In vitro selection of salt tolerant *Morus alba* and its field performance with bioinoculants

S. Kashyap, S. Sharma

Centre for Rural Development and Technology, Indian Institute of Technology, New Delhi, India

ABSTRACT: *In vitro* selected salt tolerant saplings of *Morus alba* (cv. Sujanpuri) were raised from nodal explants with axillary buds collected during three different periods of the year. The growth and shoot/root multiplication of the nodal explants collected between November to February and July to October were found to be better than those collected between March to June. In cultures, shoot multiplication was induced by the application of 2.5 mg/l of 6-benzylaminopurine (BAP) and 0.3 mg/l of gibberellic acid (GA_3), while rooting by 1.0 mg/l of indolebutyric acid (IBA). Sodium chloride (NaCl) was added to induce salt stress and its concentration was gradually increased from 0.1% (w/v) onwards. The salt tolerance was observed up to 0.4% (w/v) NaCl and 100% mortality of explants was noted above this concentration. The inclusion of Arbuscular Mycorrhizal (AM) fungi and *Azotobacter chroococcum* to tissue culture of raised saplings during acclimatization enhanced their survival and resulted in a significant increase of plant growth. After the transfer of plants to salt affected wasteland, only NaCl-treated saplings survived, whereas those developed without NaCl resulted in 100% mortality.

Keywords: *in vitro*; mulberry; NaCl; AM fungi; *Azotobacter*

Soil salinity is a major constraint to food and biomass production since it limits the land's capability for supporting optimum plant growth. Worldwide, there are 952 million ha of salt affected land and out of this, 10.1 million ha are in India (Yadav 2000). The growing demands of the expanding population for various biomass products have necessitated an exploitation of these soils. Salinity tolerance would therefore be a highly desirable characteristic to be induced in economically important multipurpose plant species like *Morus alba.*

M. alba is the best known species for sericulture as its leaves are used for rearing silkworms (*Bombyx mori*). The soil with crumb structure having higher microbial activity, organic matter and sufficient moisture, having soil pH in the range of 7.5 to 8.5 is ideal for *M. alba* growth but it does not grow well under rainfed conditions and salinity (Dorcus, Vivekanandan 1991). The high degree of cross-pollination, heterozygosity, polyploidy and the dioecious nature of the genus is a major problem in developing salt tolerant cultivars by conventional techniques (Sharma, Madan 1994). In this context, the use of *in vitro* methods may prove beneficial as the plant tissue culture techniques take a considerably shorter period of time, reduce time between generations and generate large variations, which is useful for inducing the desired traits. The inoculation of tissue cultured plantlets with suitable bioinoculants like Arbuscular Mycorrhiza (AM) fungi (Monticelli et al. 2000; Yano-Melo et al. 2003) and *Azotobacter* (Carletti 2000) could not only protect them from transplant shocks and lower their total mortality percentage but also improve the biomass quality. The inoculation of plant roots with AM fungi helps in nutrient recycling, water absorption, production of phytohormones, biocontrolling of pathogens, stress tolerance and soil fertility improvement (Sharma et al. 1997). Similarly, the ability of *Azotobacter* to synthesize auxins, vitamins and antifungal antibiotics is well recognized (Subba Rao 1982). The beneficial effects of dual inoculation i.e. AM fungi and *Azotobacter* in certain plant species have been reported (Paroha et al. 2000; Kashyap et al. 2004). It appears that all these advantages of *Azotobacter* and mycorrhizal associations could be used to reduce the mortality rate of tissue cultured plantlets under salt stress. Further, transplanting of the developed saplings on saline land may test the field performance of *in vitro* developed salt tolerant plants.

Therefore, it was thought worthwhile to determine if salt tolerance in *Morus alba* (cv. Sujanpuri) could be induced through tissue culture by gradually ap-

plying salt (NaCl) stress and if exploiting AM fungi and *Azotobacter* during hardening could enhance the survival percentage of developed plants on salt affected land.

MATERIALS AND METHODS

Explant collection: Growing shoots of *Morus alba* L. cv. Sujanpuri were collected from one year old mulberry plants cultivated on normal soil (pH = 7.5, EC = 0.110 mmhos/cm) at Micromodel, IIT, Delhi during the following months: A) March to June, B) July to October, C) November to February. Nodal segments with one axillary bud each (1.5 to 2.0 cm) were excised, washed and disinfected first with 0.7% (w/v) bleach solution (sodium hypochloride) for 10 min and with 0.1% aqueous mercuric chloride solution for next 10 minutes and then rinsed 5 to 6 times with sterile distilled water.

Shoot multiplication: The Murashige and Skoog's (MS) (1962) medium containing 3% (w/v) sucrose, 2.5 mg/l of BAP and 0.3 mg/l of GA_3 with 1.0% agar and pH 5.8 was used as shoot multiplication medium. For induction of salinity stress, the medium was supplemented with 0.1% NaCl. The surface sterilized explants were inoculated vertically onto the culture medium with one explant per test tube and twenty test tubes were kept for each treatment.

Cultures were grown under cool white fluorescent tubes with irradiance of 24 μmol m^2/s, 16 h photoperiod and at the temperature of 25 ± 1°C. After multiple shoot induction, the NaCl concentration of medium was gradually increased from 0.1 to 0.4% till 100% mortality of explants observed and average shoot length and number of shoots per explant was noted.

Root induction: For rhizogenesis, well developed shoots (5.0 cm long) with 2–3 leaves were excised from cultures and cultured on MS supplemented with 1.0 mg/l of IBA, 3.0% sucrose and NaCl (from 0.1 to 0.4%). For root production, shoots were inoculated on medium with the same concentration of NaCl that was used during shoot multiplication. For each treatment, twenty shoots were used. Numbers of roots/shoot and root length were recorded at the end of the experiment.

Acclimatization/hardening and transfer of tissue-cultured saplings to field: Small plastic pots (5 cm diameter × 7 cm height) were filled with autoclaved mix of vermicompost: soil (1:3), 500 g mix/pot with and without NaCl. The NaCl was added ranging from 0.1 to 0.4%. Plantlets with well-developed roots were removed from culture medium with different NaCl concentrations. The roots were washed thoroughly under tap water to remove agar particles. Plantlets were then transferred to plastic pots with and without NaCl, at the same concentration as they were exposed to *in vitro* with following bioinoculants:

i) AM fungi (M),
ii) *Azotobacter chroococcum* (A),
iii) AM fungi + *A. chroococcum* (M + A),
iv) control (C).

For each treatment and NaCl concentration, 20 replicates were maintained. A consortium of AM spores of *Glomus* and *Gigaspora* species (*Glomus mosseae*, *G. microcarpum*, *G. macrocarpum*, *G. fasciculatum*, *Gigaspora margarita* and *G. heterogama*) was obtained from the mycorrhizal bank at Micromodel, IIT, Delhi. The spores were sterilized with 2% (w/v) chloramine T and 200 ppm streptomycin for 10 minutes and then rinsed thoroughly in autoclaved water before inoculation in pots. About 50 surface sterilized AM spores were then added to each pot. For the *Azotobacter* treatment, thoroughly washed roots of plantlets were dipped in *A. chroococcum* solution (with 10^7 to 10^9 cells/ml) for ten minutes before plantlets were transferred to the pots. For the dual inoculation, both AM fungi and *Azotobacter* inocula were added as above. The pots without bioinoculants were used as controls.

Potted plantlets were grown at 60–70% relative humidity, 25 ± 2°C and irradiance of 24 μmol m^2/s and watered with 1/10 strength MS basal salt solution devoid of sucrose at 4-day intervals for a period of 4 weeks. Precautions were taken to avoid drainage of salts.

Inoculated plantlets were further transplanted to pots (15 cm diameter × 20 cm height) containing the same substrates and treatments and kept under shade for another four weeks before being transferred to the field. The rhizosphere of acclimatized plants was analyzed for *Azotobacter* cell count/g soil, AM colonization in roots and AM spore count/100 g pot soil. For analyzing *Azotobacter* and AM fungi, standard methods were followed (Subba Rao 1982; Phillips, Hayman 1970; Gerdemann, Nicolson 1963). The data regarding stem height, number of nodes, stem girth 30 cm above ground, survival percentage, fresh and dry weight, electrical conductivity (EC) and pH of pot soil was noted after four months.

Transplantation of salt tolerant saplings to wasteland: After proper acclimatization, the salt tolerant saplings (10 plants per salt concentration with bioinoculant treatment) were transplanted in wasteland of village Jamalpur (soil EC – 5.2 mmhos/cm)

Table 1. Selection of NaCl tolerant *Morus alba* from nodal explants collected during three periods of the year (4 months data for each salt concentration)

NaCl (%)	Shoot multiplication (mean values)						Root production (mean values)					
	number of shoots/explant			shoot length (cm)			number of roots/shoot			root length (cm)		
	A	B	C	A	B	C	A	B	C	A	B	C
0.0	$5^{a\,A} \pm 1.1$	$8^{a\,B} \pm 2.1$	$10^{a\,B} \pm 2$	$1.7^{a\,A} \pm 0.1$	$1.75^{a\,A} \pm 0.2$	$1.8^{a\,A} \pm 0.3$	$9^{a\,A} \pm 1.4$	$12^{a\,B} \pm 1.8$	$12^{a\,B} \pm 2$	$4.2^{a\,A} \pm 0.2$	$4.5^{a\,A} \pm 0.3$	$5.2^{a\,B} \pm 0.3$
0.1	$5^{a\,A} \pm 1.3$	$8^{a\,B} \pm 1.8$	$10^{a\,B} \pm 3$	$1.6^{a\,A} \pm 0.1$	$1.55^{a\,A} \pm 0.3$	$1.7^{a\,A} \pm 0.2$	$8^{A} \pm 1.5$	$11^{a\,B} \pm 1.5$	$12^{a\,B} \pm 3$	$4.0^{a\,A} \pm 0.3$	$4.0^{b\,A} \pm 0.2$	$4.2^{b\,A} \pm 0.3$
0.2	$3^{b\,A} \pm 1.9$	$7^{a\,B} \pm 1.5$	$8^{a\,B} \pm 1$	$1.5^{ab\,A} \pm 0.1$	$1.52^{a\,A} \pm 0.3$	$1.6^{a\,A} \pm 0.3$	$8^{A} \pm 1.3$	$10^{B} \pm 1.9$	$10^{a\,B} \pm 1$	$3.5^{ab\,A} \pm 0.4$	$3.7^{b\,A} \pm 0.3$	$3.8^{b\,A} \pm 0.2$
0.3	$2^{b\,A} \pm 0.9$	$5^{b\,B} \pm 1.4$	$7^{b\,C} \pm 1$	$1.5^{ab\,A} \pm 0.1$	$1.40^{a\,A} \pm 0.2$	$1.6^{a\,A} \pm 0.5$	$7^{b\,A} \pm 1.3$	$10^{B} \pm 1.9$	$9^{b\,B} \pm 2$	$3.0^{b\,A} \pm 0.4$	$3.5^{b\,B} \pm 0.4$	$3.5^{c\,B} \pm 0.4$
0.4	$1^{c\,A} \pm 0.7$	$3^{b\,B} \pm 1.0$	$5^{b\,C} \pm 1$	$1.2^{b\,A} \pm 0.1$	$1.3^{b\,A} \pm 0.2$	$1.5^{a\,A} \pm 0.3$	–	$8^{b\,A} \pm 1.5$	$8^{b\,A} \pm 1$	–	$3.0^{c\,A} \pm 0.3$	$3.0^{c\,A} \pm 0.3$
0.5	–	$1^{c} \pm 0.5$	$1^{c} \pm 0$	–	$1.0^{b\,A} \pm 0.3$	$1.2^{b\,A} \pm 0.2$	–	–	–	–	–	–

A – collected between March and June, B – collected between July and October, C – collected between November and February; – 100% mortality; small letter superscript (with the same letter in a column) explains a non-significant difference (at $p = 0.05$ level) among divers NaCl concentrations; capital letter superscript (with the same letter in a row under the same growth parameter) explains a non-significant difference (at $p = 0.05$ level) among divers time-periods

in Haryana. After four months of growth in waste-land, data pertaining to survival percentage, growth parameters, *Azotobacter* cell count/g of soil, AM spore count/100 g soil and AM colonization (%) of roots was recorded.

Statistical analysis: The data was analyzed by computer using the SPSS for Windows 9.0 package. Categorical data was compared using χ^2-analysis and Fisher's exact test when indicated (expected frequency of less than 5 in any cell). ANOVA was applied on quantitative variables with multiple groups followed by Duncan's multiple comparison tests. Quantitative variables with normal distribution and equal variance were compared by two-tailed *t*-test. The Mann-Whitney *U* nonparameteric test was used for non-normal data.

RESULTS

Effect of seasons: The effects of NaCl stress on explants collected during different months of the year are presented in Table 1. The growth and proliferation of the nodal explants was influenced by collection date with the maximum number of shoots produced from explants collected between July to October (8/explant with no NaCl and 0.1% NaCl) and November to February (10/explant with no NaCl and 0.1% NaCl). Also, a significant increase of roots/shoot number was observed on explants collected during these periods as compared to those collected between March and June. However, no significant difference in shoot length was observed.

Effect of NaCl on shoot and root development: With an increase of the NaCl concentration *in vitro*, bud sprouting, in general, was found to be delayed and the number of shoots per explant and average shoot length decreased significantly. At 0.5% NaCl, some explants collected from July to October and from November to February showed a late shoot production (Table 1), but turned brown after two weeks of growth. Similarly, a significant reduction in root length and number of roots per shoot was observed with the increase of NaCl concentration (Table 1). As the survivability was observed up to 0.4% NaCl, salt tolerant saplings developed up to these concentrations were selected for further work.

Acclimatization/hardening of *in vitro* selected salt tolerant saplings: The acclimatization of *Morus alba* plantlets, with respect to shoot height, number of nodes/plant, fresh and dry weight and survival rate (%) was found best on M + A (Table 2), however increased concentrations of NaCl affected all parameters in all treatments. The EC of pot soils exceeded from 4.037 to 6.378 mmhos/cm after 4 months with different concentrations of NaCl in all treatments (Table 2). The pH value was found to be almost normal (between 7.5–7.57 with/without different NaCl treatments). The results of microbial analysis of acclimatized saplings are summarized in Table 3. Maximum AM colonization

Table 2. Acclimatization of salt tolerant saplings of *Morus alba* with different bioinoculant treatments (4 months data)

NaCl (%)	Treatments	Growth parameters (mean values)			Survival (%)	Mean fresh wt/plant (g)	Mean dry wt/ plant (g)	pH level of soil	EC of soil (mmhos/cm)
		shoot height (cm)	number of nodes/plant	stem girth (cm)					
0.0	M	50.4 ± 2.4	28 ± 2.2^{a}	2.65 ± 0.2^{a}	90	140 ± 3.9^{a}	46.2 ± 1.8^{a}	7.50	0.110
	A	49.1 ± 4.0	30 ± 3.7^{a}	2.5 ± 0.3	85	135 ± 3.7^{b}	45.0 ± 3.1^{a}	7.50	0.110
	M + A	52.5 ± 1.9^{a}	32 ± 3.0^{b}	2.8 ± 0.3^{a}	95	144 ± 3.5^{a}	49.3 ± 2.2^{b}	7.50	0.110
	C	46.8 ± 3.7^{b}	26 ± 2.3^{a}	2.2 ± 0.3^{b}	80	132 ± 2.4^{b}	42.0 ± 3.2^{b}	7.50	0.110
0.1	M	48.7 ± 2.8^{a}	25 ± 4.7	2.55 ± 0.3	85	132.5 ± 5.3^{a}	43.7 ± 1.8^{ab}	7.51	4.134
	A	49.0 ± 2.2^{a}	28 ± 3.3	2.52 ± 0.3	80	135 ± 2.6^{a}	44.8 ± 1.7^{b}	7.54	4.408
	M + A	52.5 ± 1.5^{b}	28 ± 1.9	2.7 ± 0.2	85	143.2 ± 1.3^{b}	48.2 ± 2.7^{c}	7.50	4.037
	C	42.7 ± 3.2^{e}	25 ± 1.9	2.50 ± 0.3	70	130 ± 2.7^{c}	40.5 ± 4.0^{a}	7.55	4.518
0.2	M	43.1 ± 2.2^{a}	24 ± 3.0^{a}	2.52 ± 0.3	80	125 ± 4.6^{a}	41.8 ± 1.6^{a}	7.54	5.931
	A	45.4 ± 4.8^{a}	27 ± 2.1^{ab}	2.48 ± 0.04	75	130 ± 3.2^{b}	45.0 ± 2.6^{b}	7.52	5.974
	M + A	50.9 ± 4.2^{b}	28 ± 2.9^{b}	2.6 ± 0.3	85	142 ± 2.4^{c}	47.4 ± 1.8^{b}	7.57	4.725
	C	39.5 ± 3.3^{c}	20 ± 1.5^{c}	2.41 ± 0.03	65	115 ± 3.7^{d}	38.2 ± 1.0^{c}	7.51	5.958
0.3	M	39.2 ± 3.6^{a}	23 ± 1.6^{a}	2.48 ± 0.04	80	110.5 ± 7.3^{a}	38.2 ± 2.8^{a}	7.52	6.108
	A	38.7 ± 2.9^{a}	25 ± 1.5^{ab}	2.3 ± 0.3	75	122 ± 2.7^{b}	40.7 ± 2.3^{a}	7.51	6.163
	M + A	43.8 ± 2.6^{b}	27 ± 1.6^{b}	2.52 ± 0.3	80	133.1 ± 4.1^{c}	44.2 ± 1.3^{b}	7.51	5.838
	C	34.1 ± 4.1^{c}	20 ± 2.4^{c}	2.28 ± 0.03	65	92.8 ± 1.7^{d}	30.5 ± 4.2^{c}	7.50	6.238
0.4	M	36.1 ± 3.4	24 ± 2.4^{ab}	2.36 ± 0.03^{a}	75	100 ± 6.9^{a}	35.1 ± 2.3^{a}	7.51	6.174
	A	34.2 ± 2.1^{a}	22 ± 2.4^{a}	1.95 ± 0.4^{b}	70	95 ± 3.7^{b}	31.8 ± 2.5^{b}	7.55	6.267
	M + A	38.7 ± 2.9^{b}	26 ± 1.8^{b}	2.4 ± 0.3^{c}	80	120.8 ± 4.4^{c}	40.2 ± 3.1^{c}	7.57	6.032
	C	32.5 ± 4.4^{a}	18 ± 2.4^{c}	1.8 ± 0.2^{d}	60	90 ± 2.5^{b}	30.0 ± 3.4^{d}	7.52	6.378

Mean and percentage followed by the same letter or without a letter in a column are not significantly different (at $p = 0.05$ level) within the same concentration of NaCl between treatments (M, A, M + A, C). M – AM fungi, A – *Azotobacter*, C – control

Table 3. Microbial analysis of rhizosphere soil of acclimatized saplings of *Morus alba*

NaCl (%)	Treatments	*Azotobacter* cell count/g of soil		AM colonization (%)		AM spore count/100 g soil	
		A	B	A	B	A	B
0.0	M	–	–	50	70	40 ± 1.4[a]	42 ± 1.8[a]
	A	7.5×10^2	10.7×10^2	–55	–	–	–
	M + A	8.2×10^2	12.2×10^2	55	72	45 ± 2.1[b]	45 ± 2.1[b]
	C	–	–	–	–	–	–
0.1	M	–	–	35	65	32 ± 1.6[a]	55 ± 3.6[a]
	A	7.2×10^2	9.3×10^2	–	–	–	–
	M + A	7.8×10^2	10.8×10^2	35	66	35 ± 3.0[b]	60 ± 2.9[b]
	C	–	–	–	–	–	–
0.2	M	–	–	30	62	30 ± 2.3	50 ± 3.0[a]
	A	5.3×10^2	7.4×10^2	–	–	–	–
	M + A	6.2×10^2	8.0×10^2	30	64	32 ± 1.9	58 ± 2.1[b]
	C	–	–	–	–	–	–
0.3	M	–	–	28	58	25 ± 2.9[a]	48 ± 3.4[a]
	A	4.7×10^2	6.2×10^2	–	–	–	–
	M + A	5.8×10^2	7.8×10^2	32	60	30 ± 3.3[b]	52 ± 2.2[b]
	C	–	–	–	–	–	–
0.4	M	–	–	28	52	22 ± 3.0[a]	45 ± 2.9[a]
	A	4.0×10^2	6.0×10^2	–	–	–	–
	M + A	5.0×10^2	7.5×10^2	30	58	28 ± 1.5[b]	50 ± 2.9[b]
	C	–	–	–	–	–	–

A – after 2 months, B – after 4 months; M – AM fungi, A – *Azotobacter,* C – control; mean and percentage followed by the same letter or without a letter in a column are not significantly different (at $p = 0.05$ level) within the same concentration of NaCl between treatments (M, A, M + A, C)

(72%) was observed in M + A treatment without NaCl. With addition of NaCl from 0.1 to 0.4%, the AM root colonization was reduced from 66 to 58% in M + A. Similarly, the AM spore count/100 g pot soil and *Azotobacter* cell count/g pot soil were also found to be decreased with salinity.

Transplantation of salt tolerant saplings to wasteland: The results pertaining to transplantation of *in vitro* developed saplings in village Jamalpur, District Gurgaon, Haryana are depicted in Table 4. The transplantation of saplings developed without NaCl (–NaCl) to wasteland resulted in 100% mortality. Only *in vitro* developed salt tolerant saplings survived there, though the survival percentage was very poor (Table 4). It was noted that salt tolerant saplings developed at higher NaCl levels (0.3%, 0.4%) had a better growth rate than saplings developed at lower NaCl levels (0.1%, 0.2%). Significant differences were observed between the treatments. The dual inoculated (M + A) plants were found to be the best with respect to all parameters studied, i.e. survival (%), shoot length, number of nodes/plant, stem girth, AM colonization (%) and rhizospheric *Azotobacter* cell count/g soil and AM spore count/100 g soil; however, the number of the rhizospheric microflora decreased as compared to the hardening period. Control plants (C) without NaCl did not survive at all, while the plants treated with 0.2 to 0.4% NaCl had 10–30% survival rate (Table 4).

DISCUSSION

Effect of seasons: The seasonal influence on bud sprouting and micropropagation rate in different plant species is reported (Vijaya Chitra, Padmaja 2002; Civinova, Sladsky 1990; Pattanaik et al. 1996). The nodal explant proliferation was greatly influenced by the time of explant collection in *Morus serrata* and November to February was found to be the best time of the year according to the studies of Pattnaik and Chand (1997). Similarly, summer was found better than rainy and winter season for four cultivars of mulberry both *in vitro* and *in vivo* (Vijaya Chitra, Padmaja 2002). Quraishi et al.

Table 4. Effect of bioinoculants on *in vitro* selected saplings of *Morus alba* in Jamalpur wasteland recorded after 4-month growth

NaCl* (%)	Treatments**	Growth parameters (mean values)			Survival (%)	*Azotobacter* cell count/g soil	AM colonization (%)	AM spore count/ 100 g soil
		shoot length (cm)	number of nodes/ plant	stem girth (cm)				
0.0	M	–	–	–	–	–	–	–
	A	–	–	–	–	–	–	–
	M + A	–	–	–	–	–	–	–
	C	–	–	–	–	–	–	–
0.1	M	94.8 ± 3.0	70 ± 2.5^{a}	4.2 ± 0.5	20	2×10^2	66	50 ± 5.2^{a}
	A	93.7 ± 2.0	70 ± 1.3^{a}	4.2 ± 0.2	20	7.0×10^2	50	48 ± 6.3^{a}
	M + A	95.7 ± 2.7	75 ± 1.9^{b}	4.5 ± 0.3	20	8.0×10^2	62	58 ± 2.0^{b}
	C	–	–	–	–	–	–	–
0.2	M	96.0 ± 2.8^{a}	70 ± 2.7^{a}	4.3 ± 0.3^{a}	20	2×10^2	65^{a}	52 ± 4.0^{a}
	A	95.8 ± 2.4^{a}	72 ± 1.4^{ab}	4.2 ± 0.2^{a}	20	7.0×10^2	50^{a}	48 ± 7.2^{ac}
	M + A	98.0 ± 2.8^{a}	75 ± 2.3^{b}	4.6 ± 0.2^{a}	20	8.4×10^2	62^{a}	60 ± 3.4^{b}
	C	90.7 ± 2.1^{b}	50 ± 2.4^{c}	3.8 ± 0.5^{b}	10	1.8×10^2	40^{b}	42 ± 4.3^{c}
0.3	M	98.3 ± 2.5	70 ± 2.0^{a}	4.5 ± 0.4^{a}	20	2.5×10^2	66^{a}	54 ± 3.2^{a}
	A	98.0 ± 2.5	72 ± 2.5^{ab}	4.4 ± 0.5^{a}	20	7.0×10^2	50^{a}	50 ± 4.2^{a}
	M + A	100.2 ± 2.5^{a}	75 ± 2.0^{b}	5.0 ± 0.4^{a}	30	8.5×10^2	64^{a}	60 ± 4.5^{a}
	C	92.8 ± 2.2^{b}	52 ± 2.2^{c}	4.0 ± 0.7^{b}	10	2.0×10^2	40^{b}	45 ± 4.5^{b}
0.4	M	100.5 ± 2.3	72 ± 1.9^{a}	4.5 ± 0.2^{a}	20	2.5×10^2	66^{a}	55 ± 3.5^{a}
	A	100.3 ± 3.0	73 ± 2.6^{a}	4.5 ± 0.3^{a}	20	7.2×10^2	52^{a}	50 ± 4.5^{a}
	M + A	100.7 ± 2.4	80 ± 2.5^{b}	5.1 ± 0.4^{a}	30	8.5×10^2	66^{a}	62 ± 3.7^{b}
	C	95.4 ± 2.3	54 ± 2.1	4.0 ± 0.3^{b}	10	2.0×10^2	40^{b}	45 ± 3.8^{a}

100% mortality * *in vitro* selected saplings tolerant to different NaCl levels
M – AM fungi, A – *Azotobacter,* C – control, ** bioinoculant treatments given during acclimatization of saplings
Mean and percentage followed by the same letter or without a letter in a column are not significantly different at $p = 0.05$ level (comparison was done among similar concentrations of NaCl between treatments)

(1996) reported April–June as the best period for shoot culture initiation in *Cleistanthus collinus* and the nodal segments produced significantly more shoots in this period. Good shoot multiplication of *Morus cathayana* and *M. lhou* was recorded with nodal explants collected between July and October (Pattnaik et al. 1996). In the present work as well, the time of the year influenced the growth of nodal explants *in vitro* and those collected between July to October and November to February were found to be significantly superior over those collected between March to June. The differences in the physiological condition, antioxidant activity, anthocyanin and total phenolic constituents in stem tissues of the stock plants grown under natural environmental conditions might be the reason for differential growth responses *in vitro* observed by culturing explants collected in different seasons (Vijaya Chitra, Padmaja 2002; Sývacý, Sökmen 2004).

Effect of NaCl on shoot and root development: The delay in sprouting may be due to the increased osmotic potential of the saline medium affecting water and nutrient uptake, which may in turn inhibit the metabolic activities necessary for bud initiation and growth (Cherian, Reddy 2003). High salinity causes hyperosmotic stress and ion disequilibrium in cells producing secondary effects like reduction of turgor below the yield threshold of cell wall resulting in growth cessation (Yokoi et al. 2002). An increase of osmotic pressure is caused by the increase of osmolytes and osmoprotectants like sugars, sugar alcohols (Elavumoottil et al. 2003), quarternary amino acid derivatives (proline, glycine betaine, β-alanine betaine, praline betaine), tertiary amines (1,4,5,6-tetrahydro-2-methyl-4-carboxyl pyrimidine), and sulfonium compounds (Cherian, Reddy 2003) in the cells to enhance stress tolerance. Energy required for salt tolerance is obtained mainly at the expense of growth as observed in present study. An adaptive biochemical function of osmoprotectant is the scavenging of reactive oxygen species disrupting normal metabolism through an oxidative damage of lipids, proteins and nucleic acids (Sudhakar et al. 2001), thus resulting in reduced plant growth by different concentrations of NaCl. Differences in antioxidant activity in stem tissue of *M. alba* during different seasons (Sývacý, Sökmen 2004; Sudhakar et al. 2001) might have helped in resistance to the oxidative damage and have enhanced the shoot and root multiplication of certain explants collected between November to February and July to October under NaCl stress and might have helped in selection of such salt tolerant saplings *in vitro.*

Acclimatization/hardening of *in vitro* selected salt tolerant saplings: The beneficial effects of inoculation with AM fungi in association with *Azotobacter* in terms of increased plant growth, leaf biomass and P-content in leaf have been reported in different crop plants (Paroha et al. 2000).

Azotobacter, being a group of nitrogen fixing beneficial rhizosphere bacteria is known to increase plant growth by production of growth regulators in rhizosphere and antagonism towards pathogenic organisms even under high salinity (Carletti 2000). The positive response of *A. chroococcum* was investigated *in vitro* culture establishment as well as in the acclimatization of plants (Carletti 2000).

Similarly, survival increase of tissue-cultured saplings by inoculating AM fungi is documented (Monticelli et al. 2000; Yano-Melo et al. 2003). The two main deficiencies of *in vitro* grown plants are: 1) a poor control of water loss, and 2) heterotrophic mode of nutrition (Bhojwani, Razdan 1996). Also, roots penetrating the nutrient medium often lack root hairs. Thus the inoculation of AM fungi might have helped plantlets in enhancing uptake of water and nutrients, especially phosphorus (Tian et al. 2004) from soil even in the absence of root hairs, thereby alleviating salt stress and avoiding transient transplant shock and stunted growth on transfer to the field. Besides, the inoculation of AM fungi improved certain physiological processes like increased carbon dioxide exchange rate, transpiration, stomatal conductance and water use efficiency of the plants (Ruiz-Lozano et al. 1996). The addition of *Azotobacter* to AM fungi further enhanced the survivability of saplings, however salinity affected *Azotobacter* cell count/g soil, AM spore count/100 g soil and AM colonization (%) in rhizosphere of developed saplings**.** AM hyphae growth in saline soil require energy to maintain ionic balance in the mycelium and internal water potential to maintain turgor (Juniper, Abott 1993), which might have reduced AM colonization (%) in saplings grown on soil with NaCl.

Overall, during acclimatization of saplings, the dual inoculation of these microorganisms displayed a synergistic effect resulting in maximum growth of plants might be through better N and P uptake and due to the secretion of phosphoenzyme and other growth promoting substances by AM and *Azotobacter,* respectively (Habte, Manjunath 1987).

Transplantation of salt tolerant saplings to wasteland: Although higher survival percentage of *in vitro* developed saplings of *M. alba* (with different NaCl concentrations), as compared to

the saplings developed without NaCl, was noted on wasteland in present studies, the reason is not clear by which plants acquire incremental improvement in their salt tolerance. It could be either due to a pre-existing variability or new genetic changes. Genotype of the parent plant is known as a strong determinant of variability in cultures (Skirvin, Janick 1976; Cummings et al. 1976). A ploidy of the donor plant may determine susceptibility of cells to *in vitro* changes. Polyploids generally tend to show higher variation in culture than diploid genotypes of the same species. Under a normal situation, the genetic variability induced in somatic cells by polysomaty (the state of having reduplicated chromatin in the nucleus) or any other kind of genetic changes remains unnoticed, as these cells do not divide. However under culture conditions these cells may be induced to divide and undergo a re-differentiation and express the inherent variability at whole plant level. Such type of variability induced *in vitro* as well as polyploid nature of *M. alba* may be responsible for inducing survivability of saplings on wasteland.

The increased survivability of M + A inoculated plants on wasteland in present study is supported by earlier studies (Paroha et al. 2000; Kashyap et al. 2004). Mycorrhizal symbiosis resulted in significant changes in root system morphology, acclimatization and transplant survival in micropropagated plants (Monticelli et al 2000; Yano-Melo et al. 2003). It plays an important role in water economy of plants by improving the hydraulic conductivity of the roots and contributing towards a better uptake of water by the plants even in extremely dry conditions (Al-Karaki et al. 2001). Plants colonized by AM fungi acquire a greater percentage of photoassimilates than non-mycorrhizal ones. Consequently, they exhibit higher root/shoot ratios (Clapperton, Reid 1992). The AM fungi also improve the resistant capacity to osmotic stress by increasing soluble sugars and electrolyte concentrations in roots (Feng et al. 2002) in order to compensate for decreased soil water potential. Similarly the *Azotobacter* improves survival of transplanted plants by a production of plant growth regulators, antifungal substances and nutrients (Subba Rao 1982).

In the present work it was noted that although AM fungi and *Azotobacter* assisted saplings in their establishment under field conditions, their growth got reduced considerably as compared to hardening period which may be due to complexity of soil environment in field and scarcity of nutrients in wasteland soil.

Acknowledgements

The authors are highly grateful to ICAR (Indian Council of Agricultural Research) for providing the financial assistance.

References

AL-KARAKI G.N., HAMMAD R., RUSAN M., 2001. Response of two tomato cultivars differing in salt tolerance to inoculation with mycorrhizal fungi under salt stress. Mycorrhiza, *11*: 41–47.

BHOJWANI S.S., RAZDAN M.K., 1996. Plant Tissue Culture: Theory and Practice., Elsevier Science Publications: 502.

CARLETTI S., 2000. Use of plant growth – promoting rhizobacteria in plant micropropagation. In: Fifth International PGPR Workshop, Villa Carlos Paz: 295.

CHERIAN S., REDDY M.P., 2003. Evaluation of NaCl tolerance in the callus cultures of *Suaeda nudiflora* Moq. Biologia Plantarum, *46*: 193–198.

CIVINOVA B., SLADSKY Z., 1990. Stimulation of the regeneration capacity of tree segment explants *in vitro*. Biologia Plantarum, *32*: 407–413.

CLAPPERTON M.J., REID D.M., 1992. Effects of low-concentration sulphur dioxide fumigation and vesicular-arbuscular mycorrhizas on ^{14}C-partitioning in *Phleum pratense* L. New Phytologist, *120*: 381–387.

CUMMINGS P.D., GREEN C.E., STUTHMAN D.D., 1976. Callus induction and plant regeneration in oats. Crop Science, *16*: 465–470.

DORCUS D., VIVEKANANDAN M., 1991. Screening of mulberry varieties for rainfed conditions. Sericologia, *31*: 233–241.

ELAVUMOOTTIL O.C., MARTIN J.P., MORENO M.L., 2003. Changes in sugars, sucrose synthase activity and proteins in salinity tolerant callus and cell suspension cultures of *Brassica oleracea* L. Biologia Plantarum, *46*: 7–12.

FENG G., ZHANG F.S., LI X.L., TIAN C.Y., TANG C., REMGEL Z., 2002. Improved tolerance of maize plants to salt stress by arbuscular mycorrhiza is related to higher accumulation of soluble sugars in roots. Mycorrhiza, *12*: 185–190.

GERDEMANN J.W., NICOLSON T.H., 1963. Spores of mycorrhizal endogene species extracted from soils by wet sieving and decanting. Transactions of British Mycological Society, *46*: 235–244.

HABTE M., MANJUNATH A., 1987. Soil solution P status and mycorrhizal dependency in *L. leucocephala*. Applied and Environmental Microbiology, *53*: 797–801.

JUNIPER S., ABOTT L.K., 1993. Vesicular-arbuscular mycorrhizas and soil salinity. Mycorrhiza, *4*: 45–57.

KASHYAP S., SHARMA S., VASUDEVAN P., 2004. Role of bioinoculants in development of salt resistant saplings of

Morus alba (var. Sujanpuri) *in vivo.* Scientia Horticulturae, *100*: 291–307.

MONTICELLI S., PUPPI G., DAMIANO C., 2000. Effects of *in vivo* mycorrhization on micropropagated fruit tree rootstocks. Applied Soil Ecology, *15*: 105–111.

MURASHIGE T., SKOOG F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, *15*: 463–497.

PAROHA S., CHANDRA K.K., TIWARI K.P., 2000. Synergistic role of VAM and *Azotobacter* inoculation on growth and biomass production in forestry species. Journal of Tropical Forestry, *16*: 13–21.

PATTNAIK S.K., CHAND P.K., 1997. Rapid clonal propagation of three mulberries, *Morus cathayana* Hemsl., *M. lhow* Koiz. and *M. serrata* Roxb., through *in vitro* culture of apical shoot buds and nodal explants from mature trees. Plant Cell Reports, *16*: 503–508.

PATTNAIK S.K., SAHOO Y., CHAND P.K., 1996. Micropropagation of a fruit tree, *Morus australis* Poir. Syn. *M. acidosa* Griff. Plant Cell Reports, *15*: 841–885.

PHILLIPS J.M., HAYMAN D.S., 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions British Mycological Society, *55*: 158–161.

QURAISHI A., KOCHE V., MISHRA S.K., 1996. *In vitro* micropropagation from nodal explants of *Cleistanthus collinus.* Plant Cell Tissue Organ Culture, *45*: 87–91.

RUIZ-LOZANO J.M., AZCON R., GOMEZ M., 1996. Alleviation of salt stress by arbuscular-mycorrhizal *Glomus* species in *Lactuca sativa* plants. Physiologia Plantarum, *98*: 767–772.

SHARMA S., MADAN M., 1994. Potential of mulberry (*Morus alba*) biomass. Journal of Scientific and Industrial Research, *53*: 710–714.

SHARMA S., MADAN M., VASUDEVAN P., 1997. Biology and applications of mycorrhizal fungi. Microbiologia SEM, *13*: 427–436.

SKIRVIN R.M., JANICK J., 1976. Tissue culture induced variation in scented *Pelargonium* spp. Journal of American Society of Horticultural Science, *101*: 281–290.

SUBBA RAO N.S., 1982. Biofertilizers in Agriculture. New Delhi, Oxford and IBH Publishing Co. Pvt. Ltd.: 230.

SUDHAKAR C., LAKSHMI A., GIRIDARAKUMAR S., 2001. Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. Plant Science, *161*: 613–619.

SÝVACÝ A., SÖKMEN M., 2004. Seasonal changes in antioxidant activity, total phenolic and anthocyanin constituent of the stems of two *Morus* species (*Morus alba* L. and *Morus nigra* L.) Plant Growth Regulation, *44*: 251–256.

TIAN C.Y., FENG G., LI X.L., ZHANG F.S., 2004. Different effects of arbuscular mycorrhizal fungal isolates from saline or non-saline soil on salinity tolerance of plants. Applied Soil Ecology, *26*: 143–148.

VIJAYA CHITRA D.S., PADMAJA G., 2002. Seasonal influence on axillary bud sprouting and micropropagation of elite cultivars of mulberry. Scientia Horticulturae, *92*: 55–68.

YADAV J.S.P., 2000. Management of salt affected soils for sustainable production. In: GAWANDE S.P., BALI J.S., DAS D.C., SARKAR T.K., DAS D.K., NARAYANASWAMY G. (eds.), Advances in Land Resource Management for 21st Century. Soil Conservation Society of India. New Delhi, Angkor Publishers: 253–264.

YANO-MELO A.M., SAGGIN JR. O.J., MAIA L.C., 2003. Tolerance of mycorrhized banana (*Musa* sp. cv. Pacovan) plantlets to saline stress. Agriculture Ecosystem and Environment, *95*: 343–348.

YOKOI S., RAY A.B., HASEGAWA P.M., 2002. Salt stress tolerance of plants. JIRCAS Working Report: 25–33.

Received for publication May 6, 2005
Accepted after corrections September 12, 2005

Selekce na zasolení u *Morus alba* v podmínkách *in vitro* a její výsledky v polních podmínkách při použití bioinokula

ABSTRAKT: Výpěstky *Morus alba* (cv. Sujanpuri), selektované v podmínkách *in vitro* na toleranci k zasolení, byly získány z nodálních explantátů s axilárními pupeny odebranými během tří různých ročních období. Růst a multiplikace výhonků nebo kořínků byly lepší u explantátů odebraných v období mezi listopadem a únorem než u explantátů odebraných v období mezi březnem a červnem. Multiplikace výhonků v kultuře byla indukována po aplikaci 2,5 mg/l 6-benzylaminopurinu (BAP) a 0,3 mg/l kyseliny giberelové (GA_3), kdežto zakořeňování pomocí 1,0 mg/l kyseliny indolylmáselné (IBA). Chlorid sodný (NaCl) byl dodáván za účelem indukce stresu zasolením a jeho koncentrace byly postupně zvyšovány od 0,1 % (w/v) výše. Tolerance k zasolení byla pozorována až do 0,4 % (w/v) NaCl, avšak nad tuto

hranici již docházelo ke 100% mortalitě explantátů. Dodání mykorhizních hub Arbuscular Mycorrhiza (AM) a *Azotobacter chroococcum* do tkáňové kultury zvyšovalo výtěžek dopěstovaných rostlin během jejich aklimatizace a významně zvýšilo další růst těchto rostlin. Po přesazení rostlin na zasolený pozemek přežívaly pouze ty výpěstky, které byly selektovány při použití NaCl, kdežto výpěstky vypěstované bez tohoto ošetření 100% hynuly.

Klíčová slova: *in vitro*; morušovník; NaCl; AM houby; *Azotobacter*

Corresponding author:

Dr. Suman Kashyap, Centre for Rural Development and Technology, Indian Institute of Technology, New Delhi 110016, India
tel.: + 91 11 265 962 29, + 91 11 263 033 61, fax: + 91 11 265 911 21, e-mail: skkashyap@hotmail.com