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Satoshi Ishii · Thomas E. Loynachan

Rapid and reliable DNA extraction techniques from trypan-blue-stained mycorrhizal roots: comparison of two methods

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Abstract Two improved DNA extraction techniques from trypan-blue-stained root fragments were developed and compared for rapid and reliable analyses. In Method A, 1 cm trypan-blue-stained mycorrhizal root fragments were individually isolated, crushed by bead beating, and purified with Chelex-100 (Bio-Rad). In Method B, DNA extraction was carried out using an UltraClean microbial DNA isolation kit (MoBio Laboratories). DNA was extracted from the mycorrhizal roots of four plant species, quantified by UV absorbance, and PCR-amplified with primers specific to arbuscular mycorrhizal fungi. Although PCR inhibitors might still exist when using Method A, appropriate dilution and employment of nested-PCR overcame this problem. Method B removed PCR inhibitors, but sometimes, depending on the mycorrhizal colonization within the root fragments, it also required nested PCR. In conclusion, both methods enabled us to handle many samples in a short time. Method B provided greater reliability and Method A provided better cost performance. Both techniques can be useful for PCR-based applications to identify species and estimate species composition after measuring mycorrhizal colonization rate with trypan blue staining.

Keywords Arbuscular mycorrhizal fungi · DNA extraction · Glomales · Trypan blue staining

S. Ishii · T. E. Loynachan (✉)
Department of Agronomy, Iowa State University ,
1126 Agronomy Hall,
Ames, IA 50011, USA
e-mail: teloynac@iastate.edu
Tel.: +1-515-2943064
Fax: +1-515-2948146

Present address:
S. Ishii
Department of Soil, Water, and Climate, University of Minnesota,
439 Borlaug Hall, 1991 Upper Buford Circle,
MN 55108 St. Paul, USA

Introduction

Arbuscular mycorrhizal (AM) fungi provide their host plants with nutrients, especially nutrients with poor soil mobility, such as P and Zn, and in return they receive carbohydrate for their energy source (Smith and Read 1997). AM fungi associate with various plant species, and positively—sometimes negatively—influence their host plants (Johnson et al. 1997); therefore, AM fungi have a strong influence on plant productivity. In natural ecosystems, diverse AM fungal species coexist in plants and soil. Efficiency of AM fungi in nutrient acquisition is different among species; some species can provide more P, while others do not promote, and may even reduce, plant growth (Smith et al. 2000). Therefore, species composition of AM fungi in plants and soil might have important consequences for plant productivity (van der Heijden et al. 1998).

The effects of AM fungi have been studied along with their colonization rates in plant roots. Conventionally, AM fungal colonization rates are measured microscopically. The most common method is the grid-line intersect method (Giovannetti and Mosse 1980), in which mycorrhizal roots are stained with trypan blue. This method, however, does not provide species identification because morphological characteristics of some AM fungi in roots are similar. To overcome this problem, several methods have been applied to detect AM fungi in planta. Antibodies (Hahn et al. 1993), isozyme patterns (Hepper et al. 1988; Thingstrup and Rosendahl 1994), lipid profiles (Bentivenga and Morton 1994; Madan et al. 2002), and molecular techniques based on polymerase chain reaction (PCR) have been tested. Hepper et al. (1988) showed that isozyme patterns enabled detection of metabolically active fungi, but detection sensitivity differed among fungal species. Lipid profiles have potential as a biomass indicator for AM fungi but are not specific at the species level (Madan et al. 2002). PCR-based molecular techniques are the most sensitive method to identify AM fungi at the species level, but several problems exist with this technique. Among these, DNA can be amplified from

metabolically inactive AM fungi such as dormant or dead spores and dead mycorrhizal roots. Also, the amount of PCR product does not necessarily reflect species biomass. Quantitative PCR can estimate the amount of template DNA and thus has the potential to estimate species biomass (Edwards et al. 1997), but species-specific primers are necessary for this and, therefore, the technique is not adequate for a complex AM fungal community study. The relative proportion of AM fungal DNA to host plant DNA derived from quantitative PCR with AM fungal specific primers and plant specific primers, however, might alter the mycorrhizal colonization percentage, as Winton et al. (2002) have shown with pathogenic fungi, and this needs further study.

Microscopy with stained roots is the best method currently available to measure mycorrhizal colonization rates in roots, but it cannot identify species, while DNA-based molecular techniques are likely to provide information regarding species composition. Therefore, a combination of microscopy and DNA-based molecular techniques can provide both mycorrhizal colonization percentage and colonizer species information. Further, if species from several pieces (e.g. ten pieces) of mycorrhizal fragments per plant are identified with DNA-based molecular techniques after measuring colonization percentage, the relative proportion of each AM fungus colonizing the same host can be estimated (Jacquot et al. 2000). Since this estimation requires DNA extraction and PCR-amplification of many samples, it is necessary to develop rapid and reliable AM fungal DNA extraction methods from trypan-blue-stained mycorrhizal roots.

Several researchers have developed AM fungal DNA extraction techniques from mycorrhizal roots, but some of these methods are quite laborious because they include manual grinding (van Tuinen et al. 1998; Jacquot et al. 2000; Turnau et al. 2001), homogenization in liquid N (Simon et al. 1992; Lanfranco et al. 1995; Edwards et al. 1997), razor-blade chopping (Redecker 2000), heating (Simon et al. 1992; Di Bonito et al. 1995; Redecker 2000), cell lyses and protein removal with enzymes and/or chemicals (Claassen et al. 1996; Lanfranco et al. 1995), and/or phenol/chloroform extraction of impurities (Simon et al. 1992; Clapp et al. 1995; Lanfranco et al. 1995; Edwards et al. 1997). Kit-based DNA extraction techniques have been applied to soil (Chelius and Triprett 1999; Dickie et al. 2002), ectomycorrhizal root samples (Koide and Dickie 2002), and endomycorrhizal roots (Jansa et al. 2003), but they are costly when a large number of samples should be handled. Here, we improved upon existing DNA extraction techniques and report two rapid, reliable, and relatively inexpensive techniques to extract DNA from trypan-blue-stained mycorrhizal roots.

Materials and methods

AM fungus, host plants, and culture

Glomus mosseae (Nicol. & Gerd.) Gerd. & Trappe isolate BEG83 (=DN990) was obtained from the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM, Morgantown, W.Va.). Four plant species were used for mycorrhizal host: alfalfa (*Medicago sativa* L. subsp. sativa), corn (*Zea mays* L.), soybean (*Glycine max* [L.] Merr.), and sudan grass (*Sorghum sudanense* [Piper] Staph). Plant seeds were surface disinfected by soaking in 70% ethanol for 5 min, and rinsed with sterilized water five times. Two seeds of soybean, three seeds of corn, 20 seeds of sudan grass, or 30 seeds of alfalfa were planted in individual pots containing 2 kg soil:sand mixture and grown for 4 weeks in the greenhouse. There were three pots of soybean and two pots of other species. The soil was collected at the Agronomy and Agricultural Engineering Research Farm, Iowa State University, located in Boone County, Iowa, which contained 41% clay, 32% silt, and 27% sand; with a pH of 6.9; 54 g kg^{-1} organic matter; 38 mg kg^{-1} Bray 1-extractable P; and 136 mg kg^{-1} 1 M NH_4OAc-extractable K. The soil was passed through a 2-mm mesh sieve and autoclaved twice with 1 day duration between autoclavings, and mixed with autoclaved silica sand (60:40, soil:sand w:w).

The growth conditions were 30°C in daytime (15 h) with natural sunlight supplemented with artificial light and 25°C at night (9 h). The light intensity at the bench surface was 960 μmol photon m^{-2} s^{-1}. The plants were watered daily with sterile distilled water. Matric potential in soil was maintained between ca. 7.5 and 30 kPa based on a soil moisture characteristic curve (Ozbek 1998). Three weeks after planting, sterile nutrient solution (Broughton and Dilworth 1971) was applied instead of sterile distilled water. The P level was one-fifth of the recommended concentration to stimulate mycorrhizal colonization.

Plant roots were harvested after 4 weeks of growth in the greenhouse. They were gently washed in distilled water to remove soil particles and stained following the protocol of Brundrett et al. (1996) with minor modification. Briefly, roots of each seedling were placed in a 50-ml flask and bleached with 10% (w/v) KOH at 90°C for 90 min. They were rinsed with sterile distilled water three times, and stained with 0.05% (w/v) trypan blue in lactoglycerol (lactic acid:glycerol:H_2O=1:1:1 v:v:v) at 90°C for 30 min. They were destained with sterile 50% (v/v) water:glycerol several times and stored at 4°C. Mycorrhizal colonization rates were ca. 23, 39, 18, and 27% in alfalfa, corn, soybean, and sudan grass, respectively.

DNA extraction

Genomic DNA was extracted from individual 1-cm fragments of trypan-blue-stained mycorrhizal roots using several extraction methods. Crushing of the roots was carried out in a microcentrifuge tube by manual grinding in 40 μl Tris buffer (100 mM Tris-HCl, pH 8) with a micropestle (van Tuinen et al. 1998), manual grinding and powdering with liquid N_2 with a micropestle, or bead beating (described below). Although root grinding in liquid N_2 provided the highest amount of DNA, we did not use it further because of its laboriousness. Since manual grinding in the Tris buffer was also time- and labor-intensive and did not provide higher DNA amounts than bead beating (data not shown), we used bead beating for further analyses.

Purification of the crude DNA extract proceeded by phenol-chloroform extraction of impurities followed by precipitation of DNA with 3 M sodium acetate (pH 5.2) and 2-propanol, chelation using 5% (w/v, final concentration) of Chelex-100 (Bio-Rad, Hercules, Calif.) at 90°C for 10 min (John 1992; Di Bonito et al. 1995; Sanders et al. 1995; van Tuinen et al. 1998; Jacquot et al. 2000; Turnau et al. 2001), or use of silica spin column contained in an "UltraClean Microbial DNA Isolation Kit" (MoBio Laboratories, Solana Beach, Calif.) (Koide and Dickie 2002) with minor modification (described below). Although phenol-chloroform pur-

ification somewhat improved DNA purity (data not shown), this process lost much DNA and took a lot of time and labor. Therefore, this purification step was not included in further preparations.

After preliminary comparative work, we concluded that two DNA extraction techniques would work for rapid, reliable, and relatively inexpensive analyses. In the first method (Method A), 1 cm trypan-blue-stained mycorrhizal root fragments were individually isolated, rinsed in sterile H_2O, and placed in a microcentrifuge tube. Each root fragment was crushed by bead beating for 10 min using approximately 50 µl (30–40 beads) 1-mm zirconia beads (BioSpec Products, Bartlesville, Okla.) in 240 µl Tris-HCl buffer (100 mM Tris-HCl, pH 8.0). Bead beating was carried out for 10 min using a MaxMix voltex (Fisher Scientific, Pittsburgh, Penn.) with an adaptor that enabled us to handle up to 24 samples. Crude DNA extract was incubated at 90°C for 10 min with 60 µl 10% (w/v) Chelex-100. Chelex resin chelates heavy metals that inhibit enzymatic activity in PCR. Following the incubation, sample tubes were cooled on ice for approximately 1 min and centrifuged at 12,000 *g* for 5 min; 10 µl supernatant was diluted to 100 µl with sterile water and served as DNA template in the PCR reaction. This method takes 40–50 min to process 24 samples.

In the second method (Method B), DNA was extracted by bead beating with silica spin column purification using the UltraClean Microbial DNA Isolation Kit following the protocol of Koide and Dickie (2002) with some modification. Root samples were also 1 cm trypan-blue-stained mycorrhizal root fragments. Each root fragment was crushed by bead beating for 10 min using approximately 50 µl (30–40 beads) 1-mm zirconia beads in 300 µl bead solution (MoBio), 50 µl M1 (detergent-based extraction solution, MoBio), and 50 µl IRS (inhibitor removal solution, MoBio). Large and uniform-sized beads (1 mm), compared with those supplemented in the kit (approximately 0.1 mm), were used to efficiently crush plant roots (these beads were smaller than the beads used by Koide and Dickie (2002; 2.4 mm). IRS is a component of the UltraClean Soil DNA Isolation Kit (MoBio) and inactivates phenolic compounds present in soil organic matter and plant tissues that inhibit PCR reactions (Tebbe and Vahjen 1993). The supernatant (300–350 µl) was transferred to a new tube, 100 µl M2 (an acetate-based solution, MoBio) was added, and held at −20°C for 15 min to precipitate impurities. Other steps followed the manufacturer's recommendation except the washing step with ethanol-based solution M4 (MoBio) was performed twice. The DNA solution was used directly for PCR. This method takes 90–120 min to process 24 samples.

DNA quantification

Subsamples of DNA extracted from Method A and B were diluted 2.5- and 10-fold, respectively, with purified H_2O, and used for DNA quantification based on the reading of UV-absorbance at 260 nm measured by a Cary 50 UV-visible spectrophotometer (Varian, Palo Alto, Calif.). The purity of DNA was also estimated by the ratio between the UV-absorbance at 260 nm and 280 nm (A_{260}/A_{280}). All numerical data were statistically analyzed by one-way ANOVA.

Polymerase chain reaction

Nested-PCR was performed using a MiniCycler (MJ Research, Waltham, Md.) to amplify rRNA genes including the ITS region. The universal eukaryotic primers, NS5 (White et al. 1990, 5′-AACTTAAAGGAATTGACGGAAG-3′) and ITS4 (White et al. 1990, 5′-TCCTCCGCTTATTGATATGC-3′), were used in the first step, and a primer specific to *Glomus mosseae* and the *G. intraradices* group, GLOM1310 primer (Redecker 2000, 5′-AGCTAGGCTTAACATTGTTA-3′), was used in combination with ITS4 in the second step (Integrated DNA Technologies, Coralville, Iowa). The first step reaction had a total volume of 7.5 µl containing 0.2 mM each dNTP, 0.5 µM each primer, 1.5 mM $MgCl_2$, 0.2 µg $µl^{-1}$ bovine serum albumin (BSA), 0.02 U $µl^{-1}$ Platinum*Taq* DNA polymerase (Invitrogen, Carlsbad, Calif.), the manufacturer's reaction buffer, and 1 µl DNA template. As a negative control, 1 µl sterile H_2O was added instead of DNA template. Platinum*Taq* polymerase accommodates hot-start PCR to minimize undesired amplification, such as primer dimmers. PCR cycles were programmed as follows: initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 90 s, followed by final extension at 72°C for 5 min.

The amplified product of the first step PCR was diluted 1/100, and 1 µl was used as DNA template in the second step reaction containing the same reaction mixture with a total volume of 12.5 µl. The same PCR cycle program was used in the second step. Aliquots of 3.0 µl were run on a 1.2% (w/v) agarose gel and stained with ethidium bromide to confirm the amplification of products with the desired size (approximately 1,000 bp).

Results

The amount and purity of DNA extracted from trypan-blue-stained mycorrhizal roots using Methods A and B were compared (Table 1). Method A yielded a greater amount of DNA per sample (per 1-cm trypan-blue-stained mycorrhizal root fragment) than Method B. DNA purity (A_{260}/A_{280}), however, was lower with Method A compared with Method B (pure preparation of DNA has an A_{260}/A_{280} ratio of 1.6–1.8), suggesting impurities such as proteins and phenolic compounds might exist in the extract when using Method A. Since proteins, RNA, and phenolic compounds also absorb UV at 260 nm, the amount of DNA shown in Table 1 might be overestimated, especially in Method A. All these observations were consistent in the four host plants.

Although the amount of DNA in Method A was greater than that in Method B, the final concentration of DNA extracted by Method A was lower (Table 1). The final volume of Method A was 300 µl and it was diluted 10-fold, whereas the final volume of Method B was 50 µl and no dilution was applied. Since 1.0 µl each was used as a template for PCR reactions, the amount of DNA in PCR reactions was ca. 0.3–0.6 ng per reaction in Method A and ca. 6–10 ng per reaction in Method B. Theoretically, these amounts of DNA are enough for PCR, but presence of

Table 1 Amount, purity, and final concentration of DNA extracted by Methods A and B (mean ±SEM, n=3). P: *, **, *** indicate significant difference between Methods A and B at the 0.05, 0.01, and 0.001 probability levels, respectively

	Amount (µg/sample)			Purity (A_{260}/A_{280})			Final concentration (µg/ml)		
	A	B	P	A	B	P	A	B	P
Alfalfa	1.46±0.08	0.34±0.04	**	1.15±0.03	1.82±0.32	**	0.49±0.03	6.8±0.75	**
Corn	1.06±0.15	0.49±0.03	*	1.26±0.03	1.35±0.04	*	0.36±0.05	9.9±0.67	
Soybean	1.67±0.36	0.36±0.04		1.23±0.03	1.82±0.04	**	0.56±0.12	7.3±0.75	*
Sudan grass	1.19±0.24	0.43±0.03		1.23±0.03	1.62±0.02		0.40±0.08	8.5±0.50	***

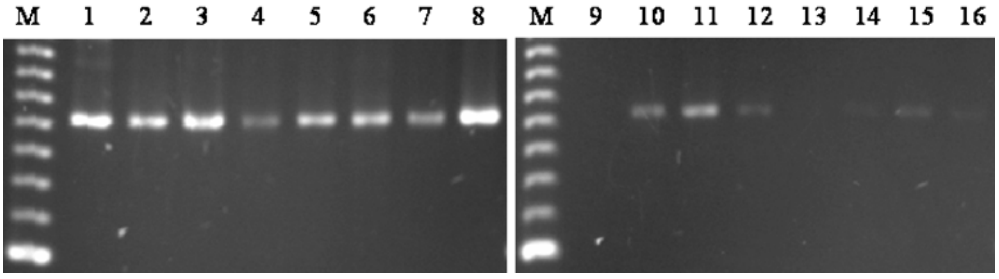

Fig. 1 PCR products amplified with primers GLOM1310/ITS4 by nested-PCR (lanes *1–8*) and one-step PCR (*9–16*) on 1.2% agarose gel stained with ethidium bromide. Method B from alfalfa (*1*, *9*), corn (*2*,*10*), soybean (*3*, *11*), and sudan grass (*4*, *12*). Method A from alfalfa (*5*, *13*), corn (*6*, *14*), soybean (*7*, *15*), and sudan grass (*8*, *16*). *M* 100-bp ladder; from bottom to top, 600, 700, 800, 900, 1,000, 1,100, 1,200, and 1,300 bp

PCR inhibitors required us to employ nested-PCR. PCR products on 1.2% (w/v) agarose gel are shown in Fig. 1.

Methods A and B both provided strong bands after nested-PCR (Fig. 1). The percentage of samples successfully amplified by PCR was 71% (107/151) in Method A and 94% (17/18) in Method B. In Method A, further dilution (100-fold) of DNA solution provided successful amplification in 18 out of 23 attempts (78%) for DNA samples not amplified when they were diluted 10-fold. Negative controls were always clean (no amplification observed).

Discussion

In this paper, Method A was simplified from the current DNA extraction protocol (van Tuinen et al. 1998) by incorporating bead beating to crush roots. Since bead beating enabled us to simultaneously handle up to 24 samples by attachment to a voltex mixer, and even up to 192 samples with a commercially available bead beater (e.g., Mini-BeadBeater-96, BioSpec), this method is useful especially when many samples need to be analyzed. Purification using Chelex-100 did not remove all PCR inhibitors, but appropriate dilution (10- or 100-fold) overcame inhibition of the PCR reaction in most cases. *Taq* DNA polymerase is sensitive with respect to DNA template; therefore, dilution of the DNA extract does not in itself affect DNA amplification in many cases.

We also modified the DNA extraction method proposed by Koide and Dickie (2002) to make it suitable for trypan-blue-stained mycorrhizal roots (Method B). We used smaller beads than they proposed to crush the short and soft roots efficiently, and several steps such as root freezing and bead beating without solution were avoided to shorten the processing time. Kit-based methods are generally expensive, but the proposed DNA extraction method is ca. 50, 35, and 25% less expensive than the methods of Chelius and Triprett (1999), Dickie et al. (2002), and Jansa et al. (2003), respectively, based on current component catalog prices. This method also worked well with unstained mycorrhizal roots of corn and four weed species (Timling, University of Minnesota, personal communication).

Comparison between Method A and B showed that Method B provided a purer DNA solution of higher final concentration (Table 1). Method A, however, was over 100 times less expensive and around 3 times less time consuming. Both methods provided strong bands with nested-PCR (Fig. 1), but the percentage of successful amplification was greater in Method B. The difference in percentage of successful amplification might be due to the different amount of PCR inhibitors in the DNA extract. In neither method did the final DNA concentration contain enough AM fungal DNA to overcome the PCR inhibition problem for one-step PCR when extracted from a 1-cm length of trypan-blue-stained mycorrhizal root fragment (Fig. 1). Longer root fragments may overcome this problem.

In conclusion, bead beating was a rapid and simple root crushing method, especially useful when many samples were analyzed. Method B provided greater reliability, and Method A provided better cost performance. Both techniques can be useful for PCR-based applications, such as restriction fragment length polymorphism, to identify species and estimate species composition after measuring mycorrhizal colonization rate with trypan-blue staining.

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