Effect of NaCl on growth and ion relations in two salt-tolerant strains of *Paxillus involutus*

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Abstract The effect of NaCl on growth, biomass and ion relations of two salt-tolerant isolates of *Paxillus involutus*, MAJ and NAU were investigated. The two *Paxillus* strains were exposed to the following concentrations of NaCl: 0, 100, 200 and 500 mmol·L⁻¹. Growth of MAJ and NAU was enhanced by 100 mmol·L⁻¹ NaCl but severely inhibited at the concentration of 500 mmol·L⁻¹. NAU exhibited a greater capacity to exclude Na⁺ and Cl⁻ under all salinity levels, whereas the salt-includer MAJ had a higher capacity in nutrient uptake under salt stress. The ratios Na⁺/K⁺, Na⁺/Ca²⁺ and Na⁺/Mg²⁺ in NaCl-treated MAJ and NAU did not increase at levels of 100 and 200 mmol·L⁻¹ NaCl but markedly increased at 500 mmol·L⁻¹. This suggests that the two strains, especially MAJ, enhanced nutrient uptake corresponding to the increased Na⁺ influx at moderate salinity. We conclude that both MAJ and NAU are able to tolerate 500 mmol·L⁻¹ NaCl but there are species-specific differences in retaining ionic homeostasis in the two *Paxillus* strains. NAU is a salt-excluder, MAJ is a salt-includer but retains a high capacity in nutrient selectivity under saline conditions. Their definite mechanisms to enhance salt tolerance of mycorrhizal hosts need further study.

Key words NaCl, *Paxillus involutus*, growth, salt ions, nutrients

1 Introduction

Most plants in temperate forest form mutualistic arbor associations with ectomycorrhiza (EcM) fungi. It is estimated that at least 30% of the microbial biomass in boreal forest soils is the extraradical hyphae of EcM fungi (Högberg and Högberg, 2002). The positive effects of EcM fungi on plant production and in terrestrial ecosystem maintenance have been widely recognized. EcM are the dominant nutrient-gathering organ in temperate ecosystems and these structures provide host with nutrition, especially nitrogen and phosphorus (Pella, 1990; Bücking et al., 2002; Tibbett and Sanders, 2002). Moreover, EcM protect plants from diseases and heavy metal contamination (Shaw et al., 1990; Leyval et al., 1997; Blaudez et al., 2000).

The interactions between fungi and plant roots are rather complex and the influence of ectomycorrhizas on plant and environment is enormous. It is suggested that over 50% of CO₂ released from boreal forest soils is accounted for by the respiration of tree roots and their associated EcM fungi (Högberg et al., 2001). In

the microcosm, 20%–30% of current assimilate from seedling hosts is allocated to EcM fungi (Söderström, 2002).

Paxillus involutus is an emerging model system for the molecular analysis of EcM fungi. Its nuclear genome is relatively small, with a haploid size of 23 Mb, and contains a predicted total number of about 7,700 genes (LeQuéré et al., 2002). A strain of *P. involutus*, MAJ, is an abundant species in a number of forest ecosystems, which forms EcM with both gymnosperms and angiosperms species (Baum et al., 2000). MAJ forms a hyphae mantle of interwoven fungal on the surface of fine roots and a Hartig's net between the cells in the root (Gafur et al., 2003). The broad host range and relative ease of culture of MAJ lead to an increased studies of ectomycorrhizal symbioses. Unlike MAJ, another *P. involutus* strain, NAU is incapable of mycorrhizal synthesis under axenic conditions. Moreover, NAU was not able to penetrate the host cell walls and caused thickenings of the outer cell walls of the host, but it led to higher H₂O₂ levels in the outer epidermal wall (Gafur et al., 2003).

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Soil salinity is a worldwide problem, especially in arid and semi-arid regions. It has been found that *P. involutus* mycorrhiza attenuated NaCl-stress responses in the salt-sensitive hybrid poplar *Populus × canescens* (Langenfeld-Heyser et al., 2007). However, the contribution of *P. involutus* to ionic homeostasis in host plants and the relevance to salt stress is inconclusive. In this study, the authors used two salt-tolerant *Paxillus involutus* isolates, MAJ and NAU to clarify their differences in growth, biomass and ion relations under a wide range of salinity conditions. The results in this research indicate that the two tested *Paxillus* strains, MAJ and NAU are both salt-tolerant fungi in term of their growth response to increasing salinity. However, there are species-specific differences in retaining ionic homeostasis in the two *Paxillus* strains.

2 Materials and methods

2.1 Fungal cultures

The EcM fungi used in this study were *P. involutus* isolates, which were obtained from the Institute of Forest Botany, Göttingen University (Germany). *P. involutus* isolates were cultured in modified Melin Norkrans (MMN) agar medium. The medium contained following components in 1 L: KH_2PO_4 0.500 g, $(\text{NH}_4)_2\text{SO}_4$ 0.250 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.150 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.050 g, NaCl 0.025 g, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.01 g, glucose 10 g, malz extract 3 g and thiamine HCl 100 μg . The pH was adjusted to 4.8 using NaOH ($1 \text{ mol} \cdot \text{L}^{-1}$) before autoclaving.

2.2 Salt treatment

For salt treatment, 100, 200, and 500 $\text{mmol} \cdot \text{L}^{-1}$ NaCl were added to the culture medium, respectively. EcM fungi controls were grown at the absence of NaCl. At the initiation of salt treatment, a regular “lawn” was placed on the surface of medium (ca. 10 mm × 10 mm in size with a tooth-pick aseptically). The Petri dish was closed, sealed with a strip of ParafilmTM. All controls and salinized *P. involutus* isolates were cultured on Petri dishes (diameter 90 mm) containing the modified MMN medium (30–35 mL for each) with or without NaCl. Fungi isolates were kept in permanent darkness at 20°C and relative humidity was 60%. The experiment was repeated three times at each salinity level, 0, 100, 200 and 500 $\text{mmol} \cdot \text{L}^{-1}$ NaCl. Fungi were harvested after 30 days of salt treatment.

2.3 Growth measurement of fungi

The two strains on culture medium grew radially and the diameter reflected the growth rate of fungi during

the observation period. The diameter was measured in perpendicular direction every six days.

2.4 The biomass of mycelium

Fungi samples were dissolved in 200 mL deionized water at 60°C, and the solution was immediately filtrated with qualitative filter paper. The obtained mycelium was washed with deionized water (6°C) to remove culture medium, and then air-dried at room temperature (48 h), and dry weight was obtained.

2.5 Ion analysis

2.5.1 Chloride

Fungi samples were extracted with $1 \text{ mmol} \cdot \text{L}^{-1}$ HNO_3 as described in Storey (1995), and Cl^- was determined by a modified method of silver titration (Chen et al., 2001b). A brief description is given as follows. Abundant AgNO_3 solution ($0.025 \text{ mmol} \cdot \text{L}^{-1}$) was used to precipitate chloride of aqueous extracts and excessive Ag^+ was estimated by NH_4SCN ($0.029,76 \text{ mmol} \cdot \text{L}^{-1}$) titration. $\text{NH}_4\text{Fe}(\text{SO}_4)_2$ was used as a color indicator for ionic point determination. Chloride concentration was calculated using the following formula:

$$\text{Cl}^- (\text{mmol} \cdot \text{g}^{-1} \text{ DW}) = (N_{\text{AgNO}_3} V_1 - N_{\text{NH}_4\text{SCN}} V_2) / DW$$

where N is the molar concentration, DW (g) is the dry weight of fungi, V_1 (mL) represents the total volume of AgNO_3 solution in Cl^- extracts and V_2 (mL) is the volume of NH_4SCN solution used for excessive Ag^+ precipitation.

2.5.2 Cations

Duplicated 0.04 g were digested by $\text{H}_2\text{SO}_4\text{-H}_2\text{O}_2$, Na^+ and K^+ content were determined by a flame spectrometer (Perkin-Elmer 2280) at 589.0 and 766.5 nm, respectively. Ca^{2+} and Mg^{2+} concentrations were measured by an atomic absorption spectrophotometer (HITACHI-180) at 422.7 and 285.2 nm respectively.

3 Results

3.1 Growth

Non-salt control strains of the two *P. involutus* exhibited a vigorous growth after transformation onto new medium and maintained at a higher growth rate until day 18 (Fig. 1). The two strains, MAJ and NAU survived concentrations of NaCl up to 500 $\text{mmol} \cdot \text{L}^{-1}$,

Fig. 1 Growth of *P. involutus* strain MAJ (A) and NAU (B) under NaCl stress. Each point is the mean of their measurements and bars present the standard error of the mean.

although their growth was markedly reduced by that salinity (Fig. 1). Noteworthy, 100 mmol·L⁻¹ NaCl enhanced the growth of mycelium in both strains (Fig. 1). The same trend was observed when the two strains were subjected to 200 mmol·L⁻¹ NaCl (Fig. 1). However, growth rates of the two strains were reduced by 200 mmol·L⁻¹ NaCl after 12 days of salt treatment, in which stressed MAJ returned to control levels and salinized NAU were lower than controls (Fig. 1).

3.2 Biomass

Dry weight (DW) of NAU and MAJ were accelerated by 100 mmol·L⁻¹ NaCl after 30 days of treatment (Fig. 2). In contrast to controls, DW of MAJ and NAU increased by 19.8% and 46.5%, respectively (Fig. 2). DW of the two stains grown under 200 mmol·L⁻¹ NaCl was similar to that in controls (Fig. 2). In this study, salt-induced decline of DW was only observed at 500 mmol·L⁻¹ NaCl (Fig. 2).

3.3 Na⁺ and Cl⁻ in two fungi strains

The strain MAJ had typically higher Na⁺ and Cl⁻ than NAU under non-salt control conditions (Fig. 3A, B). Cl⁻ concentration in strain MAJ increased corresponding to the increased external saline (Fig. 3A). After exposure to 100 mmol·L⁻¹ NaCl, Na⁺ in MAJ

abruptly increased to the highest level, up to 1.13 mmol·g⁻¹ DW, and then maintained at this level under increasing salinity (Fig. 3B). In contrast to MAJ, Na⁺ and Cl⁻ in NAU did not significantly increase at 100 and 200 mmol·L⁻¹ NaCl, but markedly elevated at 500 mmol·L⁻¹ NaCl (Fig. 3A, B).

3.4 K⁺, Ca²⁺ and Mg²⁺ in two fungi strains

Control MAJ exhibited typically higher K⁺ than the strain NAU, but Ca²⁺ and Mg²⁺ levels were similar in the two strains (Fig. 3C–E). K⁺ level in MAJ increased by 56.7%–54.0% after the fungi was exposed to 100 and 200 mmol·L⁻¹ NaCl, but it returned to control levels under 500 mmol·L⁻¹ saline (Fig. 3C). Ca²⁺ and Mg²⁺ in MAJ exhibited a trend similar to that of K⁺ except that their concentrations were typically lower than K⁺ (Fig. 3C–E). In contrast to MAJ, increasing salt stress did not significantly change K⁺, Ca²⁺ and Mg²⁺ concentrations in NAU (Fig. 3C–E).

3.5 Na⁺/K⁺, Na⁺/Ca²⁺ and Na⁺/Mg²⁺ in two fungi strains

Compared with controls, Na⁺/K⁺ ratio of the two strains did not elevate under 100–200 mmol·L⁻¹ NaCl, but it increased upon 500 mmol·L⁻¹ NaCl (Fig. 4A). Na⁺/Ca²⁺ and Na⁺/Mg²⁺ resembled the trend of Na⁺/K⁺ in MAJ and NAU (Fig. 4B, C).

Fig. 2 Biomass of *P. involutus* strains MAJ and NAU under NaCl stress. Each column is the mean of their measurements and bars present the standard error of the mean.

4 Discussion

4.1 Growth and salt-tolerance of *P. involutus* fungi under salinity

In this study, 100 mmol·L⁻¹ NaCl enhanced growth rate of the tested strains, MAJ and NAU, as well as their biomass (Figs. 1 and 2). The inhibition effect of NaCl on growth was observed when the two ectomycorrhizal fungi (EcM fungi) were subjected to 500 mmol·L⁻¹ NaCl (Figs. 1 and 2). Result is accordant

Fig. 3 Concentrations of Cl⁻ (A), Na⁺ (B), K⁺ (C), Ca²⁺ (D), Mg²⁺ (E) in *P. involutus* strain MAJ and NAU under NaCl stress. Each point is the mean of their measurements and bars present the standard error of the mean.

Fig. 4 Na⁺/K⁺ (A), Na⁺/Ca²⁺ (B) and Na⁺/Mg²⁺ (C) in *P. involutus* strain MAJ and NAU under NaCl stress. Each point is the mean of their measurements and bars present the standard error of the mean.

with other studies in which a moderate salinity had positive effect on the metabolism of cytoplasm (Nagarajan and Nagarajan, 1999) whereas a high salinity decreased the content of protein and restricted the growth of mycelium (Kernaghan et al., 2002). Blomberg and Adler (1993) found that NaCl decreased the growth of both mycelium and the host plants at 50–200 mmol·L⁻¹ salinity. A variety of different *Pisolithus* species showed growth reduction in the presence of 200 mmol·L⁻¹ (Chen et al., 2001a). MAJ and NAU were salt-tolerant than the above-mentioned fungus species since the both *P. involutus* strains remained a growth rate similar to that in controls at 200 mmol·L⁻¹ NaCl (Figs. 1 and 2).

4.2 Ion relations and ionic homeostasis in fungi

There are species-specific differences in response to NaCl although NAU and MAJ are both defined as salt-tolerant fungi. Compared with NAU, MAJ exhibited a greater capacity for Na⁺ and Cl⁻ absorption under the same saline conditions (Fig. 3A, B). Therefore, MAJ is defined as a salt-includer whereas NAU is a salt-excluder.

K⁺, Ca²⁺ and Mg²⁺ in MAJ were markedly in-

creased by 100–200 mmol·L⁻¹ NaCl, whereas there were no corresponding changes in the strain NAU (Fig. 3). Results suggest that MAJ had a higher capacity in nutrient uptake under salt stress, as compared to NAU. Na⁺/K⁺, Na⁺/Ca²⁺ and Na⁺/Mg²⁺ ratios in fungi furnish direct evidence of selective ion uptake under the saline conditions. Na⁺/K⁺, Na⁺/Ca²⁺ and Na⁺/Mg²⁺ in MAJ and NAU increased upon 500 mmol·L⁻¹ NaCl stress (Fig. 4), suggesting that these strains took up more Na⁺ as compared to K⁺, Ca²⁺ and Mg²⁺. Noteworthy, Na⁺/K⁺, Na⁺/Ca²⁺ and Na⁺/Mg²⁺ in NaCl-treated MAJ and NAU did not significantly increase during the period of moderate salt stress (100–200 mmol·L⁻¹ NaCl) (Fig. 4), suggesting that the two strains enhanced nutrient uptake corresponding to the increased Na⁺ influx, especially MAJ.

4.3 Salt-tolerant mechanism in fungi and mycorrhizal host

Based on the alternate strategies to remain ionic homeostasis in MAJ and NAU, we suppose that the two tested *Paxillus* strains may enhance salt tolerance of host via different pathways:

1) NAU is a salt-excluder, presumably, this strain

may increase salt tolerance of host plants by salt exclusion. It is widely established that the capacity to exclude Cl^- and Na^+ is a key determinant of salt tolerance in glycophytes, e.g. poplars (Chen et al., 2002, 2003). Mycorrhization with a salt tolerant fungus would protect the host from uptake of excessive NaCl (Muhsin and Zwiazek, 2002; Scheloske et al., 2004).

2) MAJ is a salt-includer but remains a higher capacity to take up nutrients under saline conditions. Accordingly, host plants may benefit from the nutrient uptake assisted by the fungi MAJ. Our German colleagues found that the performance of host plant was positively affected by the *Paxillus* fungus since total biomass was greater and leaves accumulated less Na^+ than non-mycorrhizal plants (Langenfeld-Heyser et al., 2007). EDX-TEM analysis of the influence of mycorrhization on the subcellular localisation of Na^+ and Cl^- in roots showed that the hyphal mantle did not diminish salt accumulation in root cell walls, indicating that mycorrhization did not provide a physical barrier against excessive salinity (Langenfeld-Heyser et al., 2007). They conclude that improved performance of mycorrhizal poplar under salt stress may result from diminished xylem loading of Na^+ and increased supply with K^+ (Langenfeld-Heyser et al., 2007). In accordance, AM (arbuscular mycorrhizal) fungi enhanced uptake of K^+ , Mg^{2+} and Ca^{2+} and improved nutrient status of host plants under salinity conditions (Rao and Tak, 2002; Rabie and Almadini, 2005).

In conclusion, the two tested *Paxillus* strains, MAJ and NAU were both salt-tolerant fungi in term of their growth response to increasing salinity. However, there are species-specific differences in retaining ionic homeostasis in the two *Paxillus* strains. NAU is a salt-excluder, MAJ is a salt-includer but remains a higher capacity to take up nutrients under saline conditions. The two *Paxillus* strains may enhance salt tolerance of host via different pathways. In our view, the results of this study will benefit to elucidate the mechanism of mycorrhizal plants in salt tolerance.

Acknowledgements

The research was supported jointly by the Alexander von Humboldt-Stiftung/Foundation (Germany), German Science Foundation through Poplar Research Group Germany (PRG), the key project of National Natural Science Foundation of China (Grant No. 30430430), the Hi-Tech Research and Development Program of China ("863" Program, 2006AA10Z131), a Foundation for the Author of National Excellent Doctoral Dissertation of P. R. China (200152), the Teaching and Research Award Program for Outstanding Young Teachers in Higher Education Institution of MOE, PRC (2002-323). Ms. Gabriele LEHMANN is greatly acknowledged for assistance in fungi culture.

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(Received October 15, 2007 Accepted December 28, 2007)