Polish Journal of Environmental Studies Vol. 8, No. 3 (1999), 161-163

Identification by PCR-RFLP of a Fungus Isolated from Mycorrhizal Roots of a Distinguishable Birch Growing in Areas Disturbed by Industry

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Received 18 December, 1998
Accepted 6 February, 1999

Abstract

Different protective mechanisms are induced in plants against pollutants when they grow at extreme life conditions in contaminated areas. Ectomycorrhizal mutualistic plant-fungus symbiosis is one of these mechanisms. Some trees are better adapted to these unfavourable conditions than others. The main reason for this phenomenon may be success in finding the proper symbiotic fungus. The result presents the process of determination of fungal species forming ectomycorrhiza with roots of a birch. The birch was distinguished by good growth and development, in spite of the fact the tree grew in the vicinity of a zinc and lead plant. The identification of mycorrhizal fungus was performed comparing a restriction fragments' pattern of the fungal ITS region with DNA isolated from fruiting bodies of *Basidiomycetes* fungi growing in the experimental area. Restriction analysis was preceded by PCR amplification of the ITS region.

Keywords: PCR, ectomycorrhizae, mycorrhizal fungi.

Introduction

Extreme conditions for growth and development of plants are often found in areas surrounding nonferrous plants, industrial works emitting heavy metal compounds into the atmosphere and in metallurgic heaps. Trees growing there are mostly dwarf and deformed. However, some single specimens distinguishable by good growth and appearance may be found in these areas. All trees originate from the same sowable material. Initial field experiments have revealed that the fine growing specimens showed higher mycorrhizal frequency than the dwarf trees. A positive role of mycorrhizal fungi is commonly known [1, 13, 14]. It may be conjectured that symbiotic interactions with proper fungi may be responsible for better growth and development of trees growing in degraded areas. It is interesting to learn about fungal species that best coexist with trees in polluted habitats. The aim of this study was to identify the species of fungus which symbiotically coexists with birch, distinguishable by good growth and appearance (A,), growing in the vicinity of a nonferrous plant.

Materials and Methods

The experimental field incorporated birch planted in industrial waste surrounding the nonferrous plant Miasteczko Slaskie. The trees growing here were 14 years old and they originated from homogenous sowable material. The root morphological and anatomical analysis of birch $A_{(}$ to determine the fungal species forming mycorrhiza was unsuccessful. Therefore, a comparative identification at DNA level was decided. Fruiting bodies of fungi belonging to *Basidiomycetes* that grew in the forest region around the plant Miasteczko Sla.skie were harvested in autumn 1997. The clean cultures of vegetative mycelium were isolated from the fruiting bodies by the plate method (Table 1), according to Pachlewski [9].

The mycorrhizal fungus of birch A, was harvested in spring 1998. The fungal DNA was isolated from axenic agar cultures by the method of Raeder and Broda [11]; Doyle and Doyle [2]. The mycelium was homogenized in CTAB buffer and then a fine suspension was placed in Eppendorf vials [3]. The vials were incubated at 60°C for

Table 1. Species of fungi belonging to *Basidiomycetes* isolated from fruitbodies growing around the plant Miasteczko Slaskie.

Simbild	Species	Date of isolation
011	*Amanita muscaria*	20.08.1997
012	*Xerocomus badius*	25.08.1997
014	*Amanita citrina*	20.08.1997
017	*Paxillus involutus*	02.09.1997
018	*Hebeloma crustuliniforme*	02.09.1997

30 min and then a mixture of chloroform-isoamyl alcohol (24:1) was added. After short centrifugation (1600 g) a top phase was collected into vials. DNA was precipitated with isopropanol and after incubation for 20 min at 20°C it was centrifuged for 15 min at 18,300 g. The pellet was washed with 70% ethanol, dried and resuspended in 10 1 sterile distilled water.

Amplification of Allelic Fragments of DNA

An allelic region of DNA used for identification of fungal taxa is a nonencoding fragment between 18S rRNA and 5.8S rRNA called ITS (internal transcribed spacer) [15, 3]. It was decided to amplify this fragment by PCR. Reaction mixture for PCR contained 35 µl of distilled water, 5 µl of PCR buffer (Promega), 4 µl of 25 mM $MgCl_2$ (Promega), 4 µl of each deoxynucleoside triphosphate at a concentration of 10 nM (Boehringer Mannheim), 1 µl of each primer (ITS4 and ITS5) at a concentration of 50 pM (Gibko BRL) and 1 µl of isolated DNA (30 ng). The sequences of primers ITS4 and ITS5 were 5'-TCCTCCGCTTATTGATATGC-3' and 5'-GGAAGTAAAAGTCGTAACAAGG-3' respectively. The reaction was performed in Personal Cycler Version 3.02 (Biometra) with initial denaturation of DNA for 10 min at 95°C and then 44 cycles consisting of denaturation for 90 s at 95°C, primer annealing for 30 s at 54°C, *Taq* polimerase annealing for 90 s at 92°C, and extension for 60 s at 72°C.

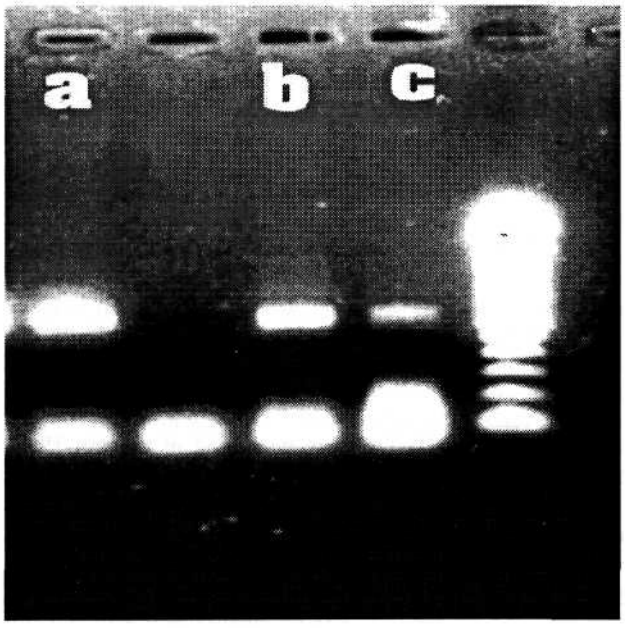

Fig. 1. Amplification products of a ITS region isolated from *H. crustuliniforme* (a), *A. muscaria* (b), an unknown mycorrhizal fungus (c), separated on 1% agarose.

Analysis of PCR Products

An aliquot (8 µl) of PCR reaction mixture with added 2 1 of brillant blue buffer was analyzed on 1% agarose gel in the presence of a 100 base pair marker (Gibko BRL) for 40 min, and then the gel was viewed and photographed under UV light.

Restriction Analysis of PCR Products

An aliquot (8 µl) of positive PCR product was incubated with the addition of appropriate restriction enzyme (Table 2).

After incubation, an aliquot of 2.5 µl of brillant blue buffer was added to the reaction mixture and analyzed for products by electrophoresis on 2% agarose gel.

Results and Discussion

The result of correct PCR was 600 bp fragment of fungal DNA. The results for *H. crustuliniforme*, *A. muscaria* and the unknown species (X) isolated from roots are presented in Figure I.

The reaction of digestion of PCR products by restriction enzymes allowed us to obtain the patterns specific for each fungal species. Comparison of received patterns of known fungal species with that of the unknown root isolate (X) facilitated identification of the unknown X as *H. crustuliniforme* (Fig. 2).

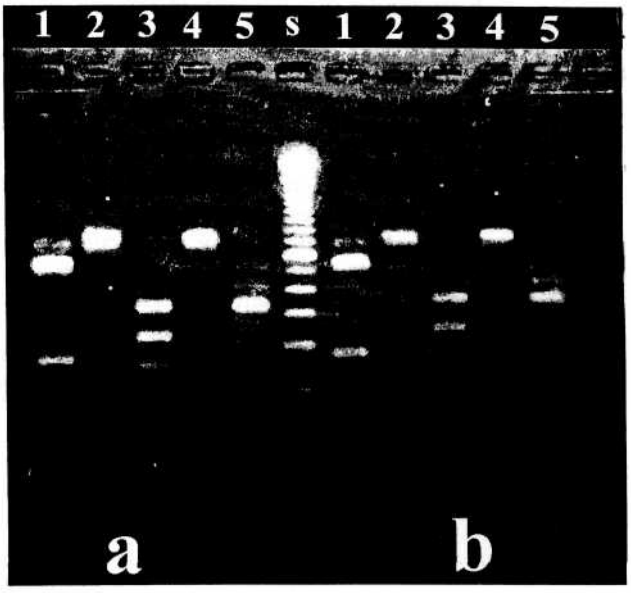

Fig. 2. Comparison of patterns obtained after restriction of PCR products with enzymes: 1. Alu I, 2. Bsur I, 3. Hinf I, 4. Hpa II, 5. Taq I.

a. *H. crustuliniforme* b. an unknown fungus X1

Table 2. Restriction enzymes, buffers and incubation temperatures used in the restriction analysis of PCR products.

Restriction enzymes	Buffers	Incubation temperatures [°C]
Alu I (MBI Fermentas)	Y + BSA (MBI Ferments)	37
Bsur I (MBI Fermentas)	React 2 (Gibco)	37
Hinf I (MBI Fermentas)	R + BSA (MBI Fermentas)	37
Hpa II (MBI Fermentas)	B (MBI Fermentas)	37
Taq I (New England Biolabs Inc.)	React 2 (Gibco)	65

Results of these experiments should be treated as an initial stage in studies aimed at checking the PCR-RLFP method for identification of mycorrhizal fungi successfully coexisting with trees in habitats contaminated by heavy metals. The unknown symbiotic fungus of distinguishable birch A| (identified as *Hebeloma* sp. in this work) has been known as a perfect mycorrhizal fungus of pine [10, 4]. Good coexistence of this fungus with birch in areas disturbed by industry would give *H. crustuliniforme* the status of universal fungus, which could be a perfect material for artificial inoculations of tree seedlings.

However, this phenomenon requires further extensive studies. It was noteworthy that fruitbodies of *H. crustuliniforme* did not grow in great numbers in the experimental field. This contradicts the theory that trees coexist successfully with those fungi that form many fruitbodies.

The PCR method used in this study becomes a common technique for identification of bacteria and fungi's taxa. To identify species of *Basidiomycetes,* different regions of fungal genome have been used, e.g. the gene responsible for chitin synthesis [8] or the *gpd* gene encoding glyceraldehyde-3-phosphate dehydrogenase [5, 6]. In this study, amplification of specific region ITS of fungal rDNA, commonly exploited for identification of fungi was used [15]. Gardes [3] indicated special usefulness of this allelic DNA region for identification of fungi belonging to *Basidiomycetes.* The ITS region and PCR-RFLP analysis were successfully used for identification of ectomycorrhizal morphotypes of *Scots pine* by Timonen [12]. The correct identification of the unknown symbiotic fungus of birch permits us to conclude that the PCR-RFLP method may be useful for identification of mycorrhizal fungi. It may be also applied as a tool for monitoring of fungal colonization of tree roots growing in polluted environments.

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