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Full Length Research Paper

Molecular characterisation of a mycorrhizal inoculant that enhances *Trifolium alexandrium* resistance under water stress conditions

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The occurrence of drought is an economically important problem in Morocco. The use of mycorrhizal technology offers a possibility to overcome this problem. A mycorrhizal fungal inoculum "Aoufous Complex" isolated in Morocco was shown to enhance *Trifolium alexandrium* resistance in water deficit situation. The efficiency of this inoculum was confirmed in this study. In order to identify this inoculum, specific primers were used to amplify the 18S subunit. The *Alu*I RFLP typing of the PCR products revealed a single pattern showing no diversity. Phylogenetic analyses of seven sequences including other glomeromycetes allowed an unambiguous identification of the "Aoufous Complex" as *Glomus mosseae* strains.

Keywords: Water stress, mycorrhiza, Aoufous Complex.

INTRODUCTION

Of all stresses having negative effects on plant growth, water stress is the most lethal. It is a crucial limiting condition occurring in Morocco notably in regions with oasis where hydrous stress continuously prevails. Therefore, this is an economically important problem since productivity has drastically decreased this decade. Different approaches can be used to overcome such situation. Of them, the integration of mycorrhization is of fundamental interest. Mycorrhizae are a symbiosis between the roots of most land plants (Newman and Redell, 1987; Simon et al., 1992) and some soil fungi belonging to Glomeromycota (Schüssler et al., 2001). In fact the mycorrhizal symbiosis helps plants to acquire phosphate and other nutrients from soil. Moreover, the endosymbiosis is known to increase plant water resistance (Fitter, 1988; Smith and Read 1996, Smith et al. 1993). Under many natural or man made water stress conditions, arbuscular mycorrhizal fungi can play an important role in maintaining plant populations and crop yields (Bethlenfalvay and Linderman, 1992; Barea et al., 1993). The contribution of mycorrhization to the plant water stress resistance depends on the efficiency of the inoculum. There is strong evidence that drastic water limiting conditions can affect the mycorrhizal rate in the root of plants. That is why there is a need to make a preliminary selection of efficient mycorhizal inoculum. In Morocco, efficient mycorrhizal inoculum (Oihabi and Meddich, 1996) that improves *Trifolium alexandrium* water resistance (Meddich et al., 2000) has been selected from the desert. Therefore, the use of mycorrhizal biotechnology to improve plant water resistance is being anticipated in Morocco. However, this implies a quick and efficient identification of the fungal incolum. Among the mycorrhizal fungi tested as inoculum to improve *T. alexandrium* water resistance in water stress condition (Meddich et al., 2000), is the so called "Aoufous Complex" that has been shown to have a higher potential. For this reason, it was important to identify this efficient mycorrhizal inoculum for a better monitoring if it is to be used.

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In this paper, we confirmed the ability of the "Aoufus Complex" inoculum to enhance *T. alexandrium* plants resistance when a severe water stress condition is applied. Molecular techniques (Helgason et al., 1999) were adapted to identify the "Aoufous Complex" inoculum.

MATERIAL AND METHODS

Plant material

One week-old *T. alexandrium* seedlings were inoculated with the arbuscular mycorrhizal inoculum originated from the same natural environment as well as the *T. alexandrium* plants. Inoculated and uninoculated *T. alexandrium* were planted in buckets (16 cm height and 20 cm diameter) filled with an autoclaved mixture of peat and sand (2:1) and placed in a greenhouse.

Hydrous constraints application

In order to estimate the influence of the mycorrhizal inoculant on plant growth in water deficit conditions, water was supplied at 30% equivalent to the soil capacity. The hydrous constraints at 30% were applied to plants as previously described by Tobar et al. (1994). The weight of each bucket containing plants was first measured and the soil was watered until saturation. The bucket was weighted again after draining excess water by gravity. The difference between the two measures corresponds to soil field capacity (M). In order to apply a hydrous constraint at 30%, each bucket was filled with water volumes corresponding to 0.30 x M. The buckets were weighted again and watered two times a day up to the initial soil field capacity volume corresponding to 30% during two months.

Measuring water deficit tolerance

After two months, five *T. alexandrium* plants were collected in order to determine the effect of the hydrous constraints on their development in presence or absence of the mycorrhizal inoculant. The extent of colonisation in roots of mycorrhizal plants were analysed as described by Trouvelot et al. (1986). In order to estimate plant resistance to the water deficit conditions applied, leave numbers, length of aerial parts and biomasses were measured. Water contents and relative water contents were also determined as described by Turner (1981). Proline a stress indicator, was also measured according to Dreier and Goring (1974). Statistical analyses were performed with STATITCF software using the Newman and Keuls test at 5% threshold.

Molecular identification of the mycorrhizal inoculant

DNA was extracted from roots of mycorrhizal *T. alexandrium* using DNeasy^R (QIAGEN) DNA extraction kit according to the supplier. Partial SSU DNA fragments were amplified with Taqplus precision (Promega) using a universal eukaryotic primer NS31 (Simon et al., 1992) and a general fungal primer AM1 (Heglason et al., 1999) designed to exclude plant DNA sequences. The PCR was conducted as described by Hegalson et al. (1999). Resulting PCR products were cloned into a pGEM-T vector (Promega) according to the manufacturer. The cloned products were transformed into *Escherichia coli* JM109 competent cells from Promega according to the manufacturer. Transformed cells (100µl) were plated on LB containing 100µg/ml ampicillin, 0.5mM IPTG and 40µg/ml X-Gal. In order to select positive clones for the presence of inserts, teeth sticks were used to harvest cells from white colonies in 100µl of sterile water. After boiling for 5 min, 5µl were used for PCR amplification in a volume of 50µl using primers as described above. Putative positive inserts were classified by RFLP using *Alu*I. A representative of each RFLP type was sequenced and phylogenetically identified using ClustalW (Thompson et al., 1994).

RESULTS

The mycorrhizal inoculant is benefical to *T. alexandrium* plants under water deficit conditions

The growth of *T. alexandrium* plant under water deficit conditions for two months was estimated by measuring different parameters. No mycorrhizal fungal structures could be detected in non-inoculated roots while inoculated plants harboured 75% root colonisation. The numbers of leaves from mycorrhizal and nonmycorhizal *T. alexandrium* are shown in Table 1. These results clearly show that mycorrhizal *T. alexandrium* plants developed more leaves than uninoculated plants. The inoculated plants showed a more extensive growth of the aerial parts compared to the non-inoculated plants (Table 1). Moreover, the plant biomass was significantly increased in mycorrhizal plants. It was shown that mycorrhizal plants had higher water content than nonmycorrhizal plants under the same limited water conditions supplied (Table 2).

Molecular identification of the mycorrhizal inoculant

As part of an integrated effort to compete drought in Morocco, mycorrhizal fungi are being used as a sustainable strategy. A collection of mycorrhizal fungi originnating from a region in Morocco where drought is the most prevalent environmental stress causing low crop poduction is under way. It means that identification of these collected mycorrhizal fungi are of importance. A quick and effecient molecular method was needed. The mycorrhizal inoculum used in this study was initially selected because of its positive influence on plant growth. The *T. alexandrium* plants and the mycorrhizal inoculum used as biological models originated from the same desert area. In order to identify this mycorrhizal fungal inoculum, a molecular approach developed by Helgason et al. (1999) was adapted. The small subunit 18S from the roots of mycorrhizal *T. alexandrium* was amplified using primers NS31 and AM1. An expected 583 bp PCR products was obtained (Figure.1). After cloning, putative positive clones were PCR-amplified (an example is shown in Figure 2) and digested with the enzyme *Alu*I. A single RFLP type was identified (Figure. 3). In order to differentiate between these *Alu*I types, seven fragments were randomly chosen for complete sequencing. Sequence alignment of seven representatives of the *Alu*I pattern identified showed a 97-100% nucleotide similarity. Phylogenetic analyses including other mycorrhizal

Table 1. Effects of the water stress condition, on leave numbers, aerial part length, mass of fresh and dry matter in mycorrhzal and nonmycorrhizal *T. alexandrium*.

	Nonmycorrhizal plants	mycorrhizal plants
Leave numbers	5a	11.5b
Length of aerial parts	18.25a	34.38b
Mass of fresh matter (g)	0.2a	1.55b
Mass of dry matter (g)	0.05a	0.25b

Values followed by the same letter are not significantly different P<0.05 (Table have to be read by line).

Table 2. Water and proline content in mycorrhizal and nonmycorrhizal *T. alexandrium* plants.

	Nonmycorrhizal plants	mycorrhizal plants
Water content (g/g DM)	3.03a	4.74b
Quantity of proline (µg/g FM)	43.06a	23.06b

Values followed by the same letter are not significantly different at p<0.05 (Table have to be read by line) .
FM, Fresh matter; and DM, dry matter.

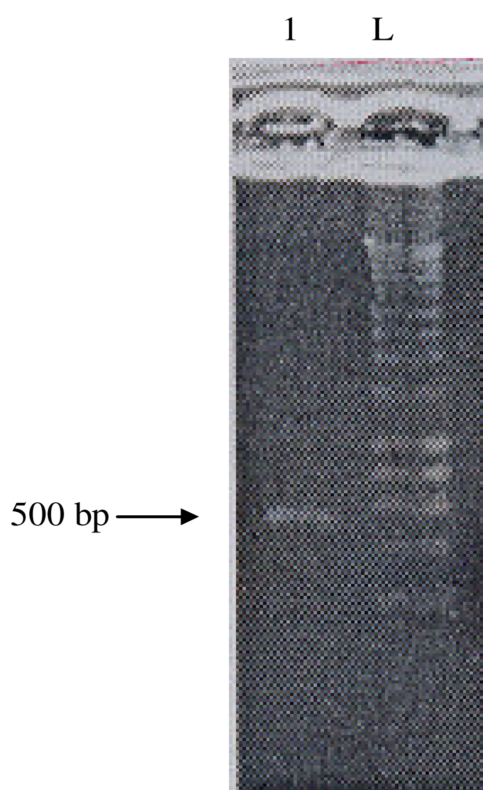

Figure 1. Amplification with primers NS31 and AM1. Lane 1 represents PCR products amplified from total DNA of a mycorrhizal *Trifolium alexandrium* roots. Lane L is the 100 bp ladder.

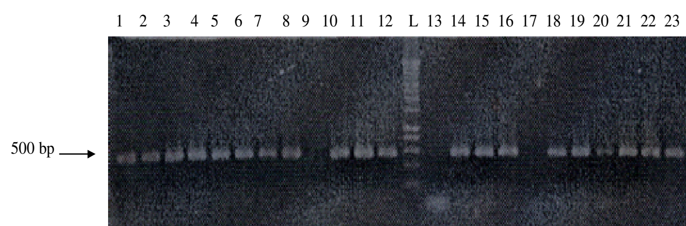

Figure 2. Amplification of putative positive clones using primers NS31 and AM1. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 14, 15, 16, 18, 19, 20, 21, 22, 23 represent genuine positive clones while lanes 9, 13, 17 represent negative ones. Lane L represents 100 bp ladder.

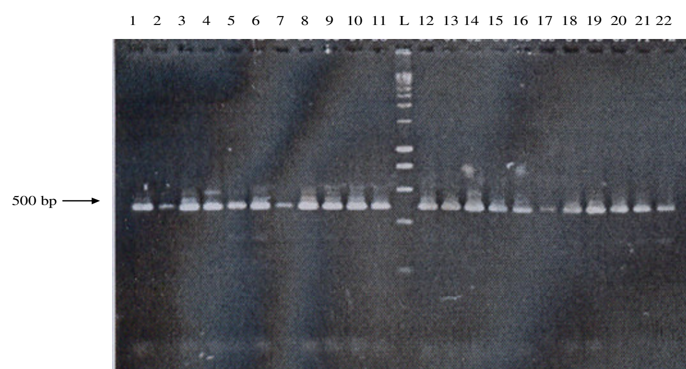

Figure 3. Electrophoresis on 4% Nusieve agarose gel of *Alu*I digested fragments. Lanes 1-22 represent digested products. Lane L represents 100 bp ladder.

fungi showed that these sequences (Figure 4) cluster with well supported bootstrap values demonstrating that the mycorrhizal inoculum is essentially composed of *Glomus mosseae* strains.

DISCUSSION

The “Aoufous complex inoculum” was used to assess water deficit tolerance of *T. alexandrium* mycorrhizal

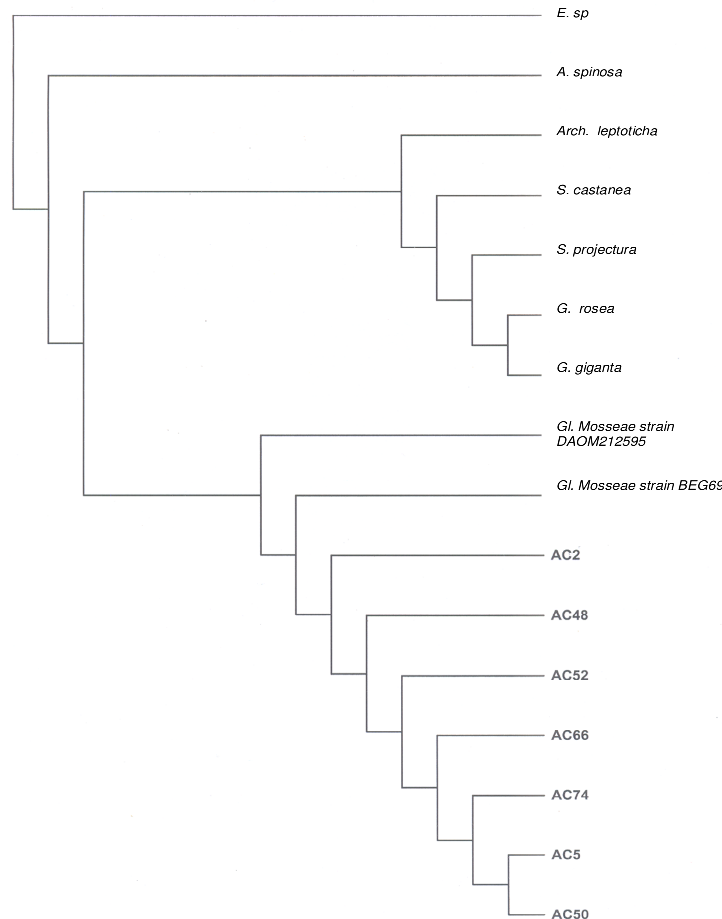

Figure 4. Dendrogram showing the identification of the mycorrhizal inoculum cloned fragments (AC2, AC48, AC52, AC66, AC74, AC5 and AC50). Glomeromycete sequences from databases including U96141 (*Glomus mosseae* BEG69), U96143 (*Glomus mosseae* DAOM212595), Z14010 (*Gigaspora giganta*), X58726 (*Gigaspora rosea*), AJ242729 (*Scutellospora projecturata*), AF038590 (*Scutellospora castanea*), AJOO6466 (*Archaeospora leptoticha*), and Z14004 (*Acaulospora spinosa*), an outgroup Z14011(*Entrophospora sp.*) were used for sequence comparison using ClustalW (Thompson et al. 1994).

plants. It was shown that *T. alexandrium* mycorrhizal plants had a better resistance to water stress compared to nonmycorrhizal plants (Meddich et al., 2000). In this work, we confirmed the potential of this inoculum. For this purpose, the water limiting condition applied at 30% was shown to be severe for the nonmycorrhizal plants asindicated by the quantity of proline, a plant stress indicator (Table 2). It was shown that proline is highly produced in nonmycorrhizal plants, indicating the severity of the water stress. In contrast, mycorrhizal plants showed a lower proline quantity and higher water content. Consequently, the presence of the mycorrhizal inoculant reduces the effect of the water stress in *T. alexandrium* plants. The molecular method (Helgason et al., 1999) is confirmed to be a powerful strategy to quickly and efficiently identify mycorrhizal fungi. Different genera representing glomeromyctes and *Entrophospor*a sp. as outgroup were used to identify the “Aoufous Complex”. It allowed us to unambiguously identify the mycorrhizal inoculum as *G. mosseae*. However the seven sequences analysed may represent different strains because discrete sequence groups could be identified (Figure 4). It means that the “Aoufous complex” inoculum is composed of many *G. mosseae* strains which act synergically. That may contribute to the efficiency of this inoculum allowing a better resistance of *T. alexandrium* plants to drought.

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