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Research Article

STRATEGIES TO IMPROVE ROOTING AND ACCLIMATIZATION OF *ALBIZIA AMARA (ROXB.) BOIV.*

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ABSTRACT

The success of acclimatization and plant survival greatly depends on the quality of roots. The present study explores a reproducible and efficient protocol for enhancing the acclimatization of micropropagated plants of *Albizia amara* by *in vitro* root induction using auxin dip followed by *ex vitro* root development and biohardening. Successful *in vitro* root induction by the basal dipping of microshoots in an auxin solution for short periods and subsequent transfer to growth regulator-free medium in dark conditions for 6 to13 days at a temperature of 26-28°C has been reported. In the present study, Indole-3-Acetic Acid (IAA), Indole-3-Butyric Acid (IBA), and α-Naphthalene Acetic Acid (NAA), and different strengths of MS medium (full, half and 1/4) were tested. Shoots of size 2-3 cm were dipped in various concentrations (30, 40, 50, 60, 70, 90, 100 mg/l) of auxin solution for different time periods (15min, 30min, 60 min for each conc.). Highest root induction was observed on quarter strength MS medium and of the different auxins tested maximum root induction (92%) was achieved when shoots were dipped in 60mg/l NAA for one hour. Shoots with root primordia were placed in a sterilized potting mix (Soil:Peat:Vermiculite:Perlite in 2:1:1:1) supplemented with bioinoculants for root elongation and biohardening. This strategy of employing a combination of both *in vitro* and *ex vitro* methods for root development enhanced the plant survival rate to a maximum of 82%.

Keywords: *Albizia amara,* Microshoots, *In vitro*, *Ex vitro*, Auxin dip, Root induction

1. INTRODUCTION

Albizia amara (Nallaregoo, Chigaraku) belonging to the family leguminaceae is a valuable economic medicinal and multipurpose drought-tolerant tree commonly found in dry forests of India. The wood of *Albizia amara* is purplish brown with lighter bands, very hard, and strong used for cabinets in building and agriculture purposes [1]. The extracts from plant parts are used extensively in traditional medicine [2].

In the present investigation, this timber yielding leguminous tree *Albizia amara* has been selected owing to its importance as a plant with potential medicinal value [3]. There are few preliminary reports on the tissue culture of *Albizia amara* [4]. Ramamurthy and Savithramma [5] described shoot bud regeneration from leaf explants. Regeneration of true to type plantlets directly from seedling explants via cotyledonary bud proliferation was described in *A. amara* but limitations were observed during complete in vitro rooting and acclimatization [6]. The present proposal explores a reproducible and efficient protocol for enhancing the acclimatization of micropropagated plants by *in vitro* root induction using auxin dip followed by *ex- vitro* root elongation and biohardening.

Micro cutting may be rooted in two ways [7]. After a dip in a concentrated solution of auxin, they may be directly planted which is referred to as *ex vitro* rooting. Micro cutting may also be rooted completely *in vitro* by culture on medium with auxin. It should be noted that *in vitro* and *ex vitro* treatments have major dissimilarities. The exposure to auxin is for a short time in *ex vitro* rooting, but very long during *in vitro* rooting. Apart from these two methods in the present study, a combination of both *in vitro* and *ex vitro* method is used for rooting *i.e* root induction *in vitro* and root elongation *ex vitro*.

According to De Klerk [8], the division of the rooting process into phases has proven to be fruitful for improving the rooting treatment of Microcuttings. The process of root formation can be divided into at least two developmental stages: the initiation of primordia following cutting or wounding, and the stage of root emergence and growth. It is considered that in the first stage auxin acts as gene activator, *i.e.,* triggers the early formation of root primordia. For root

elongation, exogenous auxin is usually not required or is even inhibitory. Auxins should be distinctively applied during different stages of root formation (induction, initiation, root emergence, and elongation). The auxinic stimulation is generally at its maximum when high concentrations are applied immediately or just after the cutting process [9]. Once the roots are induced the removal of ethylene gas accumulated in the headspace of the culture vessel promotes division of the root meristem [10]. Fabbri and Bartolini [11] established that it is important to transfer shoots to the acclimatization substrate before roots emerge because roots developed *in vitro* are structurally different from those developed in soil. The *ex vitro* root development, however, resulted in favourable root development and shoot growth without producing the callus when root elongation is done on substrates other than agar [12, 13]. Debergh et al. [14] reported that Microcuttings of many woody plants can be transferred to greenhouse conditions with only root primordia as planting becomes more convenient, and can even be easily automated.

The success of acclimatization and plant survival greatly depends on the quality of roots. Roots developed completely under *in vitro* conditions lack well defined vascular bundles that are necessary for the supply of water and nutrients to the aerial part of the plant. *Ex vitro* formed roots usually are structurally and functionally better than *in vitro* formed roots and give higher survival rates of plants [15-19].

The strategy to obviate this inhibitory effect of auxins is the shoot transfer from induction medium with auxins to development medium devoid of these growth regulators. A better method is the shoots basal immersion in auxin for a limited period, followed by culture in an auxin-free medium. This method has been successfully employed with various species [20]. The objective of this study was to determine the effect of high auxin dip and nutrient strength on root induction (*in vitro*) followed by *ex vitro* root elongation and bio hardening of *A.amara* microshoots.

2. MATERIAL AND METHODS

2.1. Establishment of aseptic cultures

In vitro propagated plantlets of *Albizia amara* were used in the experiment. Cotyledonary node explants obtained from 15-d-old aseptically raised seedlings of *A.amara* were used for multiple shoot induction on Murashige and Skoog (MS) medium containing 2.0% sucrose, 0.8% agar supplemented with BAP (1mg L^{-1}). Microshoots of size 2-3 cm were excised from the proliferating shoot cultures and used for rooting experiments in the present study.

2.2. Effect of nutrient strength & darkness on rooting

The effect of inorganic salt concentration and darkness on the *in vitro* root induction of *A.amara* by using MS culture medium of different strength (full, half- and quarter strength) was tested. Healthy shoots from the shoot multiplication medium after auxin dip were taken and cultured on different strength MS media containing 2.0% sucrose and 0.8% agar, incubated in darkness for 2-3 weeks at 26$\pm$2°C and RH of 55%. NAA at a concentration of 60 mg/l for 30 minutes was used for auxin dip treatments. The same experiment was repeated in continuous light for 2-3 weeks to check the efficiency of dark/light conditions on root induction. For the dark treatment of shoots, culture vessels were wrapped with tin foil and placed on shelves without light.

2.3. Effect of various auxins on root induction

The microshoots of 2-3cm in length were excised carefully from the multiple shoots proliferated from the cotyledonary nodal explant of aseptically raised seedlings of *A.amara*. The base of the microshoots was immediately dipped in high concentrated auxin solution for 30 minutes. Dipping was carried out by placing shoots in culture vessels containing auxinic solution covered with a layer of perlite (for support).

The treatments tested were types of auxin (IAA, IBA, and NAA) at different concentrations (30, 40, 50, 60, 70, 90, 100 mg/l).After this, the Microcuttings were grown in 1/4 strength MS auxin free media in dark conditions for 2 weeks at a temperature of 26-28°C and RH of 55% for root induction. This helped in screening the auxin which promoted maximum root induction.

2.4. Effect of NAA on root induction

The basal ends of microshoots were dipped in NAA solution of various concentrations (30, 40, 50, 60, 70, 90, 100 mg/l) at different time periods (15min, 30min, 60 min for each conc.). After this, the Microcuttings were grown in 1/4 strength MS auxin free media in dark conditions for one week for root induction at a temperature of 26-28°C and RH of 55%. Shoots were grown in the dark for one week and then placed under

normal light conditions (16 hr photoperiod) for the next week. Experiments were carried out to determine the optimum NAA concentration, the optimum duration of dipping on root induction. The percentage of shoots that had rooted, was determined two weeks after the root-induction treatment. Each experiment consisted of three replicates, and 20 Microcuttings were used per treatment. The rooting response was expressed in terms of root induction percentage (Number of rooted shoots / Total number of shoots kept for rooting x100).

2.5. *Ex vitro* root development & acclimatization

The microshoots with root primordia were taken out from the culture vessel, thoroughly washed with tap water to remove adhering agar, and transferred them to the sterilized potting mixture (soil: peat: vermiculite: perlite in 2:1:1:1) in plastic pots. The microshoots were treated with bioinoculants namely - *Pseudomonas fluorescens* and *Trichoderma viride* immediately after transplantation to potting mix by the soil-drenching method. The plantlets were covered with plastic bags to maintain high humidity and kept in shade at 28±2°C with 13/11 hours light/dark regime with 65-70% RH. The potting mix was moistened daily with ¼ MS salt solution. Humidity was gradually reduced by making larger holes in the bags, later their covers were opened gradually. Un-inoculated plants of similar size were also maintained in the potting mix as control. For successful acclimatization hardening of plantlets under high humidity during first four weeks was found to be essential. After one month the root colonized plantlets were transplanted into earthen pots containing soil, sand, and farmyard manure (1:1:1) and were allowed to grow under nursery shade conditions. At the time of transplantation, various growth parameters were recorded from treated and control samples. Each experiment consisted of three replicates and 20 plantlets were used per treatment. Data was recorded 60 days after the transfer of plants to pots.

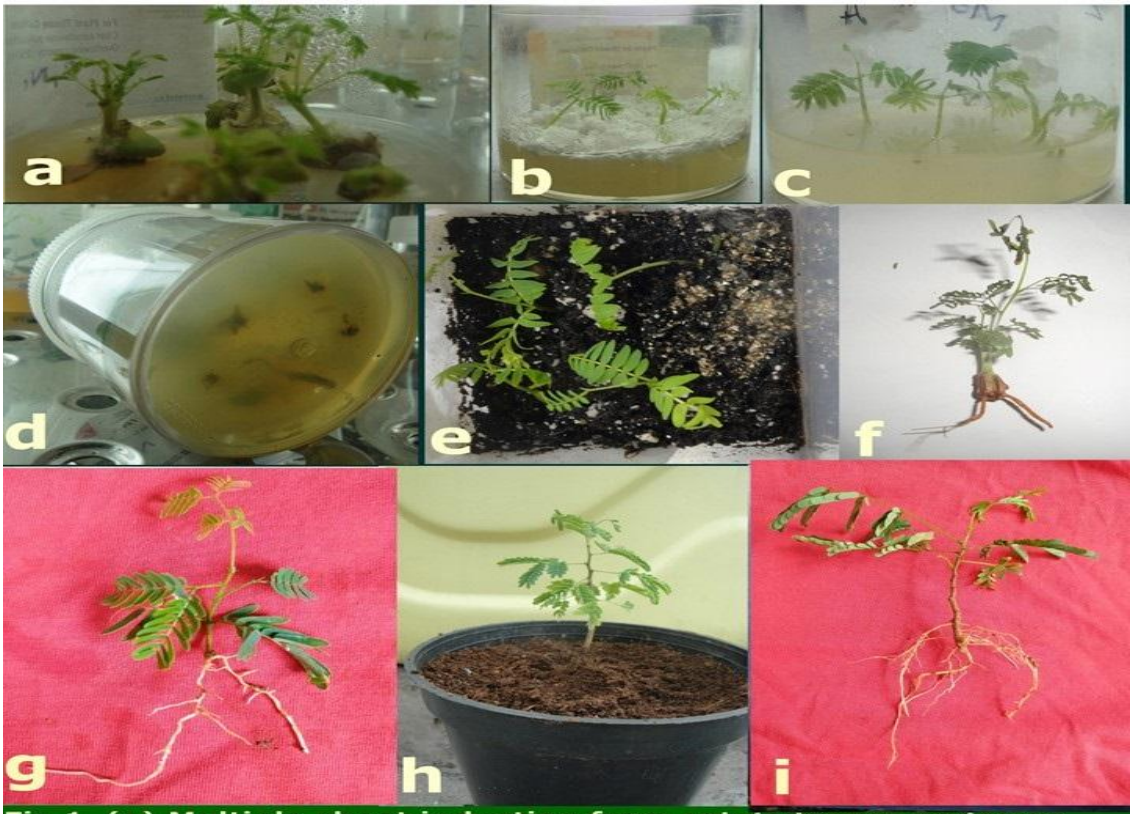

Fig.1. (a) Multiple shoot induction from cotyledonary nodes on MS medium fortified with BAP 1mg/l (b) Microshoots subjected to Auxin dip - NAA 60 mg/l for 60 minutes (c) In vitro root induction on ¼ MS medium + 2% sucrose+ 0.8 % agar (d) Root primordia- Bottom view (e) Ex vitro root development in potting mix after treatment with bioinoculants (f) Two week old bio-hardened plant (g) Four week old bio-hardened plant (h) Two month old potted plants (i) Two month old hardened plants with lateral roots.

3. RESULTS

3.1. Effect of Darkness and Nutrient strength on rooting

Dark treatment had a significantly beneficial effect on the rooting of *A.amara*. Root initials were visible at the cut ends of microshoots between the 6th and 13th day. Shoots kept in dark were slightly pale, thin, and lengthy with narrow leaves. But on transfer to light, they attained good growth. Minimum callus was formed at shoot bases in darkness. Root induction is less in continuous light as compared to dark. A maximum rooting of 34.54% was achieved in light conditions with less callus on ¼ MS medium compared to half and full-strength MS medium. Thus in *A.amara*, light treatment-induced negligible rooting, whereas in dark treated shoots, root primordia were visible on 6th day onwards and the highest percentage of root induction (82.04 %) was found after dark treatment between 6-13 days in all the treatments tested.

Root development was much better on the low-nutrient medium (1/4 strength MS) than on the standard medium (Table 1). After 2 weeks in culture, the highest percentage of root induction (82.04%) was found in ¼ strength MS medium. 1/4 MS growth regulator-free medium was superior to Half-strength (65.34 %) and full-strength MS (33.52 %) for root development. Most shoots failed to develop roots on the full-strength growth regulator-free medium. The presence of nutrients at lower concentrations in ¼ strength MS medium facilitated better rhizogenesis.

Table 1: Effect of Darkness and Nutrient strength on root induction of *A.amara* microshoots after auxin dip in NAA (60mg/L for 30 minutes)

Types of medium	Percentage of Root Induction		No.of Days for root induction		Basal Callus Induction*	
	Dark	Light	Dark	Light	Dark	Light
MS Full strength	33.52	21.4	13	20	++	+++
MS 1/2 strength	65.34	30.18	10	15	+	+++
MS 1/4 strength	82.04	34.54	6	12	-	++

3.2. Effect of high auxin dip on root induction of Microcuttings

In control without auxin treatment, only 5% of the plantlets formed roots, whereas in auxin treatments regardless of its concentration, most of the microshoots showed root induction. The three auxins tested have a positive effect on root induction. In all cases, at an auxin concentration ranging 50- 80 mg/l produced root primordia. At low concentrations, the percentage of root induction was very low, but at a high concentration above 80 mg/l triggered basal callus formation.

It is evident from Fig. 2 that of all the auxins tested, the maximum root induction (86%) was achieved when shoots were dipped in NAA at 70 mg/l for 30 minutes. The percentage root induction for IBA was maximum (60.21%) at 80 mg/l and for IAA it was maximum (28.43%) at 60mg/l for 30 minutes auxin dip treatment. NAA-treated shoots started to initiate root primordia after 6 days, while with IBA and IAA, root initiation commenced after 10 days and 13 days respectively. Of all the auxins tested, NAA was the most effective for *in vitro* root induction, followed by IBA and IAA, in descending order.

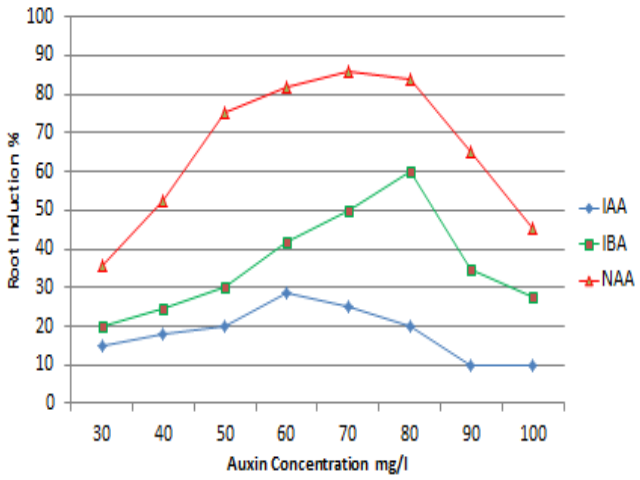

Fig. 2: Effect of high auxin dip for 30 minutes on root induction of A. amara microshoots

3.3. Effect of NAA on root induction of Microcuttings

These experiments were carried out to optimize the conditions of root induction (NAA concentration and dipping time). It is evident from Figure 3 that the maximum root induction (92%) was achieved when shoots were dipped in 60 mg/l NAA for 60 minutes.

NAA levels above 90 mg l^{-1} showed decreased rate of rooting with more callus at the shoot base, whereas below 40 mg l^{-1} showed low root induction. NAA at 70 and 80 mg l^{-1} concentration also resulted in good rooting The dipping time of 30 & 60 minutes was favourable in most of the NAA concentrations tested. Reducing the concentration of NAA below 40 mg/l caused a significant drop in the rooting rate and for a concentration of NAA above 80mg/l dipping gave significantly worse results by the basal callus formation.

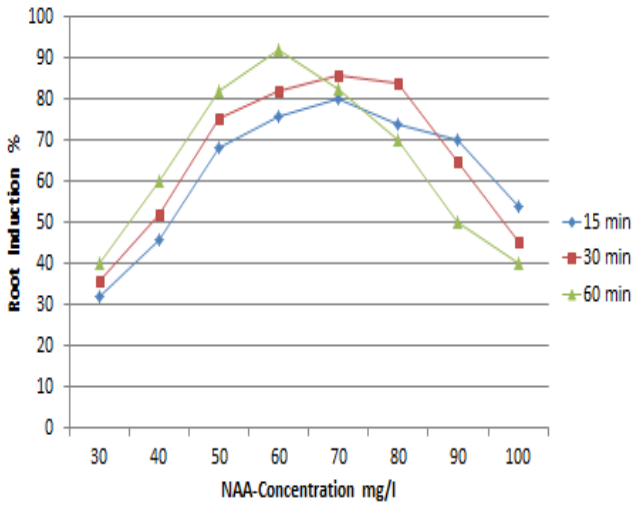

Fig. 3: Effect of NAA auxin dip on root induction of A. amara microshoots

3.4. *Ex vitro* root development and acclimatization

An increase in shoot length and root length was recorded in bioinoculants added treatments over control. After 60 days of growth in nursery shade, a significant increase in root length and number of lateral roots was observed over uninoculated control. Plantlets inoculated with bioinoculants attained maximum shoot and root length of 12.5 cm and 10.8 cm as compared to control plantlets with 4.24 cm and 2.32 cm on an average per plantlet respectively. Bioinoculant treated plantlets also showed a significant increase in total plant biomass fresh weight (1.12 g) and dry weight (0.28g) as compared to control plantlets with fresh weight (0.32 g) and dry weight (0.06 g) on an average per plantlet.

4. DISCUSSION

4.1. Effect of Nutrient strength on rooting

It was observed that lower concentrations of MS nutrients contributed to improvement in *in vitro* root induction of *A.amara*. Excised shoots failed to develop roots on the full-strength growth regulator-free medium due to callus formation. The presence of nutrients at lower concentrations in 1/4 MS medium facilitated better rhizogenesis.

The results observed in the present study are in coincidence with other reports. Adventitious shoots cultured on half MS produced almost the double number of roots than when grown on full MS medium in *Mentha arvensis* regenerants [21]. Tetsumura et al. [22] also observed that a reduction in the strength of MS medium resulted in the increase of *in vitro* shoot and root formation from high bush blueberry. Low salt concentrations (1/10 MS strength) favoured rooting of *Philodendron erubescens* microshoots as opposed to full strength MS which inhibits rooting [23]. Other researchers have also reported the beneficial effect of a reduction of the strength of the culture medium on the *in vitro* initiation of roots of plant species such as rose, ginger, stevia, spearmint, and globe artichoke [24-30].

4.2. Effect of Darkness

The effect of light and darkness has considerable importance during the stages of root formation, including the sequence of these regimes, intensity, and quality of light. Since light inhibited root formation as it does in many species including certain recalcitrant juvenile clones of Camellia, an immediately post-dip dark period was considered essential for rooting [31,32]. Dark treatment had a significantly beneficial effect on the root induction of *Albizia amara*. Root initials were visible at the cut ends of microshoots 6 days after the rooting treatment. Shoots kept in dark were slightly pale, thin, and with narrow leaves. But when the culture vessels were transferred to illumination after 1-week dark incubation they attained good growth. Similar reports were observed when shoots of Prunus were kept in the dark during the first 5 days followed by transfer to light conditions [33]. Root induction was less in continuous light as compared to dark. Druart [34] observed that continuous darkness during the root inductive phase increases peroxidase activity resulting in a higher rooting rate.

Light is often considered an inhibiting factor in rooting of Microcuttings especially at the induction phase of root primordia, whereas, darkness during rooting has a rather stimulating effect on root formation [35]. Darkness applied during the first week of rooting has resulted in an enhancement of the rooting percentage, the number of roots developed per microcutting, and the length of roots in several woody species such as

apple, wild cherry, chestnut, rhododendron and cork oak [36, 37]. Hammerschlag et al. [38] worked with apple trees by maintaining a temperature of 26 °C to a dark period of 14 days to initiate root primordia . High frequency rooting under darkness was also obtained in *P.avitum* and apple [39, 40]. Hammerschlag [41] stated that a 2-week dark period was necessary for maximum rooting of Calita plum, and illumination inhibited root formation. An initial 9-day dark period imparted both a significantly higher rooting percentage (78%) and a greater number of roots per shoots of *Camellia japonica* [42].

4.3. Effect of High Auxin Dip

Today, the trend is to avoid the whole rooting process *in vitro;* instead of this, at a first step to induce the formation of root primordia *in vitro* and at a second step to promote extensive rooting ex vitro is being practiced [43]. In complete *in vitro* rooting auxins were mixed into the medium for the whole culture period, while in the present experiment the microshoots were only dipped exclusively in auxin solution for some time. The response of stem cuttings is dependent upon the plant material ontogenetic age, the auxin nature, the contact duration, and the application time [44]. Addition of auxin immediately after cutting initiates the first cellular divisions and formation of root primordia [45]. Most reports of adventitious root induction of woody species have involved treatment with exogenous auxins such as IBA, NAA, and IAA [46]. George and Sherrington [47] reported the efficacy of NAA and IBA over IAA and this is in coincidence with the present study. Moncousin [34] reported that high auxin concentrations are necessary only for the first stages of rhizogenesis as they can inhibit the evolution of the morphogenetical areas. When the auxins are added at too lower doses rhizogenesis is not stimulated and sometimes even retarded. Similar reports were observed in the present study with low and high doses of auxin.

Nissen and Sutter have shown that in tissue culture media IAA is photo-oxidized rapidly (50% in 24h) and IBA slowly (10%) and NAA is very stable [48, 49]. Thus, the different effects observed in the present study among the three auxins reflect, besides possible different affinities for auxin receptors there are differences in uptake, transport, and metabolism. With NAA treated shoots the roots started to initiate after 6 days, while on IBA & IAA treated shoots, root initiation commenced after 10 and 13 days respectively. Of the auxins tested here, NAA was the most effective for *in vitro* root induction, followed by IBA and IAA, in descending order. De-Klerk et al. found that the rate of auxin uptake varied [50]. Peeters et al. found that NAA was taken up six times faster than IAA [51], and Vander Krieken et al. found that IBA was taken up four times faster than IAA [52]. Consequently, in the present study, the efficacy of rooting in the presence of NAA may be due to its faster uptake.

Similar reports of root induction using high concentrations of auxin treatments were reported in other studies. The use of NAA at 2-8 mM increased root induction and root quality in oil palm plantlets [53]. Dipping in a solution of 100 mg/ l IBA increased the rooting percentage of *R. ponticum* to 94% [54] and a combination of IBA and IAA increased rooting percentage to 91.7% in myrtle [55]. The induction of roots by the basal dipping of shoots developed *in vitro* in an IBA solution for short periods and subsequent transfer to growth regulator-free medium has been reported in *Diospyros kaki* and *Ceratonia siliqua* [56, 57]. Das et al. and Jain et al. reported successful rooting of microshoots of tea treated with IBA at the cut ends and placed directly in the soil mix [58, 59].

4.4. Effect of NAA Auxin dip

In the present study of the different concentrations of NAA tested, maximum root induction (92%) was achieved when shoots were dipped in 60mg/l NAA for one hour. In the present study, the efficacy of rooting in the presence of NAA may be due to its faster uptake. Similar observations about NAA were found by a few workers in woody species such as *Rotula aquatica* [60]. Almedia et al. reported 96% rooting frequency and 6.2 roots per plantlet when *R. ponticum* shoots dipped in 100 m g/l NAA for 2 minutes [54].

4.5. *Ex vitro* root development and acclimatization

One of the unique features of this study was the incorporation of a bio-hardening step at the time of transplantation. The addition of bioinoculants such as *Pseudomonas fluorescens* and *Trichoderma viride* was found to be effective in improving the establishment frequency [61]. Biotization of micropropagated *A.amara* plantlets with *T.viride* and *P. fluorescens* protected micropropagated young plantlets from 'transplantation shock'. Plant survival rates were observed maximum in biohardening treatment, which must be due to the positive interaction between *T. viride* and *P. fluorescens* and their ability to enhance stress tolerance by

protecting them from subsequent 'transplantation shock'. Perhaps the increase in survival rate could also be due to the synergistic effect of *T. viride* with *P. fluorescens* as it could solubilize more P in the soil by producing organic acids [62].In the present study, bioinoculants treated plantlets showed significantly more number of lateral rootlets, an increase in root length as compared to control due to the presence of beneficial microflora, which may have produced growth-promoting substances [63]. Similar reports on the combined effect of *T. viride* and *P. fluorescens* on growth improvement was observed by other workers [64-66]. Better root system helped in more nutrient uptake, which resulted in healthy plants with more shoot biomass, as healthy roots result in a healthy plant. There was a lower percentage of survival with complete *in vitro* rooting [6] but the combination of both *in vitro* and *ex vitro* rooting using auxin dip and bioinoculants had improved the plant survival rate.

5. CONCLUSIONS

The present study has given a positive indication on the introduction of auxin dip and bioinoculants during the acclimatization phase of *in vitro* derived *A. amara* plantlets. Root induction of tissue culture-derived microshoots of *A. amara* is achieved by high auxin dip followed by incubation of cultures in 1/4 MS Medium at 26-28°C in dark conditions for 6-13 days. In the present study of the different auxins tested, maximum root induction (92%) was achieved when shoots were dipped in 60mg/l NAA for one hour. Biotization of micropropagated *A.amara* plantlets with dual inoculation of *T. viride* and *P. fluorescens* enhanced root development and plant survival (82%) than uninoculated plants. Thus *in vitro* root induction followed by *ex vitro* root development and biohardening were found to influence the survival rate, plant growth, biometric parameters, and systemic resistance.

6. FUTURE PERSPECTIVE

The probable molecular mechanism for enhancing the growth characteristics due to the application of auxin dip and bioinoculants needs to be envisaged. Development of new culture methods allowing the successful establishment of tissue culture raised plants in open fields and understanding mechanisms of signal recognition and transduction in plants under different environments both *in vitro* and *ex vitro* are probably the important areas of research to be explored.

7. ACKNOWLEDGEMENT

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