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Micropropagation of *lasora* (*Cordia myxa* Roxb.)

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ABSTRACT

A protocol for *in vitro* propagation of *lasora* (*Cordia myxa* Roxb.) was standardized using nodal segments. Single node segments, prepared from the new vegetative shoots, were cultured on Murashige and Skoog (1962) medium supplemented with 2.0, 4.0 and 6.0 mgl^{-1} kinetin and BAP alone or in combination with 0.01 mgl^{-1} NAA. The best response (93.6%) was observed with 4.0 mgl^{-1} kinetin. The regenerated shoots from nodal segments were excised aseptically and transferred onto the rooting medium supplemented with 2.0 mg/l each of NAA and IBA along with 750 mgl^{-1} activated charcoal was found superior (90.6%) over the other hormonal combinations for rooting response. Inoculation of arbuscular mycorrhizal fungi during *ex vitro* hardening stage resulted in higher survival (97%) and improved growth of micro-propagated *lasora* plantlets.

Key words: Bio-hardening, *Cordia myxa* Roxb., micropropagation, mycorrhiza.

INTRODUCTION

Lasora (*Cordia myxa* Roxb.) is a drought tolerant fruit tree spread over arid and semi-arid regions of North India. This is favoured for cultivation in marginal eco-system as it has several xerophytic characters such as deciduous nature, sunken stomata, waxy coating, hairyness on leaves and coincidence of maximal growth phase with the period of water availability. The fruits are rich in sugars but are acidless and unripe fruits are pickled. Conventional method of propagation, *i.e.*, seed propagation give rise to significant variability, while vegetative method of propagation such as budding and grafting, though successful, are ineffective for rapid multiplication of desired genotype.

Plant tissue culture has proven to be an alternative tool for rapid multiplication of elite genotypes with limited number of mother plants. In constract, the serious impediment is higher mortality of plantlets during hardening or Stage IV of micropropagation, as plants are subjected to different stresses. Besides imparting tolerance against stresses, arbuscular mycorrhizal fungi (AMF) offer a potentially efficient method to improve vigour and adaptation of plantlets for transplanting (Krishna *et al*., 10). Keeping this in view, the present investigation was carried out to develop a micropropagation protocol for maximum recovery of *in vitro* raised *lasora* plantlets.

MATERIALS AND METHODS

In order to assess the potentiality of *in vitro* culture establishment, apical shoot and nodal segments collected from field-grown trees of CIAH-1 were severed and dipped in a solution containing 2.0 g l^{-1} carbendazim and 200 mg l^{-1} 8-hydroxy quinnoline citrate for 1 h. Shoot tip and nodal segments were prepared and the ends of explant were cut in such a manner that upper cut was given just above the node (in nodal segment), while the lower one at about one cm below it (for nodal segment and shoot tip). Macro-explants (4.0-5.0 cm) were employed for culture. To alleviate the problem of explant browning, explants were pre-agitated in anti-oxidant solution of citric acid @ 50 mgl^{-1} + ascorbic acid @ 100 mg l^{-1} for 30 min. They were then surface-sterilized using 0.1% mercuric chloride (w/v) for 10 min. and rinsed 3-4 times with autoclaved double-distilled water.

The explants were inoculated on to MS medium (Murashige and Skoog, 14) supplemented with combinations of kinetin and 6-benzyl amino purine (BAP) along with 0.01 mg l^{-1} α-naphthalene acetic acid (NAA). Later for induction of rooting, explants were transferred onto medium containing different concentration (conc.) of NAA and indole-3-butyric acid (IBA) and activated charcoal (AC) @ 750 mgl^{-1}. The required amount of major and minor salts, vitamins, plant growth regulators (kinetin, BAP NAA and IBA) and sucrose were mixed together and distilled water added to a little less than the final volume. The pH of media was adjusted to 5.8 ± 0.1 and solidified with 8.0 g l^{-1} agar-agar.

All the cultures were maintained under the photoperiod of 16/8 h light and dark cycle at 26 ± 1°C. The micro-shoots were rooted on MS medium supplemented with auxins IBA and NAA. The experiments were laid out in a factorial completely randomized block design with three replication and 20 explants comprised a unit for each treatment.

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Plantlets rooted were transferred to *ex vitro* conditions for acclimatization. Initial hardening for 10 days was done in hardening chamber, a plastic chamber without a bottom, which has a dimension of 37.5 cm x 27.0 cm x 22.6 cm with two side-windows on opposite direction, which were used to regulate humidity to 85-90% by opening or closing them. This was followed by bio-hardening with AMF in poly-house for one month, with day-night temperatures maintained at 27° ± 1°C, and an extended photoperiod to 16/8 h using white fluorescent light with relative humidity maintained at 80-85% using humidifiers.

Initial hardening was done in substrate comprising autoclaved Soilrite®, perlite and cocopeat (2:2:1), while bio-hardening was done in a substrate consisting of autoclaved (1.05 kg^{-1} cm^{-2} for 2 h) mixture of soil, sand and composted farm yard manure (FYM) in the proportion of 2:2:1. A mixed culture of AMF was procured from the Division of Microbiology, Indian Agricultural Research Institute, New Delhi. Soil based AMF inoculum was used by laying the culture (approx. 20 g) below the roots. The plantlets after inoculation were immediately watered with sterile tap water and maintained under glasshouse conditions.

The experiments were laid out in a completely randomized block design with three replication and 60 explants comprised a unit for each treatment. The data were subjected to analysis of variance and mean separation was tested with Duncan's test using AGRES statistical software (1994), Version 7.01, Pascal International Software Solution, USA.

RESULTS AND DISCUSSION

Nodal segments proved to be better explants for initiation of *in vitro* culture in *lasora*. In terms of both, micro-shoot number as well as micro-shoot length, nodal segments was found superior over apical shoot segments (Table 1) when cultured on MS medium containing 2.0 mg l^{-1} kinetin + 0.01 mg l^{-1} NAA. The type of explant, apical segment versus nodal segment has been reported as a significant source of variation *in vitro* shoot proliferation. Debnath and McRae (6) in their experiments with cranberry found that nodal explants were more productive than shoot tips and the best total shoot production was obtained when nodal segments were cultured in the medium supplemented with 2.5-5.0 mg 2iP l^{-1} (12.3-24.6 µM). Kehie *et al.* (8) in their investigation with *Capsicum chinense* Jacq. cv. Naga King chili observed the greater number of shoot induction from nodal segments (12 ± 0.70) with bud-forming-capacity (BFC) index of 7.99 as compared to shoot tips (8.2 ± 0.37) with BFC index of 6.83. Likewise, Almeida *et al*. (1) noted highest shoot multiplication rate from single-nodal explants in *in vitro* multiplication of *Rhododendron ponticum* L. subsp. *baeticum*. However, Lameira *et al*. (12) noted superiority of apical shoot segment over nodal segments for culture initiation of *Cordia verbenacea* L.

Perusal of the data presented in Table 2 revealed that among the different conc. of kinetin tested 4.0 mg l^{-1} was noted to be superior over other treatments either used alone or in combination with BAP. It took shortest period for shoot induction; besides, achieving the highest shoot response of 93.6 per cent. Combinations of kinetin and BAP at 2.0 mg l^{-1} also gave comparable response. This result is in accordance with those reported by Borthakur *et al*. (4), where the best shoot multiplication and growth of *Alpinia galanga* was observed with kinetin 3.0 mg l^{-1}. Later, Datta *et al.* (5) also noted that the rate of shoot multiplication was significantly enhanced in nodal explants of *Jatropha curcas* after transfer to MS basal medium supplemented with combination of cytokinins like 2.3 µM 6-furfuryl amino purine (kinetin) and 27.8 µM adenine sulphate along with 0.5 µM indole-3-butyric acid (IBA) for 4 weeks. The poorest response was recorded with 4.0 mg l^{-1} each of kinetin and BAP. This could be attributed to the inhibitory effects of hormones at supra optimal levels. Once the shoots are induced, they are allowed to grow for about 10-12 days during, which they attain the length of 5-6 cm. Of the different treatments, combination comprising 2.0

Table 1. Influence of type of explant on number and length of shoot proliferation.

Explant type	No. of shoots/ explant	Shoot length (cm)
Apical shoot segment	2.41	8.76
Single node segment	3.13	11.59
$CD_{0.05}$	0.082	0.192

Table 2. Influence of growth regulators on shoot regeneration in *lasora*.

Kinetin (mg/l)	BAP (mg/l)	Response (%)	Time to shoot induction (days)
2.0	0.0	73.4	16.1
4.0	0.0	93.6	9.3
6.0	0.0	82.1	12.6
0.0	2.0	76.9	15.7
0.0	4.0	85.2	13.5
0.0	6.0	77.5	16.3
2.0	2.0	88.7	10.9
4.0	4.0	59.8	22.4
$CD_{0.05}$		1.75	0.43

mg l^{-1} each of NAA and IBA registered highest rooting response (85.6%) and average number of roots (4.1) followed by 1.0 mg l^{-1} each of NAA and IBA, while the least was recorded when NAA @ 1.0 mgl^{-1} was employed alone in the rooting medium (Table 3). Singh and Syamal (15) reported that the micro-shoots of rose rooted normally on the rooting medium supplemented with IBA and NAA together. The results showed that dual auxins, *i.e*., IBA + NAA, acted synergistically to produce functional roots, which helped in hardening and whole plantlet recovery. Likewise, Benniamin *et al*. (2), and Khosh-Khui and Sink (9) observed that maximum rooting (70%) of *in vitro* regenerated shoots of medicinal plant, *Crataeva magna*, was recorded in medium supplemented with 9.84 µM IBA and 0.54 µM NAA. However, Bhatia *et al.* (3) noted that mixing of two or more auxins (IAA, IBA and NAA) markedly reduced rooting percentage in micropropagated shoots of *Stackhousia tryonii* indicating antagonistic effects.

In the present studies, investigation was also carried out to assess the effect of activated charcoal on *in vitro* rooting response of *lasora*. It is evident from the data (Table 4) that AC had significant effect on rooting of *lasora* (Fig. 2). The addition of AC @ 750 mgl^{-1} resulted in a 5% increase in rooting response (Table 4). In addition, average number of roots produced per shoot also increased, while days taken for root induction was minimum (Table 4). These results are in conformity with those obtained by (Thomas, 16), who reported better root formation with addition of activated charcoal.

Table 3. Influence of growth regulators on root regeneration.

Treatment (mg/l)	Rooting (%)	Days to root induction	Av. No. of roots per shoot
IBA 1.0	28.3	17.6	1.7
IBA 2.0	48.7	12.8	2.7
NAA 1.0	21.9	19.5	1.3
NAA 2.0	37.4	15.4	2.0
IBA 1.0 + NAA 1.0	68.2	10.8	3.4
IBA 2.0 + NAA 2.0	85.6	8.2	4.1
$CD_{0.05}$	0.55	0.59	0.18

Table 4. Influence of activated charcoal on root regeneration.

Treatment (mg/l)	Rooting (%)	Days to root induction	Av. no. of roots per shoot
0	85.6	8.2	4.1
750	90.6	7.5	5.0
$CD_{0.05}$	0.74	0.59	0.18

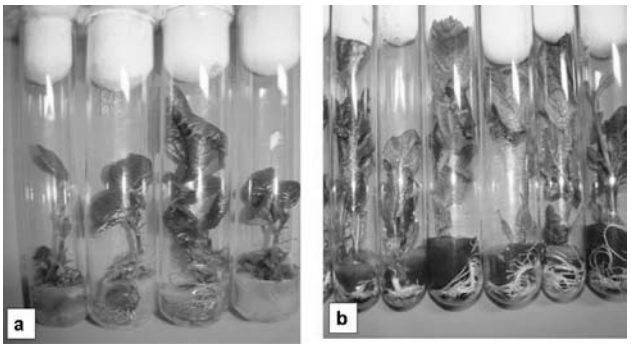

Fig. 1. Growth and rooting of micro-shoots as affected by activated charcoal. a. medium without AC; b. medium with AC.

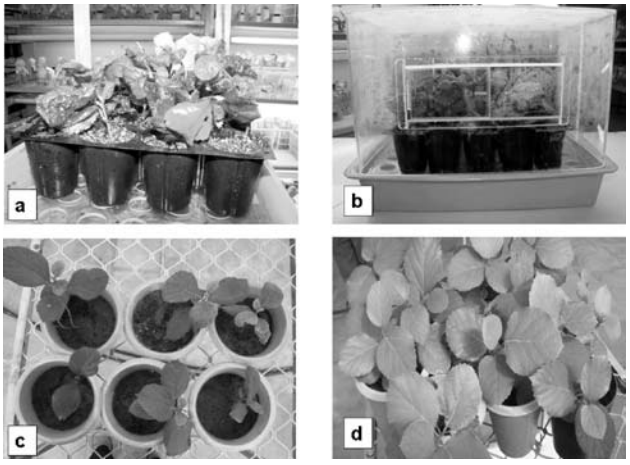

Fig. 2. Hardening of micropropagated *lasora* plants.(a) transfer to *ex vitro* conditions; (b) acclimation under hardening chamber; (c) transfer to polyhouse for bio-hardening; and (d) Successful weaning after one-month).

The rooted plants were later transferred to the hardening chamber for 10 days in hardening chamber followed by bio-hardening with AMF in poly-house for one month (Fig. 2). The mycorrhizal inoculation of micropropagated *lasora* plantlets significantly enhanced their survival (Fig. 4). This is in concurrence with those reported by Krishna *et al*. (10, 11) for *in vitro* raised grape plantlets. In the present study, the AM fungi inoculation had marked effects on growth of micropropagated plants (Table 5; Fig. 4), a result which is in line with the earlier findings of Estrada-Luna and Davies (7), and Marin *et al*. (13) in micropropagated Chile ancho pepper and persimmon plantlets, respectively. The beneficial effects of the symbiosis between the root system of tissue

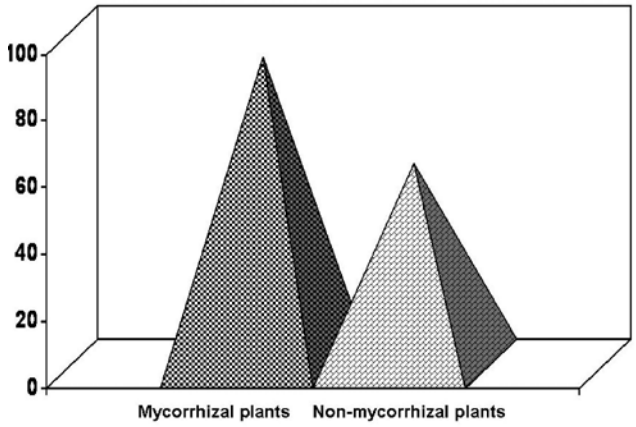

Fig. 3. Plant survival (%) in *lasora* as affected by mycorrhizal inoculation during bio-hardening.

Fig. 4. Growth response of mycorrhizal and non-mycorrhizal *lasora* plantlets.

culture-derived plants and arbuscular mycorrhizal fungi manifest themselves in the development of vigorous plants with improved physiological status and enhanced tolerance to different stress factors (Krishna *et al.*, 10). Mycorrhizal fungi are well known stress alleviator (Krishna *et al*., 11) and inoculation of AMF at an early stage of plant propagation could be a beneficial strategy to mitigate such biological and environmental constraints. These findings, further suggest their potential use of hardening agents for tissue culture raised *lasora* plantlets in order to reduce the mortality and thus to improve the overall growth and development of micropropagated plantlets during acclimatization.

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Table 5. Growth performance of mycorrhizal and non-inoculated *lasora* plants.

Treatment	No. of primary roots	Length of the longest root (cm)	Root dry weight (g)	Shoot dry weight (g)	No. of leaves	Leaf area (cm^2)
Mycorrhizal	6.9	15.3	3.83	4.67	10.3	6.7
Control	3.7	10.2	1.49	2.38	4.7	3.4
$CD_{0.05}$	0.53	0.59	0.08	0.23	0.52	0.37

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