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Note

Direct amplification of DNA from fresh and preserved ectomycorrhizal root tips

Elizabeth Bent *, D. Lee Taylor

Institute of Arctic Biology, University of Alaska, Fairbanks AK 99775, United States

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ABSTRACT

Methods are described by which DNA can be amplified directly from ectomycorrhizal root tip homogenates of a variety of plant species (*Picea mariana* (black spruce), *Betula papyrifera* (paper birch), *Populus tremuloides* (trembling aspen) and *Alnus* sp.(alder)), including root tips that have been preserved in RNA Later (Ambion, Austin, TX). In most cases for extracts and homogenates diluted 10-fold prior to PCR, and in all cases for 100-fold dilutions, direct amplification of DNA from fresh root tip homogenates yielded as many or more ng of PCR amplicon (fungal ITS region) than amplification of DNA extracted from the same tips using a commercial kit or a manual ethanol precipitation-based method. For alder root tip extracts diluted 10-fold, the commercial kit method yielded more ng of PCR amplicon than 10-fold diluted, although direct use of homogenates still resulted in amplification in all tips tested. We also demonstrate consistent amplification of DNA from homogenates of birch, spruce and aspen ectomycorrhizal root tips preserved for 4 months in RNA Later.

Community analyses of ectomycorrhizal (ECM) fungi rely on the characterization of ectomycorrhizal root tips. In order to obtain a representative sampling of the ECM fungi present on a given plant or within a given soil core, multiple tips need to be analyzed. If there are many samples, or if many tips per sample are to be analyzed, the number of tips to be analyzed can quickly make molecular analysis of individual tips expensive and unwieldy. One can pool multiple root tips from a given sample and extract and analyze DNA from the pooled sample, but this complicates the interpretation of results. Unless each tip is of similar size and contains similar numbers of gene or gene region copies (for example, the nuclear internal transcribed spacer region (ITS) between ribosomal RNA genes is frequently used) which amplify and clone with similar efficiencies, it can be difficult to assess how many root tips were colonized by fungi possessing each DNA sequence.

We wished to find a way to analyze fungal DNA from many individual root tips sampled from a variety of boreal forest plant species that would reliably produce amplicons, yet could be accommodated within a small budget. We also wished to analyze DNA from root tips that were either frozen, or preserved in RNA Later (Ambion, Austin, TX). RNA Later is useful because it preserves both sample morphology and nucleic acids for long periods of storage (Lader, 2001; data not shown). It is difficult if not impossible to process the ectomycorrhizal root tips from many fresh root samples quickly enough to avoid sample rotting, and so it is useful to be able to place sampled roots in RNA Later and subsequently analyze ectomycorrhizal root tips in terms of both morphotype (Agerer, 1987–1997) and genotype. This is the first published study to our knowledge to compare direct amplification of ectomycorrhizal root tip DNA with the amplification of DNA extracted from the same tips using different methods, and the first to report direct amplification of DNA from ectomycorrhizal root tips treated with RNA Later.

For the analysis of frozen root tissue, four root samples (one each of *Picea mariana* (black spruce), *Betula papyrifera* (paper birch), *Populus tremuloides* (trembling aspen) and *Alnus* sp. (alder)) were obtained from the campus of the University of Alaska in Fairbanks. Roots were carefully washed under tap water to remove coarse debris then examined under a dissecting microscope. Six root tips were removed from roots in each sample that could either be traced back to the stem of the plant, or in the case of the alder root sample, could be traced to a root nodule. Each root tip was frozen at −80 °C and lyophilized. We wished to compare three methods of extracting DNA from these tips, so each tip was ground in 40 µl of nanopure water within a 0.6 ml microfuge tube using a grinder and sterile pestle of appropriate size, and aliquots of this homogenate were subjected to different methods. Root homogenates were kept on ice until they could be used in DNA extraction. DNA was extracted from 20 µl of each homogenate using a commercial kit (DNEasy Plant Mini Kit, Qiagen), following the manufacturer's instructions except that DNA was eluted in 30 µl using 1/10 strength buffer AE. DNA was extracted from 10 µl of each homogenate using an N-phenacylthilazolium bromide (PTB)-based extraction method, which was chosen for study as it would be a less expensive DNA extraction method than the use of the kit. The PTB method was as follows: 10 µl of root tip homogenate was mixed with 90 µl of lysis buffer (0.1 M phosphate pH 7.2, 0.01% Tween20) and vortexed for 3 s. Ten µl of freshly made 1 M PTB (heated to 65 °C) was

* Corresponding author. Tel.: +1 907 4745805.
E-mail address: esbent@alaska.edu (E. Bent).

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added and the sample vortexed an additional 3 s. Ten μl of 0.3 M sodium acetate and 200 μl ice-cold 100% ethanol were then added, and the sample was allowed to precipitate for 14–16 h at −20 °C. Precipitated DNA was pelleted by centrifugation at 4 °C at 14,000 rpm (maximum speed) for 15 min, and the pellet washed twice with 200 μl of ice-cold 70% ethanol (4 °C, 5 min, 14,000 rpm). Pellets were dried in a Speed-Vac and stored at −20 °C until use, at which time it was resuspended in 10 μl of sterile nanopure water. The remainder of each root tip homogenate (~10 μl) was simply frozen at −20 °C until use.

Dilutions (1/10, 1/100 and 1/1000) of each homogenate or DNA extract were prepared using sterile nanopure water, and subjected to PCR amplification, as follows: fifteen μl reaction mixes were prepared containing 0.65 mM MgCl2, 0.2 mM dNTPs, 0.05 μM forward primer ITS1F (CTTGGTCATTTAGAGGAAGTAA; Gardes and Bruns, 1993), 0.05 μM reverse primer ITS4 (TCCTCCGCTTATTGATATGC; White et al., 1990), 0.06 mg/ml bovine serum albumin, 0.15 μl JumpStart RED Taq (Sigma-Aldrich), 1X JumpStart RED Taq buffer and 1.5 μl of diluted homogenate or DNA extract. Reaction mixes were prepared in 0.2 μl tubes and thermocycled in an MJ Research PTC-225 thermal cycler as follows: 96 °C for 3 min, 35 cycles of 94 °C for 30 s, 52 °C for 30 s, then 72 °C for 3 min, followed by 72 °C for 10 min. 5 μl of each reaction mix was electrophoretically separated on an agarose gel in TBE buffer (0.8% agarose, 100 V, 40 min) with 5 μl of MassRuler DNA Ladder Mix (Fermentas Life Sciences, Glen Burnie, MD) used as a standard. Gels were stained with ethidium bromide, visualized using a Kodak Gel Logic 200 Imaging System and the ng of DNA in each lane determined densitometrically from the average intensities of each band (i.e. using the MassRuler bands of known density to generate a standard curve). Amplicons generated directly from homogenates were run adjacent to amplicons generated from DNA extracts so that samples from the same root of the same dilution were in the same gel.

Whole roots of paper birch, black spruce and trembling aspen were placed in RNA Later within 1 week of harvesting, then kept for 6 months at 4 °C, after which roots were placed in dishes of nanopure water. Ectomycorrhizal root tips were identified using a dissecting microscope and individual root tips removed with forceps, frozen individually in a small amount of nanopure water and lyophilized. Twenty-one tips (8 spruce tips, 6 aspen tips, and 7 birch tips) were homogenized with a pestle in 0.6 ml tubes as described earlier, and DNA immediately extracted using a modified commercial kit protocol. The Qiagen DNEasy Plant Mini Kit was used, except that after the addition of RNAse A and incubation at 65 °C, 6 μl of a Proteinase K solution (3.24 mg/ml, in Tris-EDTA (TE) buffer pH 8.0) was added and the mixtures incubated at 55 °C for 4 h. Ambion recommends the use of Proteinase K and this length of incubation time for the extraction of DNA due to protein-DNA binding in tissues under the influence of RNA Later. The standard kit protocol resumed after this step, and the only other modification to it was that DNA was eluted in 30 μl of 1/10 strength kit buffer AE.

A 'direct' PCR method was also used on thirty additional tips (10 tips each from spruce, aspen and birch, with five of these being turgid 'healthy' tips and five being wrinkled, desiccated 'unhealthy' or old tips). These tips were homogenized and prepared as follows: each tip was ground in 20 μl of TE buffer (pH 8.0) within a 0.6 ml microfuge tube using a grinder and sterile pestle of appropriate size. To each homogenate, 6 μl of a Proteinase K solution (3.24 mg/ml, in TE buffer pH 8.0) was added. The homogenates were mixed, then incubated at 55 °C for 4 h. After this, the temperature of the water bath the mixtures were in was increased to 75 °C. Once the temperature reached 75 °C, the mixtures were incubated an additional hour, in order to inactivate the Proteinase K. Each sample was centrifuged (14,000 rpm or maximum speed for 5 min) and the supernatant transferred to a clean tube. DNA extracts and 'direct' method homogenates were diluted 1/100 in sterile nanopure water and subjected to PCR as described earlier. Amplicons were electrophoretically separated on agarose gels and stained in ethidium bromide as described earlier. For the thirty 'direct' method homogenates, the number of bands present in 'healthy' vs. 'unhealthy' tips was compared, but otherwise samples were only assessed for their ability to produce amplifiable DNA.

Table 1 shows the amount of PCR product produced per lane (reported as the relative percentage of the maximum amount from one sample) by each extraction method for 1/10 and 1/100 diluted samples from spruce, aspen, birch and alder. The amount of PCR product produced per lane was compared for each plant species and extraction method within one dilution via a Student's t pairwise analysis of the three possible paired extraction method combinations ($P<0.05$). Comparisons between plant species were not made. For samples diluted 10-fold from spruce, aspen and birch, direct amplification of DNA from a homogenate produced on average as much amplified DNA as the commercial kit method, and more than the PTB extraction method (Table 1). For samples diluted 10-fold from alder, the commercial kit method produced more amplified DNA than the PTB method and a numerically greater amount than the direct PCR method, though this difference was not significant (Table 1). For spruce, aspen and birch samples diluted 100-fold, direct amplification of DNA from a homogenate produced more DNA on average than from either of the methods involving DNA extraction. For alder samples diluted 100-fold, no difference in amplfiable DNA was seen between the direct PCR method and the commercial kit method (Table 1). Samples diluted 1000-fold demonstrated amplification for only 70% of samples, and so were not analyzed further. These results probably reflect the fact that DNA is lost during extraction, and that the PCR inhibitors expected to be present in a simple homogenate can be overcome by dilution, provided that the initial concentration of DNA is such that the dilution does not remove too much template. Alder tissue possesses an abundance of PCR-inhibitory compounds, which may explain why direct PCR amplification of a simple homogenate did not work so well with tissue from this species.

There was no difference between extraction methods and the number of bands observed for either 10-fold or 100-fold dilutions for any of the plant species tested. It was noted, however, that in samples for which there were multiple bands present in a lane, the number of bands tended to decrease as the samples were diluted (data not shown). This reflects the fact that templates present in lower quantity in the original sample can be titrated out by dilution. If a single band representing the most abundant template is desired in each lane, in

Table 1
Average ($n=6$) percent relative amplification[1] of DNA obtained from single ECM-infected root tips processed three different ways. Shared lowercase letters after values within rows (comparisons were made within each row only) indicate no significant difference (Student's t, $P<0.05$).

Plant species	Diluted 10-fold prior to PCR		
	Direct[2]	PTB[3]	Kit[4]
Spruce	92 a	48 a	76 a
Birch	86 a	66 a	75 a
Aspen	70 a	58 a	59 a
Alder	51 ab	36 a	88 b
	Diluted 100-fold prior to PCR		
	Direct[2]	PTB[3]	Kit[4]
Spruce	100 a	36 b	55 b
Birch	100 a	35 b	25 b
Aspen	80 a	17 b	51 ab
Alder	40 ab	0.1 a	69 b

[1]Relative amplification = band intensity in lane/maximum band intensity in any lane associated with the same root tip and dilution, reported here as percentages. This was calculated for each lane so that any variation in total amplifiable DNA from different root tips would not confound the results.
[2]Root tip homogenate is used directly in PCR.
[3]Root tip homogenate is processed using the PTB-based method described in the text.
[4]Root tip homogenate is processed using a commercial kit-based method described in the text.

most cases careful dilution of samples can be used to achieve this (data not shown; J. McFarland, pers. comm.).

Attempts to extract DNA from root tips treated with the preservative RNA Later without a Proteinase K digestion step failed, even when commercial kits were used (S. Hopkins, pers. comm.). Digestion with Proteinase K yielded amplifiable DNA from RNA Later-treated root tips in all cases when used in conjunction with the commercial kit. Digestion of RNA Later-treated root tip homogenates with Proteinase K, followed by a simple heat inactivation step (or, 'direct' PCR from RNA Later tips), was sufficient to release PCR-amplifiable DNA when diluted 100-fold (no other dilutions were tested) from all the spruce and birch root tips tested, and 80% of aspen root tips. There were no differences between the average number of bands observed in each lane between root tips designated as 'healthy' vs. root tips designated as 'unhealthy' (data not shown), though it should be noted that the two aspen root tips which did not produce amplifiable DNA were considered 'unhealthy'.

In summary, it is possible to use rapid and inexpensive 'direct' methods to obtain amplifiable DNA from a variety of seedling root tip tissues, even after those tissues have been preserved in RNA Later. Amplification of ectomycorrhizal DNA directly from segments of infected root tips has been reported (Iotti and Zambonelli, 2006), but to our knowledge this is the first report of an inexpensive method for the recovery of PCR-amplifiable DNA from RNA Later-treated root tissue.

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References

Agerer, R., 1987–1997. Colour Atlas of Ectomycorrhizae, 1-8 edn. Einhorn-Verlag, Germany.
Gardes, M., Bruns, T., 1993. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Molecular Ecology Notes 2, 113–118.
Iotti, M., Zambonelli, A., 2006. A quick and precise technique or identifying ectomycorrhizas by PCR. Mycological Research 110, 60–65.
Lader, E., 2001. Methods and reagents for preserving RNA in cell and tissue samples, http://www.freepatentsonline.com/6204375.html, Vol. 6204375, Ambion, Inc. (Austin, TX), United States.
White, T., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M., Gelfand, D., Sninsky, J., White, T. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, New York, pp. 315–322.