycorrhizal root extracted the boiling method combined with isopropanol precipitation.

	Jun.	Jul.		Aug.			Sep.		Oct.		Nov.
	27	11	18	6	20	28	6	30	21	29	5
Direct PCR[a)]	+	+	+	+	+	−	−	−	−	−	−
Nested PCR[b)]			+			+					+

[a)] VANS1-NS21 primer pair.

[b)] SS38-NS21 followed by VANS1-NS21 primer pair.
Blank donates not examined.

many samples at one time.

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