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In Vitro Rhizogenesis of *Morus alba* by Mycorrhizal Extracts under Saline Stress

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Summary

In vitro experiments were conducted to evaluate the efficacy of arbuscular mycorrhiza (AM) colonized root extracts of four plant species namely *Trigonella foenum graecum, Ricinus communis*, *Solanum melongena* and *Morus alba* (var. *sujanpuri*) on rhizogenesis of *Morus alba* (var. *sujanpuri*) under sodium chloride (NaCl) stress. The AM fungi (*Glomus* and *Gigaspora* species) were multiplied on above-mentioned four host plant species separately and the mycorrhizal root extracts were prepared by homogenizing well-colonized mycorrhizal roots. The extracts were added to Murashige and Skoog's (MS) media containing 0.1 to 0.4 % (w/v) NaCl. The *in vitro* grown shoot cultures of *M. alba* were transferred to different AM root extract MS media for rhizogenesis. The comparisons were made between indole butyric acid (IBA) (1.0 mg l^{-1}) and AM root extracts. The AM root extracts of *M. alba* were found to be equivalent to IBA and better than other plant extracts in promoting rhizogenesis, root growth and proliferation under both (+) and (–) NaCl conditions.

Zusammenfassung

***In Vitro* Rhizogenese von salzgestressten *Morus alba* mittels Mykorrhiza-Extrakten** Es wurden *in vitro* Experimente durchgeführt, um die Wirkung von mit arbuskularer Mycorrhiza (AM) besiedelten Wurzelextrakten der vier Pflanzenarten *Trigonella foenum graecum, Ricinus communis*, *Solanum melongena* und *Morus alba* (var. *sujanpuri*) auf die Rhizogenese von *Morus alba* (var. *sujanpuri*) unter Kochsalz- (NaCl-) Stressbedingungen zu ermitteln. Die AM-Pilze (*Glomus*- und *Gigaspora*-Arten) wurden auf den vier obengenannten Wirtspflanzenarten separat vermehrt und der Mykorrhizawurzelextrakt durch Homogenisieren der reich besiedelten Mykorrhizawurzeln hergestellt. Die Extrakte wurden dem Murashige und Skoog (MS) Medium, das 0.1–0.4 % (w/v) NaCl enthielt, zugefügt. Die *in vitro* vorkultivierten Sprosskulturen von *M. alba* wurden zur Wurzelbildung auf die mit unterschiedlichen AM-Wurzelextrakten angereicherten MS-Medien umgesetzt. Es wurde zwischen Indolbuttersäure (IBA) (1.0 mg l^{-1}) und den AM-Wurzelextrakten verglichen. Die AM-Wurzelextrakte von *M. alba* erwiesen sich sowohl unter (+) als auch unter (–) NaCl-Bedingungen im Vergleich mit IBA als gleichwertig und verglichen mit den anderen Pflanzenextrakten als besser hinsichtlich Förderung der Bewurzlung, Wurzelwachstum und Proliferation.

Key words. arbuscular mycorrhiza root extracts – NaCl – rooting – tissue culture

Introduction

Mycorrhiza, a unique symbiotic association between plant roots and beneficial fungi, often produces different growth promoting hormones, mainly auxins (ULRICH 1960; TURNER 1962; MILLER 1971; GIANINAZZI-PEARSON 1996; HIRSCH et al. 1997), affecting plant root physiology, and subsequently plant growth. These phytohormones may also play a pivotal role in establishment of AM fungal-host symbiosis (SHAUL-KEINAN et al. 2002) as an external signal or as an intracellular second messenger at the plant-fungus interface (BEYRLE 1995). The growth hormones in host roots and their exudates influence the metabolism and growth of colonized fungi (GOGALA 1991; AUDENAERT et al. 2002), affect transport of fluxes of ions (POHLEVEN and GOGALA 1986; POHLEVEN 1989, 1990) and photosynthate (CLIFFORD et al. 1986) in both plants as well as fungi and similarly colonized fungal hormones influence root morphology, metabolic changes and growth of the entire plant (GOGALA 1991). Out of all the phytohormones, auxins may trigger endogenous cAMP (cyclic adenosine monophosphate) levels, which control many physiological processes in fungi (PALL 1981), increase the activity of ATPases at the plant-fungus interface (SMITH and SMITH 1990; GOGALA 1991), thus assisting mycorrhiza to enter inside host plants and promote arbuscule formation (GUNZE and HENNESSY 1980). Mycorrhizal fungi are known to protect the plants from different types of stress (DIXON et al. 1994; HIRREL and GERDEMANN 1980; NELSON and SAFIR 1982; SELVARAJ and MANIVANNAN 1997), which may possibly be due to production of certain types of hormones (BEYRLE 1995).

The phytohormones released by this mycorrhizal association, if applied *in vitro* in form of mycorrhizal

root extracts, may influence the growth of cultures, as the hormonal content of a culture medium is crucial to any sustained growth of cultures (NARAYANASWAMY 1997). Also, the effect of *in vitro* application of mycorrhizal root extracts of different plant species on rhizogenesis of shoot cultures of a plant species may be tested. As auxins are known to be the major exudates of mycorrhiza (STRZELCZYK and POKOJSKA 1984), these are possibly contained in mycorrhizal root or spore extracts and may influence rooting in shoot cultures.

Keeping these facts in view, the present study was undertaken to observe the effect of AM root extracts of four plant species namely *Trigonella foenum graecum, Ricinus communis*, *Solanum melongena* and *Morus alba* (var. *sujanpuri*) on *in vitro* rooting of *Morus alba* (var. *sujanpuri*) shoot cultures under NaCl stress.

Materials and Methods

Production of AM colonized roots of selected plant species

The seeds of *Trigonella foenum graecum, Ricinus communis* and *Solanum melongena* (4 seeds/pot), and cuttings of *Morus alba* var. *sujanpuri* (2 cuttings/pot) were sown/planted in earthen pots (at 20 pots/plant species, pot size: 25 cm mouth diameter x 30 cm depth with soil: vermicompost, 3:1). Fifty surface sterilized (with 2 % (w/v) chloramine T and 200 ppm streptomycin for 10 minutes) AM spores of *Glomus* (*G. mosseae, G. microcarpum, G. macrocarpum, G. fasciculatum*) and *Gigaspora* (*G. margarita, G.heterogama*) species were inoculated per pot. Regular watering and weeding was done and well mycorrhiza-colonized roots (monitored by the method of PHILLIPS and HAYMAN 1970) of all plant species were used after three months for preparation of mycorrhizal root extracts.

Preparation of AM root extracts

10 g of fresh mycorrhizal roots of each plant species were excised, thoroughly washed, rinsed in autoclaved distilled water (5–6 times) and treated with 0.5 % (v/v) of bleach solution ($NaOCl_2$, sodium hypochlorite). Again these were rinsed in autoclaved distilled water for 5 to 6 times and then surface sterilized by 0.1 % (w/v) $HgCl_2$ (mercuric chloride) solution for 10 minutes. The sterilized roots were again washed in autoclaved double distilled water to remove traces of $HgCl_2$. The AM root extract was prepared by homogenizing 10 g of surface sterilized mycorrhizal roots of each plant species in 25 ml of sterilized distilled water in sterilized pestle and mortar. The homogenate was filtered through Whatman No. 2 filter paper (MUNIYAMMA et al. 2000) and collected for further experiments.

Effect of AM root extracts on rhizogenesis of M. alba (in vitro studies)

The freshly prepared filtrate of AM root extracts was added to MS medium containing NaCl concentrations of 0.0 to 0.4 % (w/v). Final volume of the media was made up to one litre. The NaCl concentration for *Morus alba* cultures was kept up to 0.4 % as shoots were able to survive only up to this concentration (0.4 %). The shoot cultures were prepared from nodal explants with axillary buds on MS media containing 2.5 mg l^{-1} of BAP (6-Benzyl Amino Purine) and 0.3 mg l^{-1} of GA_3 (Gibberellic Acid) and 0.1 to 0.4 % NaCl (w/v) concentrations. The individual shoots were transferred to different AM extract media and on MS + 1.0 mg l^{-1} IBA (Indole Butyric Acid) with/without NaCl (0.0 to 0.4 %) for comparative studies.

Statistical analysis

The data were analysed by using computer statistical data analysis software STATA 7.0.

Results and Discussion

Although the root initiation was observed within 7–10 days on MS medium supplemented with 1.0 mg l^{-1} of IBA and different NaCl concentrations (Table 1), clear root formation and growth was observed only after 30 days in cultures and 3.0–5.2 cm long roots were observed after 60 days with/without NaCl (Table 2). The data on number of roots produced per shoot and root-

Table 1. Effect of *in vitro* application of AM colonized root extracts of different plant species on root initiation in *Morus alba* (average values) with different NaCl levels.

Treatments	Root initiation time (days)				
	NaCl (0.0 %)	NaCl (0.1 %)	NaCl (0.2 %)	NaCl (0.3 %)	NaCl (0.4 %)
MS (control)	22 ± 3.8	24 ± 2.8	24 ± 2.3	26 ± 2.2	28 ± 2.5
IBA (1 mg l^{-1})	7 ± 1.2	8 ± 1.5	8 ± 1.5	10 ± 2.2	10 ± 1.5
AM *M. alba* root extract	6 ± 1.2	6 ± 0.9	7 ± 0.8	7 ± 1.1	8 ± 0.8
AM *T. foenum graecum* root extract	10 ± 1.3	12 ± 1.5	15 ± 3.6	18 ± 2.3	–
AM *S. melongena* root extract	15 ± 3.4	18 ± 3.3	20 ± 3.4	22 ± 2.7	–
AM *R. communis* root extract	17 ± 2.9	20 ± 2.7	–	–	–
CD at 0.05 %	2.38	2.56	2.35	1.71	1.29

–: No growth

Table 2. Effect of *in vitro* application of AM colonized root extracts of different plant species on root length (cm) in *Morus alba* (average values) with different NaCl levels*.

Treatments	Root length (cm)				
	NaCl (0.0 %)	NaCl (0.1 %)	NaCl (0.2 %)	NaCl (0.3 %)	NaCl (0.4 %)
MS (control)	2.5±0.6	1.5±0.6	1.5±0.8	1.4±0.3	1.3±0.2
IBA (1 mg l^{-1})	5.2±0.3	4.2±0.3	3.8±0.2	3.5±0.4	3.0±0.5
AM *M. alba* root extract	5.5±0.4	4.2±0.3	3.8±0.2	3.6±0.3	3.0±0.2
AM *T. foenum graecum* root extract	5.0±0.4	3.8±0.2	3.2±0.2	3.0±0.2	–
AM *S. melongena* root extract	4.5±0.5	3.7±0.3	3.0±0.4	2.5±0.4	–
AM *R. communis* root extract	4.0±0.5	3.5±0.4	–	–	–
CD at 0.05 %	0.20	0.27	0.35	0.36	0.26

* Data taken after 60 days
–: No growth

Table 3. Effect of *in vitro* application of AM colonized root extracts of different plant species on number of roots/shoot in *Morus alba* (average values) with different NaCl levels*.

Treatments	Number of roots/shoot				
	NaCl (0.0 %)	NaCl (0.1 %)	NaCl (0.2 %)	NaCl (0.3 %)	NaCl (0.4 %)
MS (control)	10 ± 1.9	8 ± 1.6	7 ± 1.15	6 ± 1.2	4 ± 0.6
IBA (1 mg l^{-1})	12 ± 1.15	12 ± 2.2	10 ± 1.5	9 ± 1.5	8 ± 1.15
AM *M. alba* root extract	14 ± 1.5	15 ± 1.15	12 ± 1.5	10 ± 1.4	10 ± 1.7
AM *T. foenum graecum* root extract	10 ± 2.0	8 ± 1.15	7 ± 0.8	5 ± 0.9	–
AM *S. melongena* root extract	8 ± 1.2	6 ± 0.9	5 ± 0.8	3 ± 0.8	–
AM *R. communis* root extract	6 ± 0.8	4 ± 0.8	–	–	–
CD at 0.05 %	1.07	1.07	0.88	0.95	0.90

* Data taken after 60 days
–: No growth

ing percentage with different NaCl levels are depicted in Table 3 and Fig. 1, respectively.

In case of shoots transferred to MS+AM root extract of *Morus alba*, roots initiated within 6–8 days in both cases i.e., with/without NaCl (Table 1) and the growth of the roots was found to be equivalent to IBA and faster than with other plant extracts. All parameters studied i.e. root initiation, root length, number of roots/shoot and rooting percentage were found to be superior with AM root extracts of *M. alba* and IBA. These results, pertaining to the effect of AM root extracts on rhizogenesis, are supported by MUNIYAMMA et al. (2000). The presence of optimum level of desired plant growth hormones in mycorrhizal colonized root extracts (BARROWS and RONCADORI 1977; HIRSCH et al. 1997) could have possibly promoted rooting in *in vitro* cultures. Mycorrhizal fungi are known to produce several growth promoting hormonal substances (TURNER 1962; MILLER 1971; BAREA and AZCON-AGUILAR 1982; HASAN 2002) and plant internal hormone balance may be altered with mycorrhizal colonization (GUNZE and HENNESSY 1980; LUDWIG-MULLER et al. 1997; SCAGEL and LINDERMAN 1998). Also a wide range of specific compounds like sugars, amino acids, organic acids, fatty acids, sterols, growth hormones, nucleotides, flavanones, enzymes and other miscellaneous compounds are released from plant roots as root exudates (CURL and TRUELOVE 1986). These root and fungal exudates in combination with growth promoting hormones, possibly auxins, present in mycorrhizal root extracts could have promoted rooting in *in vitro* cultures of *M. alba*.

Root initiation was late with other plant extracts at different NaCl concentrations (Table 1) and a significant decrease was observed with respect to number of roots/shoot, rooting percentage and average root length as compared to the treatment with *M. alba* root extract (Tables 1–3, Fig. 1). *R. communis* AM root extract showed very poor performance with respect to all parameters studied. Some toxic components (ricin-D, ricin-E, ricinine, alkaloid allergen and castor beanhemagglutinin) present in *R. communis* (KOGA et al. 1979; SHARMA et al. 1986), might have adversely affected rhizogenesis in the present study. The observed trend of different AM plant root extracts affecting rooting in *M. alba* was in order of *M. alba* > *Trigonella foenum*

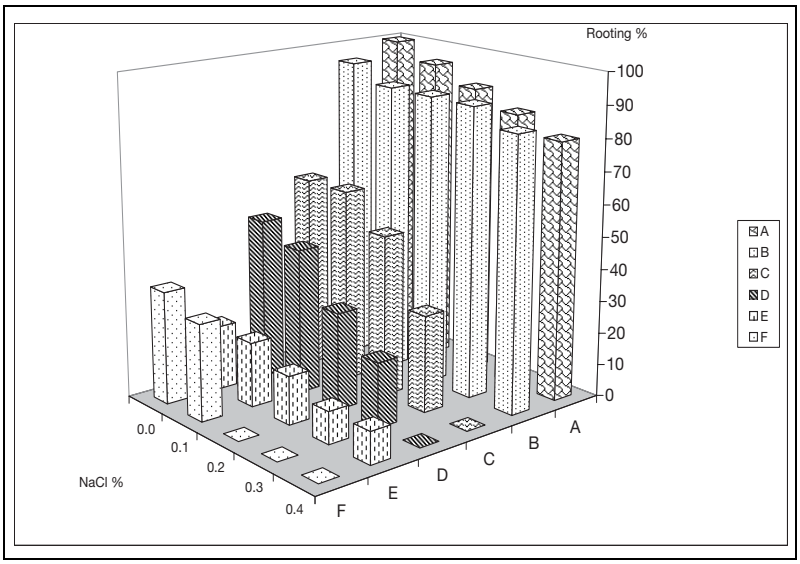


Fig. 1. Effect of in vitro inoculation of AM root extracts and IBA for rooting percentage; A = IBA (1mg l^{-1}); B= AM M.alba root extract; C= AM *T. foenum graecum* root extract ; D= AM *S. melongena* root extract; E= MS (Control); F= AM *R. communis* root extract

graecum > *Solanum melongena* > *Ricinus communis*. The best results with AM root extracts of *M. alba* may be due to similar constituents and consistency to *in vitro* grown plantlets of *M. alba* as the root exudation rate is known to be influenced by plant type, age and other factors like plant physiological and pathological responses, light, temperature, water, nutrition and the associated rhizosphere microflora (VANCURA 1964; ROVIRA 1969).

Certain phytohormone-releasing bacteria possibly associated with mycorrhizae may also influence rooting. Although the properly surface-sterilized roots were used in *in vitro* experiments, the isolation of certain bacteria from surface sterilized AM colonized roots and spores of *Glomus, Gigaspora* and *Endogone* spp. have been reported (LI and HUNG 1987; STRZELCZYK et al. 1987; TILAK et al. 1989). These microbes are reported to produce all types of growth-regulating substances and mainly auxins (TIEN et al. 1979; CROZIER et al. 1988; STRZELCZYK et al. 1994). The root promoting effect due to AM root extracts may also possibly be due to this factor.

Also, the environmental stimuli, factors or plant-internal signals such as C/N ratio, pH, ion concentrations (RUDAWSKA 1983), and the presence or absence of amino acids (KAMPERT and STRZELCZYK 1989), organic acids (POKOJSKA and STRZELCZYK 1988), vitamins (STRZELCZYK and POKOJSKA 1989) and polyphenols (LONSANE and KUMAR 1991) have been reported to induce phytohormone production (BEYRLE 1995) by AM fungi. These optimum conditions present during pot culturing might have induced auxin production in AM colonized roots.

The NaCl concentration affected root length (Table 2) and other parameters (Table 3, Fig. 1) even in presence of IBA possibly due to increasing osmotic stress. Plants growing on saline media can somewhat increase their internal osmotic concentrations by the production of organic acids or uptake of salts through the process of osmotic adjustment (BONN et al. 1985). According to NIEMAN (1980), the energy required for the operation of the salt tolerance mechanism is obtained mainly at the expense of growth resulting in stunted growth (ALI et al. 1999). The decreased length of roots with different NaCl concentrations possibly is due to this effect.

In the present *in vitro* experiments, AM *M. alba* root extract was found almost equivalent to IBA under both (–) and (+) NaCl conditions. The availability of desired auxin in AM *M. alba* root extracts could have played a role in inducing rooting in *M. alba*. The presence of different types of biochemicals including auxins in root extracts of other plant species namely *Trigonella foenum graecum, Ricinus communis* and *Solanum melongena* might have not promoted rooting in *M. alba*. It is further required to characterize the growth promoting substances present in these extracts so as to better understand their role in rhizogenesis.

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