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# Mycorrhization alleviates salt stress in grape rootstocks during *in vitro* acclimatization

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## ABSTRACT

**An *in vitro* experiment was conducted to achieve arbuscular mycorrhizal fungus (AMF) root colonization in micro-propagated grape rootstocks Salt Creek (*Vitis champini*) and Male hybrid (*Vitis vinifera*) during acclimatization and also to study the effect of AMF on alleviating salt-stress. The plantlets were grown in potting medium containing peat: vermiculite: perlite (2:1:1). In the salt-stressed medium, mycorrhizal root colonisation was significantly higher in Male hybrid (19%) than in Salt Creek (12%) rootstock. Mycorrhizal plantlets had significantly higher root (40%) and shoot (20%) dry biomass production than non-mycorrhizal plantlets raised on saline medium. The total chlorophyll (TC) content was higher in the leaves of mycorrhizal as compared to un-inoculated plantlets of Salt Creek (24%), had higher TC contents than Male hybrid (17%). Mycorrhizal tissue had significantly higher levels of P, K, Ca and Mg in addition to higher $Na^+$ and $Cl^-$ concentrations than non-mycorrhizal plantlets. Under saline conditions, Salt Creek showed a high degree of dependence on mycorrhizae, than Male hybrid. The reduction in $Na^+$/ $K^+$ ratio together with a concomitant increase in P, K, Ca and Mg absorption and high chlorophyll and proline contents in mycorrhizal plantlets may be important salt-alleviating mechanisms for plants growing in saline soils, like observed in Salt Creek rootstock.**

**Key words:** *In vitro*-mycorrhization, salt-alleviation, arbuscular mycorrhizal fungi, *Vitis* sp.

## INTRODUCTION

Mycorrhizae are of special importance to plants such as grapevines that have a coarse and poorly branched root system. Grapevines appear to be reliant on AM fungal colonisation for normal growth and development (Linderman and Davis, 8). Grapevines not only respond positively to root colonization, but may suffer in the absence of mycorrhization under natural conditions, causing mortality of tissue culture raised plants due to poor *ex vitro* acclimation.

The roots of grapevines (*Vitis* spp.) are often colonized by arbuscular mycorrhizal fungi (AMF) under field conditions. Studies conducted in sterilized soils in pots have shown that AMF enhance the uptake of P, Zn, and Cu in grapevines and that colonization of roots by AMF can increase the drought tolerance. The phenomenon of transplantation shock has been found to be reduced by inoculating the vines with AM fungi (Linderman and Davis, 8). The evidence of root-system damage suggests that AM fungi may improve water relations and nutrient access of host plants. Inoculation with AMF represents a biological solution that can result in growth enhancement. In micropropagation, the nutrient-rich growth substrate is devoid of microbes and as a result, plants free of any microbial association are produced. Mycorrhization promotes better survival and healthy vine growth, especially during hardening stage as *ex vitro* survival rate of micropropagated grapevine plantlets were reported to be almost doubled (Krishna *et al*., 7; Alizadeh, 1). These limitations are primarily due to plantlets having a poorly developed cuticle, non-functional stomata, heterotrophic habit and weak root system. In the present study, we tested the ability of AMF to colonize in two grape rootstock genotypes during *in vitro* hardening stage.

## MATERIALS AND METHODS

Salt Creek (*V. champini*) and Male hybrid (*V. vinifera*) plants maintained in Grape Germplasm Block were taken for this experiment. Full-strength MS medium (Murashige and Skoog, 9) supplemented with 3.0 mg $l^{-1}$ 6-benzyl aminopurine (BAP) and 0.25 mg $l^{-1}$ α-naphthalene acetic acid (NAA) initially developed by Singh *et al*. (14) and further standardized by Alizadeh (1) was used for culture initiation. The nodal segments, excised from newly emerged just mature vegetative shoots during April were cultured in the test tubes containing 15 ml initiation medium. The cultures were maintained under white fluorescent light (227 µmol $m^{-2}s^{-1}$) with controlled photoperiod (16/8 h) at room temperature of 25 ± 2°C. After 30 d, healthy shoot sprout was excised and sub-cultured onto the shoot elongation-cum-rooting medium (50 ml) containing conical flasks (150 ml). The medium comprised of full-strength MS medium supplemented with 1.0 mg

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$l^{-1}$ of each of IBA and NAA (Alizadeh, 1). After 60 days of inoculation, the proliferated shoot was excised into two-node micro-cuttings and then sub-cultured onto the same medium. After three sub-cultures (at 60 day interval) the plantlets multiplied were transferred onto 100 ml conical flask containing 40 ml shoot elongation-cum rooting medium for 45 day. The well grown healthy plantlets were selected for *in vitro* hardening and subsequent experimentation.

For hardening, the methodology standardized using glass jars with poly-propylene (PP) caps filled with peat: vermiculite: perlite (2:1:1) and moistened with half-strength MS salts minus organics was employed (Singh *et al*., 13). The rooted plantlets from conical flasks were first washed in sterile double-distilled water twice to clear the sticking agar-agar medium, and then immersed separately with sterile double-distilled water containing carbendazim (Bavistin®) and Ridomil® [0.1% (w/v)]. Then the plantlets were then transferred individually to the glass jars. The glass jars were then placed under controlled photoperiod and temperature conditions. The caps were loosened gradually after four weeks and during the subsequent two weeks were removed completely. Thereafter, the plantlets were misted (twice daily) with sterile distilled water containing 0.1% carbendazim (w/v) on first day and continued as per the need. At the end of second week, half population of each genotype were subjected for *in vitro* mycorrhization using 25 g soil based Pusa AMF mix (Nutrilink®) near the root zone of the plantlets at four spots with the help of sterile spatula. The soil-based mycorrhizal inocula (*Glomus mosseae*, *G. manihotis* and *Gigaspora gigantea*) were procured from the Division of Microbiology, IARI, New Delhi.

After three weeks, both AMF-inoculated and non-inoculated control plants were watered (not misted) using autoclaved distilled water containing five NaCl solutions (0, 20 40, 60 and 80 mM w/v) for 45 d. Throughout the experiment, all the plants were watered daily with 5 ml of NaCl graded water for first three weeks, and thereafter on alternate days (because water stagnation noticed at ≥60 mM NaCl due to less evapo-transpiration).

The experiment consists of 20 treatments, which comprised of three factorial combinations of two rootstocks and five NaCl levels with or without AMF-inoculation. After 45 d of salt treatment different parameters like plantlet height, leaf number, shoot and root fresh and dry weights and percentage root colonisation were estimated. Comparative shoot: root ratios were calculated on dry weight basis. Total leaf chlorophyll contents were measured by the method of Hiscox and Israelstam (6) and foliar proline was estimated according to Bates *et al*. (2). For tissue nutrient analyses, oven-dried shoot and root samples were ground, sieved and digested in nitric acid: perchloric acid (9:4). Phosphorus and sulphur were estimated by the method of Tandon (16) from di-acid digest; Na and K were quantified by flame photometer; Ca and Mg were determined using an atomic absorbance spectrophotometer; chloride was estimated after making the leaf and root sample into ash and subsequently titrating against 0.05 N $AgNO_3$ according to Yoshida *et al*. (17). The percentage of mycorrhizal root colonization was estimated after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactophenol (v/v) (Phillips and Hayman, 11). Infectivity is calculated as follows and expressed in percentage. The experiment was laid in three replications with five plants in each treatment. The percentage data was transformed using square root transformation before carrying out ANOVA. The data was analyzed with WASP-2 package for calculation of F-values and significance of means was estimated by applying LSD ($p < 0.05$) test.

## RESULTS AND DISCUSSION

In the present experiment root colonization of 12% in Salt Creek and 19% in Male hybrid grown *in vitro* on medium containing peat: vermiculite: perlite (2:1:1) was observed (Table 1). AM colonization did not occur in control treatments of both the rootstocks, which may be due to sterile media used for *in vitro* hardening. In the inoculated glass jars, percent AM colonization decreased significantly under increased salt-stress conditions of both rootstocks; however the reduction was more in Salt Creek than Male hybrid. AM colonization did not differ significantly at lower salt concentration (20 mM) in both the rootstocks however, reduced significantly thereafter and at 80 mM NaCl. AM colonization was significantly higher in Male hybrid than Salt Creek at lower salt concentrations (up to 40 mM) although no significant differences were noticed between 60 and 80 mM NaCl. Percent root infectivity reduced from about 20 to 0% in Salt Creek and 30 to 3% in Male hybrid under salt-stress. The overall percent root colonization (Fig. 1) was significantly higher in Male hybrid than Salt Creek rootstock. Ectomycorrhiza-like structures can be formed *in vitro* when perlite is used as substrate. Perlite could be a good substrate for mycorrhization, as it was easily colonized by fungal hyphae compared with agar medium and some permeable substrates such as sphagnum peat: perlite or vermiculite: soilrite have also been used (Strullu *et al*., 15).

In most salinity level except at ≤ 20 mM, there was a significant reduction in plant height, number of leaves, root and shoot dry biomass in mycorrhizal compared to non-mycorrhizal plants (Table 1) but the magnitude of growth response varied between

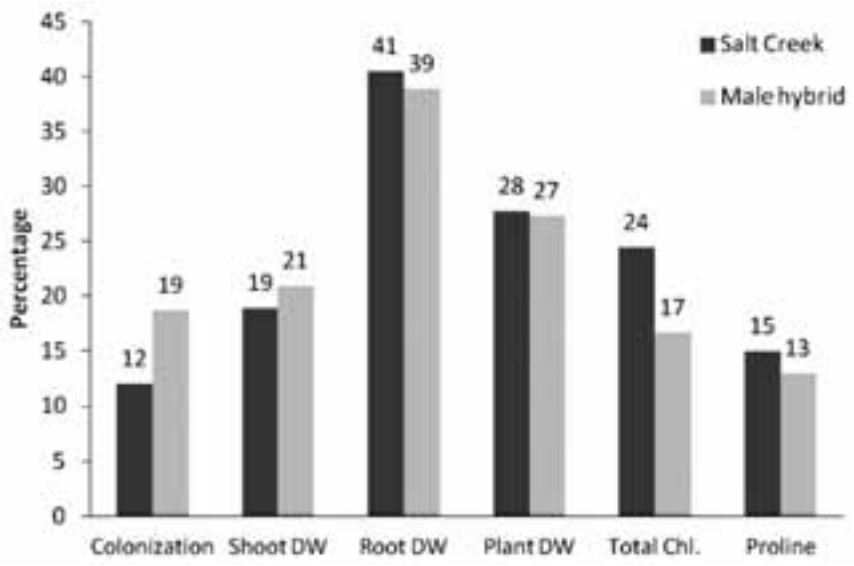


**Fig. 1.** Percent increase on root colonization, dry weight of shoot, root and whole plant, total chlorophyll and proline content of AMF + salt stressed plants over non-AMF + salt stressed plants of two grape rootstocks during *in vitro* hardening (after 45 d).

rootstocks (Fig. 3). In this study we have demonstrated that inoculation with AMF influenced mainly the root dry biomass production (about 40% increase) in addition to shoot biomass production of both rootstocks. The shoot dry weight reduced to 78% in non-mycorrhizal plants than AMF infected plants (42%) of Salt Creek under salt-stress, however in Male hybrid it was from 82 to 51%. Similarly, salt-stress decreased the root dry biomass of Salt Creek under AMF to 16% than non-AMF plant roots (46%) however, in Male hybrid the decrease of root biomass was severe from 28% in AMF-inoculated plants to 130% in non-AMF plants. Mycorrhization had almost equal effects on percent dry matter production in both the rootstocks (Fig. 1). These results indicate that use of mycorrhizal inoculation is a feasible approach during *in vitro* hardening to hasten the plantlet growth. Similar results were obtained by Alizadeh (1) when the Pusa mix AMF strains were inoculated under glasshouse conditions and recorded the highest number of roots four grape rootstocks however, various rootstock genotypes responded differently. The superiority of AMF for improving plant growth could be due to increased absorption of balanced nutrition and less degradation of chlorophyll and there by maintaining higher photosynthetic rate (Hajiboland *et al*., 5).

The total chlorophylls content in leaves of mycorrhizal Salt Creek and Male hybrid plantlets were significantly higher than non-mycorrhizal control at all salinity levels. The leaves of non-mycorrhizal plants were more chlorotic than those of mycorrhizal plantlets especially in Male hybrid rootstock under salt-stress > 60 mM (visual observation, data not shown). Mycorrhization significantly increased the total chlorophyll contents (Fig. 1) in Salt Creek than (24%) in Male hybrid (17%). The significant increase in total chlorophyll contents in (17 to 24%) AMF-inoculated rootstocks and decrease over salt-stress in our study suggests that salts interfere with chlorophyll synthesis more in non-mycorrhizal than in mycorrhizal plants. Under salinity stress there may be several reasons for low chlorophyll content in plant tissues. One explanation might be that $Na^+$ has an antagonistic effect on $Mg^{++}$ absorption. In the present investigation, a higher concentration of $Mg^{++}$ (6-7%) was observed for both rootstocks as a result of AM colonisation, which suggests that mycorrhizal fungi reduce the antagonistic effect of $Na^+$. Giri *et al*. (4) have reported that mycorrhizal fungi are effective in the absorption of $Mg^{++}$ and suppression of $Na^+$ under salt stress conditions. In addition to $Mg^{++}$, we also observed increased absorption of $K^+$, which resulted in decreased $Na^+$-$K^+$ antagonism in mycorrhizal plants, and it was more visible in Salt Creek rootstock. The proline accumulation increased significantly with increasing salinity levels in both the rootstocks. Mycorrhizal plants accumulated more proline than non-mycorrhizal plants in all the salinity levels. The percent increase in proline content (Fig. 1) was slightly higher in Salt Creek (15%) than Male hybrid (13%). An inverse relation was observed between shoot moisture content and

salinity gradients. However, the mycorrhization did not show any marked effect compared to control plants (non-AMF) for water absorption. About 14% increase in proline accumulation under salt-stress due to mycorrhization in the two grape rootstocks could be taken as an indicator for salinity tolerance. The osmo-protectant and cryo-protectant activity of proline and various betaines in cells when accumulated under stress is well documented (Murkute *et al*., 10).

Saline water irrigation significantly reduced the absorption of P in non-AMF plants in contrast; AM-inoculated plants had significantly greater concentration of P even under increased salinity up to 60 mM NaCl. Irrespective of salt stress, the overall increase in shoot P content of AM-inoculated plants was 128% in Salt Creek and 19% in Male hybrid. Salt Creek root accumulated higher P than its shoot tissue, however Male hybrid accumulated relatively less P in the roots than its shoot at lower NaCl levels. AM fungi have been shown to have a positive influence on the of uptake mineral nutrients (especially poor mobility nutrients such as P) of plants grown in salt-stress conditions. In the present study, mycorrhizal Salt Creek and Male hybrid plantlets had higher concentrations of P, *i.e*., 128 and 19% more respectively compared to non-mycorrhizal plants. Increased P uptake was found to be the primary reason for increased growth in plants showing AM colonised roots. Soil salinity significantly reduce absorption of mineral nutrients, especially P because phosphate ions precipitate with $Ca^{2+}$ ions in salt-stressed soil and become unavailable to plants (Poss *et al*., 12). Therefore, P improver/fertilisation is necessary for plant growth, which may be helpful in mitigating salt stress by overcoming the P binding capacity of the soil. In saline soil, higher absorption of P in AM-inoculated plants may improve their growth rate and salt-tolerance and suppress the adverse effect of salinity stress. In our study, the increased salt tolerance of Salt Creek over Male hybrid rootstock may be linked to increased P absorption capacity of the former. Poss *et al*. (12) also suggested that the salt-tolerance mechanism in onion is primarily related to P nutrition. Similarly, mycorrhizal fungi have the major effect on salt stress through mediation of P accumulation, besides enhanced P uptake, there are some other mechanisms such as induction of osmotica that lead to osmotic adjustment and improved salt-tolerance in mycorrhizal plants. The present study also suggest that apart from P content, the other minerals absorption was significantly higher in Salt Creek than Male hybrid, which leads to balanced nutrition in the former for better salt tolerance.

Salinity increased the uptake of $Na^+$ and $Cl^-$ in both the tissues, while $K^+$ uptake decreased significantly at higher salinity levels. However, mycorrhization significantly increased the uptake of $Na^+$ and $Cl^-$ as compared to non-AMF rootstocks. It was noteworthy that AM plants exhibited increased $K^+$ uptake in root and shoot tissues as compared to un-inoculated controls and thereby decrease of $Na^+/K^+$ ratio in the former treatment (Table 2). Shoot $Ca^{++}$ and $Mg^{++}$ also were significantly higher in AM-inoculated plants than non-AM inoculated plants for both the rootstocks. Overall the percent increase in all the mineral contents of shoot tissues were significantly higher due to mycorrhization in Salt Creek rootstocks than in Male hybrid (Fig. 2) despite more AMF infectivity in the later rootstock. It appears that the role of AMF in alleviating salt stress is partly to prevent $Na^+$ effect by increased $K^+$ absorption to root and translocation to shoot tissues. Increased $K^+$ concentration under saline conditions may help to decrease $Na^+$ uptake, which may be indirectly related to maintaining the chlorophyll content of the plant. The accumulation of $Na^+$ is strongly influenced by the form of $N^+$ available ($NO_3^-$ or $NH_4^+$) and it may also be influenced by the synthesis and storage of polyphosphate as well as by other cations, particularly $K^+$ (Giri *et al*., 4). Hence, mycorrhizal plants were less affected due to $Na^+$ intake compared to non-mycorrhizal plants. Our study also support the above findings; even though Salt Creek had higher $Na^+$ content (not a $Na^+$ excluder) than Male hybrid, the former absorbed more $K^+$ (28%) and thereby maintaining lower $Na^+/K^+$ ratio as well as higher total chlorophyll to counteract the ill effects of $Na^+$.

Increased P results in decreased $Na^+$, which is indirectly related to $Ca^{++}$ and $Mg^{++}$ uptake. Moreover, AM-inoculated onion had higher concentrations of $K^+$ in shoots and bulbs under salt stress conditions, which could be beneficial by maintaining a high $K^+/Na^+$ ratio and by influencing the ionic balance of the cytoplasm or $Na^+$ efflux from the plant. Cantrell and Linderman (3) suggested that AM fungi improve P nutrition of plants under salinity stress and reduce the negative effects of $Na^+$ and $Cl^-$ by maintaining vacuolar membrane integrity, which prevented these ions from interfering in growth metabolic pathways, facilitates compartmentalisation within vacuoles and selective ion intake. The lower leaves of non-mycorrhizal plants were affected with marginal necrosis a typical $Cl^-$ toxicity especially in Male hybrid rootstocks (Fig. 4) receiving 60 mM NaCl watering. Our study clearly shows that increased uptake of K, Ca and Mg enabled mycorrhizal-Salt Creek plants to mitigate salt-stress better than Male hybrid.

We have demonstrated that mycorrhization can be achieved during *in vitro* hardening of grape rootstocks. Mycorrhization not only improved acclimatisation but also for enhanced nutrient uptake during progressive growth stages. Under saline conditions, inoculation

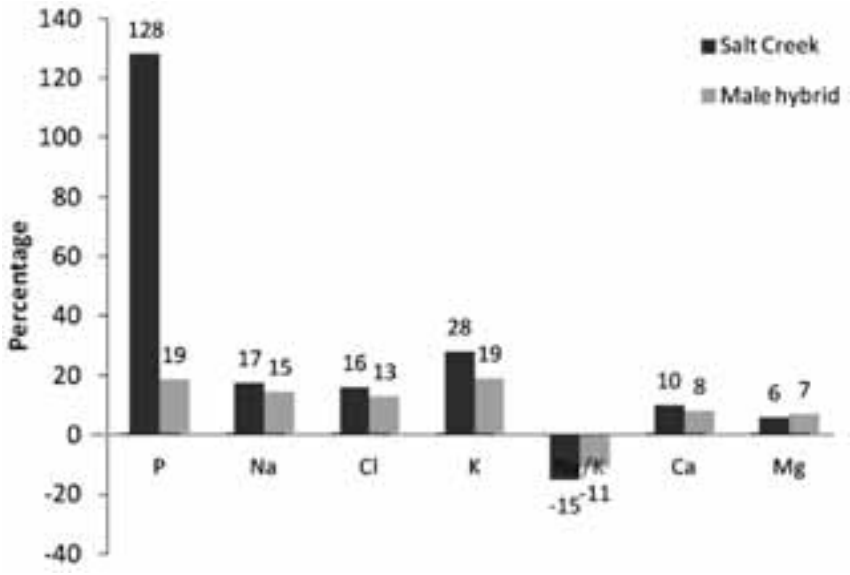


**Fig. 2.** Percent increase on various shoot mineral contents of AMF + salt stressed plants over non-AMF + salt stressed plants of two grape rootstocks during *in vitro* hardening (after 45 d).

with AMF promoted plant growth and development. There was improvement in P absorption followed by K, Ca and Mg, which contributed in containing ill effect of higher $Na^+$ and $Cl^-$ contents of mycorrhizal plants with better stability of total chlorophyll concentration. The increased proline bio-synthesis noticed in AMF-inoculated plants, revealed that its non-mediated nutritive effects that could play a role other than nutritional effects. The concentration of total chlorophyll, P, K and Ca in Male hybrid decreased severely than Salt Creek due to chloride toxicity render latter as salt-stress tolerant type. Salt Creek showed a higher degree of dependence on AM fungi than Male hybrid, as revealed in the study.

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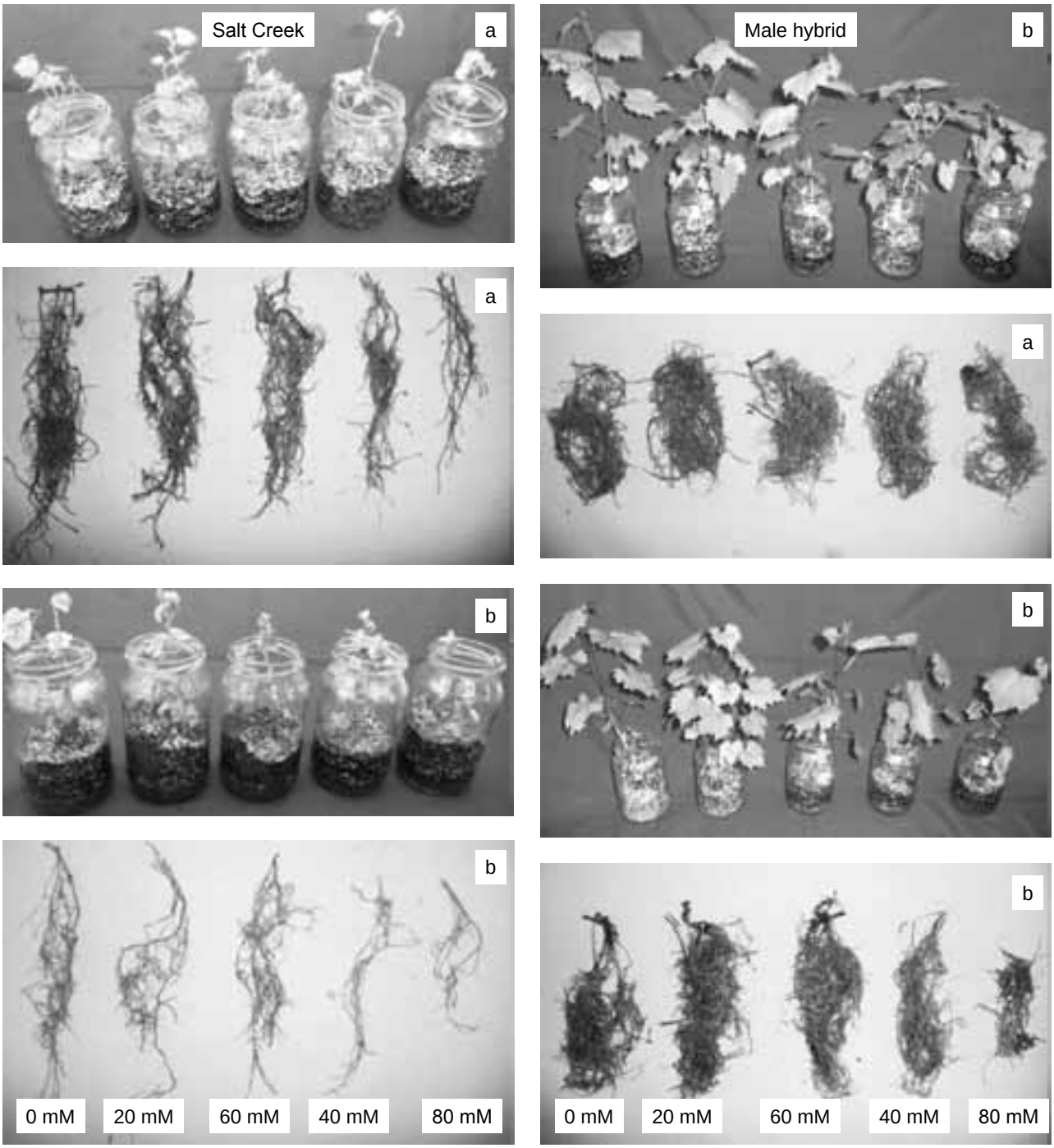


**Fig. 3.** Effect of AMF inoculation and saline (NaCl) irrigation (five levels (0-80 mM; placed L to R) on the performance of Salt Creek during *in vitro* hardening for 45 days. & (b). Shoot and root growth with AMF inoculation and saline watering; (c) & (d). Shoot and root growth without AMF inoculation and saline watering.

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**Fig. 4.** Chloride toxicity on the older leaf under *in vitro* hardening in growth chamber.

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