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Resource communication. *In vitro* plant regeneration of *Albizia lebbeck* (L.) Benth. from seed explants

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Abstract

Objectives: An efficient and reproducible regeneration protocol for rapid multiplication of *Albizia lebbeck* (L.) was developed by using intact seed explants.

Methods: Murashige and Skoog's (MS) medium supplemented with different hormones (BA, Kn, GA_3 and TDZ) was used for the induction of multiple shoots from the seed explants. *Ex-vitro* rooting was performed by using pulse treatment method in auxins (IBA and NAA) and the complete plantlets were transferred to the field.

Results: High frequency direct shoot induction was found in aseptic seed cultures of *A. lebbeck* on Murashige and Skoog medium supplemented with 5.0 µM TDZ (Thiadiazuron). Seeds were germinated after 7 days of culture and induced maximum 8 shoots from the region adjacent to the apex of the primary shoot of the seedling upto 25 days of incubation. Proliferating shoot cultures with increased shoot length was established by sub-culture of excised sprouting epicotyls on MS medium supplied with reduced concentrations of TDZ. Maximum shoot regeneration frequency (76%) with highest number of shoots (21) and shoot length (5.1 cm) per sprouting epicotyl was observed in the MS medium supplemented with 0.5 µM TDZ after 8 weeks of culture. Different concentrations of Indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA) were tested to determine the optimal conditions for *ex-vitro* rooting of the microshoots. The best treatment for maximum *ex-vitro* root induction frequency (81%) was accomplished with IBA (250 µM) pulse treatment given to the basal end of the microshoots for 30 min followed by their transfer in plastic cups containing soilrite and eventually established in normal garden soil + soilrite (1:1) with 78% survival rate. In addition, histological study was undertaken to gain a better understanding of the regenerated shoots from the epicotyl region.

Conclusion: The findings will be fruitful in getting a time saving and cost effective protocol for the i*n vitro* propagation of *Albizia lebbeck*.

Key words: woody legume, TDZ, multiplication, epicotyl segment, shoot stumps.

Introduction

Albizia lebbeck (L.) (Fabaceae) is a large, erect, unarmed, spreading tree native to deciduous and semi deciduous forests in Asia from eastern Pakistan through India and Sri Lanka to Burma (Kumar *et al*., 2007). It is a good source of fodder and green manure and used for fuel production, furniture making, erosion control and as a shade tree in tea, coffee and cardamom plantations (http://www.worldagroforestrycentre.org). The plant is reported to have anti-asthmatic, anti-inflammatory, antifertility and anti-diarrhoeal properties and an important source of chemicals of D-catechin, b-Sitosterol, Albiziahexoside etc., which are effective as antiseptic, anti-dysenteric, anti-tubercular and used in bronchitis, leprosy, paralysis, helmenth infection etc. (Kumar *et al*., 2007). It is also used for environmental management, tolerates light, frosts, and drought and also flourishes well in saline, sodic, lateritic and mining sites (Perveen *et al*., 2012). Due to its significant multipurpose properties, the tree has been overexploited, which in turn has resulted in the severe depletion of its natural population (Troup, 1986).

Conventionally, *Albizia lebbeck* is propagated from seed however, prolonged dormancy, rapid loss of viability, and low germination rates limit natural propagation (Perveen *et al*., 2011). To overpass this insufficiency and for safeguarding the species from extinc-

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Abbreviations: GA_3 (Gibberellic acid); BA (6-Benzyladenine); Kn (Kinetin); TDZ (Thidiazuron); MS (Murashige and Skoog medium); IBA (Indole-3-butyric acid); NAA (α-Naphthalene acetic acid).

tion, there is an urgent need to develop a cost effective protocol which can be utilized for large scale plant production. Of the various facets of biotechnology which demand immediate application in woody trees, the reproducible tissue culture technology for regeneration of large scale plants through intact seedling explants is outstanding and may prove to be the powerful tool for germplasm conservation and mass production of imperative plant species. The application of intact seedling method in the propagation of multipurpose plant species provides timely supply of plant materials required for the isolation of drugs by the pharmaceutical industries without depleting natural plant resources as well as for reforestation of degraded and exploited lands. Several authors (Malik *et al*., 1993; Prakash *et al*., 1994; Bhuyan *et al*., 1997; Arya *et al*., 1999; Das *et al*., 1999; Hussain *et al*., 2008) have reported the use of intact seedling method in several medicinally and economically important plant species (*Lathyrus cicera*, *Cajanus cajan*, *Murraya koenigii*, *Dendrocalamus asper*, *Litchi chinensis* and *Sterculia urens*) for quick germplasm production and conservation.

The relevance of intact seedling method in *A. lebbeck* is of two-fold. First of all, it can be used as a means of propagating many plants from just a few seeds. Secondly, and more importantly, intact seedling method can be used to produce and propagate plants quickly, with characteristics not readily found in wild populations that may be useful for forest creation and restoration. Earlier propagation of this leguminous tree species via lateral buds from mature trees and seedling explants was reported by several workers (Mamun *et al*., 2004, Perveen *et al*., 2011 and Borthakur *et al*., 2011). However, these protocols involve several stages and are rather long (ranging from 31 to 172 days).

Here, we report a very simple, reproducible and innovative protocol of culturing mature seeds of *A. lebbeck* on MS medium containing TDZ for *in vitro* propagation and multiplication. To the best of our knowledge, it is the first report of shoot multiplication using intact seedlings with a simple, reproducible, efficient and speedy regeneration system.

Materials and methods

Plant material, culture media and culture conditions

The mature fruits of *A. lebbeck* were collected from a 20 year old candidate plus tree growing in Botanical Garden of the University, Aligarh. The healthy seeds were excised from the fruits and washed thoroughly in running tap water for 30 min. These were immersed in 1% (w/v) solution of Bavistin, a fungicide, for 15 min to remove adherent particles from the surface and later treated with 5% (v/v) Teepol solution for 20 min. Thereafter, the seeds were rinsed thrice with sterile distilled water followed by surface sterilization in 0.1% (w/v) $HgCl_2$ solution under laminar flow hood for 15 min. Finally, the seeds were rinsed thrice with sterile distilled water to remove the traces of $HgCl_2$ solution. MS (Murashige and Skoog, 1962) basal medium fortified with 3% (w/v) sucrose, 0.8% (w/v) agar (Qualigens, Mumbai, India) was used in all the experiments. The medium was adjusted to pH 5.8 using 1N NaOH or HCl prior to autoclaving. The media were dispensed in 12 cm × 6 cm glass bottles (Borosil, India) for seed germination, each containing 50 mL of medium and covered with plastic caps. While for sprouting epicotyl culture (shoot induction and multiplication) 20 mL media were dispensed in each 25 mm × 150 mm test tubes and 50 mL media in each 100 ml Erlenmeyer flasks (Borosil, India) and cotton plugs (double-layered muslin cloth stuffed with non-adsorbent cotton) were used as closures. Glasswares and culture media were sterilized by autoclaving at 121°C and 1.06 Kg cm^{-2} pressure for 20 min. Cultures were incubated at 25 ± 2°C under 16/8 h (light/dark) photoperiod provided by cool white fluorescent tubes (Phillips, India) with a photon flux density of 50 μmol m^{-2} s^{-1} at 55%-60% relative humidity.

Seed germination and establishment of shoot cultures

The disinfected seeds were inoculated on MS (Murashige and Skoog, 1962) basal medium with different concentrations (1.0, 2.5, 5.0, 7.5 and 10.0 μM) of BA, Kn, GA_3 and TDZ singly for seed germination and used as explants for direct multiple shoot regeneration. Data on the frequency of seed germination, explants producing shoots, shoot number and shoot length were recorded after four weeks of culture.

Shoot proliferation, multiplication and elongation

Sprouting epicotyl segments (Fig. 1b) excised from TDZ raised, 25-day-old seedlings were cultured onto MS medium augmented with lower concentrations of TDZ (0.05, 0.1, 0.3, 0.5, 1.0 and 1.5 μM) for induction

Figure 1. *In vitro* regeneration and plantlet establishment of *A. lebbeck*. a) Seed germination and induction of multiple shoots from epicotyl region on MS + TDZ (5.0 µM). b) Sprouting epicotyl segments obtained on MS + TDZ (5.0 µM) after 25 days of culture. c) Multiplication of shoots achieved from epicotyl segments (shoot stumps) cultured on MS + TDZ (0.5 µM). d) An *ex-vitro* rooted plantlet. e) Acclimatized plants in soilrite.

and multiplication of shoots. Subculturing was performed on the same fresh media at every two weeks to avoid basal callusing. Data on the frequency of explants producing shoots, shoot number and shoot length were recorded after four weeks of culture.

Histological analysis

The histological study was performed with sprouting epicotyls in their starting stage (7 and 10 day-old) and fixed in 5:5:90 (v/v/v) formaline:acetic acid: ethanol (FAA) for 24 h and stored in 70% (v/v) ethanol, dehydrated in a graded ethanol-xylol series followed by paraffin embedding using the method described by Johansen (1940). Longitudinal sections were cut using a Spencer 820 rotary microtome (American Optical Corporation, Buffalo, NY, USA) and stained with safranin and fast-green (Johansen, 1940). Sections were examined under a light microscope (CH20i, Olympus India Pvt. Ltd. New Delhi).

Ex-vitro rooting and acclimatization

For *ex-vitro* root induction, excised shoots (4-5 cm) with four or more leaves were harvested, their basal portion dipped in different concentrations of IBA and NAA (50, 100, 150, 200, 250 and 300 µM) singly for 30 min and subsequently planted either in garden soil, vermiculite or soilrite (Keltech Pvt. Ltd. Bangalore) in plastic cups and placed in 16 h photoperiod with light intensity of 150 µmol m^{-2} s^{-1} PPFD. Potted plantlets were covered with transparent polythene bags to ensure high humidity and maintained in the culture room for 4 weeks. Thereafter, the plantlets were transferred to the mixture of soilrite + garden soil (1:1) under the similar culture room conditions. Data on percentage of rooting, mean number of roots per shoot and root length were recorded after 4 weeks of *ex vitro* transplantation. The survival rate of acclimatized plants was also assessed after 6 weeks of the transfer to the growth chamber.

Statistical analysis

The experiments were laid out according to a completely randomized design. Each treatment consisted of 10 replicates and all experiments were repeated thrice. The data were analyzed statistically using one way analysis of variance (ANOVA) and pairwise means compared using Duncan's multiple range test (p = 0.05). The results were expressed as the means ± SE of three repeated experiments.

Results and discussion

The shoot forming capacity of intact seedling explants was greatly influenced by the type of growth regulator and its concentration in the medium. However, single shoot was differentiated from the seeds cultured on MS basal medium. Similarly, the addition of different concentrations of BA and GA_3 induced seed germination. In between different tested concentrations of BA and GA_3, maximum seed germination frequency (50 % and 46 %) was found at 5.0 µM concentration with single shoot from each axillary bud of the cotyledonary node region. While Kn containing medium failed to germinate the seeds (Table 1). Similar effect of BA and GA_3 on seed germination has been reported by Mallik and Saxena (1992) under *in vitro* condition.

Table 1. Percentage of seed germination on different concentrations of BA, Kn and GA_3

Plant growth regulators (µM)	% seed germination
MS	80 ± 0.1^{a}
MS + 1.0 µM BA	20 ± 0.2^{e}
MS + 2.5 µM BA	35 ± 1.1^{c}
MS + 5.0 µM BA	50 ± 1.4^{b}
MS + 7.5 µM BA	42 ± 0.5^{bc}
MS + 10.0 µM BA	30 ± 0.9^{d}
MS + 1.0 µM Kn	00 ± 0.0^{g}
MS + 2.5 µM Kn	00 ± 0.0^{g}
MS + 5.0 µM Kn	00 ± 0.0^{g}
MS + 7.5 µM Kn	00 ± 0.0^{g}
MS + 10.0 µM Kn	00 ± 0.0^{g}
MS + 1.0 µM GA_3	18 ± 0.8^{f}
MS + 2.5 µM GA_3	30 ± 0.9^{d}
MS + 5.0 µM GA_3	46 ± 1.2^{b}
MS + 7.5 µM GA_3	32 ± 1.6^{d}
MS + 10.0 µM GA_3	26 ± 1.1^{e}

Values represent means ± SE. Means sharing the same letter within columns are not significantly different (p = 0.05) using Duncan's multiple range test.

In contrast, on a media containing different concentrations of TDZ, an enlargement and subsequent break of multiple shoots from the region adjacent to the apex of the primary shoot (epicotyl region) (Fig. 1a) was observed in all the concentrations tested within 10 days of seed inoculation. ANOVA showed a significant effect of TDZ concentrations on the frequency of seed explants forming sprouting epicotyls, on the number of shoots per epicotyl, and shoot length after 25 days on an induction medium (Fig. 2). All the viable seeds

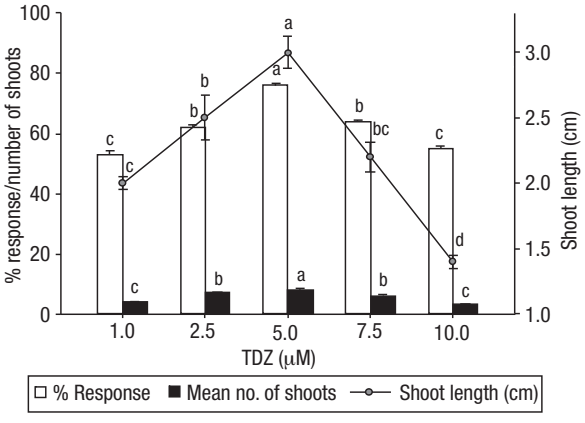

Figure 2. Effect of different concentrations of TDZ supplied in MS medium, on percentage of stump regeneration from intact seed explants, average number of shoots and shoot length obtained per stump after 25 days of culture. Bars represent the means ± SE. Bars denoted by the alphabets are significantly different (p = 0.05) using Duncan's multiple range tests.

germinated and proliferated multiple shoot containing stump (sprouting epicotyls) (Fig. 1a) in each germinated seed with two to three pairs of leaves on MS medium containing 5.0 µM TDZ with maximum 76 % sprouting epicotyls regeneration frequency, 8 number of shoots per sprouting epicotyl and a maximum of 3.0 cm shoot length within 25 days (Fig. 2) of seed culture. Similar, observation has been reported by Hussain *et al*. (2008) in *Sterculia urens*, where TDZ was found effective for direct shoot regeneration from intact seedlings. Our results are in contrast with the findings of Bhuyan *et al*. (1997) in *Murraya koenigii*, Das *et al*. (1999) in *Litchi chinensis*, Arya *et al*. (1999) in *Dendrocalamus asper,* Hussain *et al*. (2008) in *Sterculia urens* and Rani *et al*. (2010) in *Murraya Koenigii*, where BA was found most effective for direct shoot regeneration from intact seedlings. At higher concentrations of TDZ (above 5.0 µM), rate of seed germination (sprouting epicotyls induction per seedling), and shoot multiplication per sprouting epicotyls decreased significantly (Fig. 2). Similar observations have been reported in *Phaseolus* species (Mallik and Saxena, 1992 a,b), *Arachis hypogaea* (Saxena *et al*., 1992) and *Muraya koenigii* (Bhuyan *et al*., 1997).

For further shoot proliferation and multiplication, the regenerated sprouting epicotyls were excised (Fig. 1b) and subcultured onto the same fresh medium (MS + 5.0 µM TDZ), which resulted in the formation of rosette of shoots and did not elongate further. Therefore, to overcome this problem, these sprouting epicotyls were subsequently subcultured to a secondary medium containing lower concentrations of TDZ which enhanced the rate of shoot proliferation and length by three to four folds (Fig. 1c). ANOVA also showed a significant effect of TDZ on frequency of responding explants giving new shoots, on the number of shoots per epicotyls, and on the shoot length when excised sprouting epicotyls were subcultured on proliferation medium. Maximum shoot induction and multiplication with 76 % shoot regeneration frequency, maximum number of shoots (21) and shoot length (5.1 cm) was observed on the MS medium containing 0.5 µM TDZ after 8 weeks of culture (Fig. 3). Similar reports where lower concentrations of TDZ was found effective for shoot proliferation and multiplication has been reported by Siddique and Anis (2007) in *Cassia angustifolia*, Ahmad and Anis (2007) in *Vitex negundo* and Jahan and Anis (2009) in *Cardiospermum halicacabum*. While, the subculturing of shoot stumps on growth regulator free MS medium was found unproductive

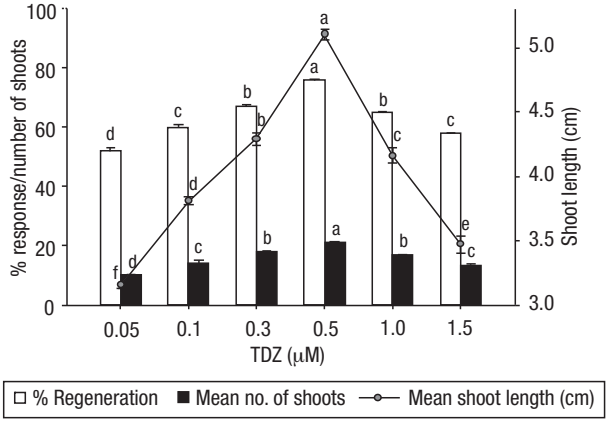

Figure 3. Effect of TDZ concentrations supplied in MS medium on percentage of stump segments showing proliferation, average number of shoots and shoot length (cm) obtained per stump segment after 8 weeks of culture. Bars represent the means ± SE. Bars denoted by the alphabets are significantly different ($p = 0.05$) using Duncan's multiple range tests.

causing fasciation and distortion of shoots leading to the death. Similarly, the synergistic effect of TDZ and auxins (NAA and IBA) was not effective in the present investigation for shoot multiplication (Data not shown).

Structure analysis is an important step in the study of the organization and changes in the plant body, and it is an extremely useful approach in the study of plant morphogenesis (Saha *et al*., 2012). Therefore, Histological studies were conducted on 7 day and 10 day-old epicotyl segments (sprouted epicotyls) to trace the origin of multiple shoots from the epicotyl region. Longitudinal section of epicotyl segment showed the development of apical shoot meristems with leaf primordia after 7 days of seed germination (Fig. 4a). While, after 10 days, adventitious buds (meristematic zones) start to develop (Fig. 4b) in the epicotyl region or in the basal region of the shoot apical meristems and subsequently differentiated into the shoots upto 25 days. Therefore, apart from shoot apical meristem, adventitious shoots also originate from epicotyl region in the intact seedling explants cultured on TDZ supplemented medium. Similar observations have been reported by Malik *et al*. (1993) in *Lathyrus* species and Poisetty *et al*. (1997) in *Cicer arietinum*, where adventitious origin of multiple shoots from epicotyl region was found directly from intact seeds cultured on different cytokinin containing medium (BA and TDZ). Borchetia *et al*. (2009) reported that the plantlets derived from adventitious mode of propagation can be genetically true to type and this mode of plant regeneration is more reliable than callus cultures.

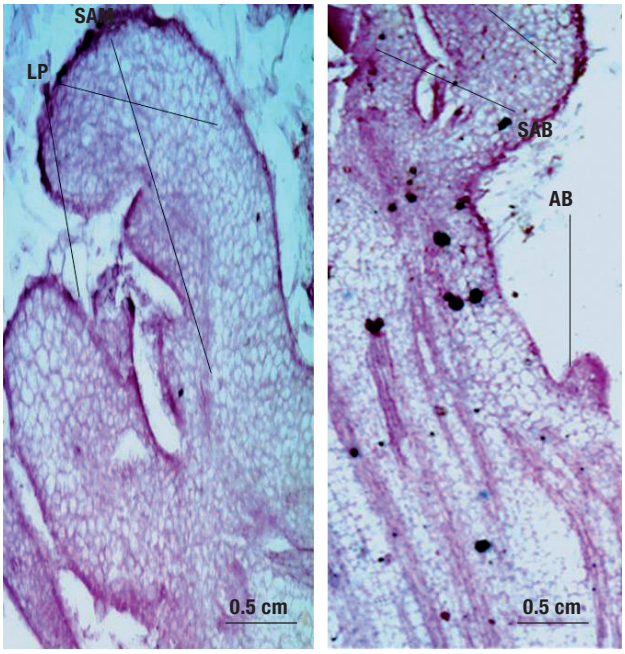

Figure 4. Histological studies conducted on 7 day and 10 day-old sprouted epicotyl segments. a) Photomicrographs of longitudinal sections of shoot apices (epicotyl segment) of *A. lebbeck* showing development of shoot apical meristem (SAM) after 7 days of seed culture. b) Longitudinal section showing lateral shoot bud (LB) proliferation after 10 days of seed culture.

Shoots produced *in vitro* were excised and treated with different concentrations of root inducing auxins namely IBA (Fig. 5) and NAA by *ex vitro* method as

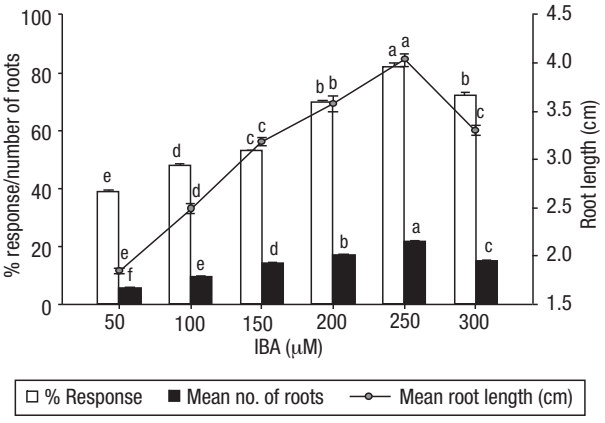

Figure 5. Effect of different IBA (dip treatment) concentrations on percentage *ex-vitro* root induction, average number of roots and root length (cm) per microshoot from isolated microshoots of *A. lebbeck* after 4 weeks of transplantation to soilrite. Bars represent the means ± SE. Bars denoted by the alphabets are significantly different ($p = 0.05$) using Duncan's multiple range tests.

described in materials and methods. Of the two, IBA treatment was found to be most effective. ANOVA revealed a significant effect of IBA on the frequency of rooted microshoots, on the number of roots per microshoot and root length. Among the different concentrations of IBA tested, the best *ex vitro* rooting response was obtained when shootlets were treated with 250 μM IBA for 30 min, where 82% of the shoots rooted well and produced the maximum number of roots per shoot (21), with the largest mean root length (4 cm) after 4 weeks of transplantation to potting mixture (Fig. 1d; 5). While, the different concentrations of NAA tested were found unproductive for *ex vitro* root regeneration (Data not shown). Higher concentration of auxin or longer duration (more than 30 min) of exposure induced undesirable callus formation at the base of the shoots (Data not shown). *Ex vitro* rooting is reported to be a promising and attractive method; used to reduce the micropropagation cost and also the time of establishment from lab to field. It involves simultaneous rooting and hardening. The main advantage of *ex vitro* rooting is that the chance of root damage is less, rooting rates are often higher and root quality is better (Bellamine *et al*., 1998). Plants rooted under *ex vitro* environment are better suited/adapted to natural climate and easy to harden. These have more vigour to tolerate stresses experienced during hardening. It has been reported that *ex vitro*-rooted plants are better suited to tolerate environmental stresses (Phulwaria *et al*., 2011). Rooting under *ex vitro* conditions has been successfully utilized for the establishment of several woody plants including *Tectona grandis* (Tiwari *et al*., 2002), *Vitex nigundo* (Ahmad and Anis, 2007), *Melia azedarach* (Husain and Anis, 2009) and *Tecomella undulata* (Varshney and Anis, 2012). Therefore, auxin dipped *ex-vitro* rooting is the most effective method for root induction in the present investigation. The stimulatory effects of IBA on the root development may be due to several factors such as its preferential uptake, transport, and stability over other auxins and sub-sequent gene activation (Ludwig-Muller, 2000). Therefore, IBA has been observed to induce strong rooting response and has been extensively used to promote rooting in a wide range of plant and specifically for woody tree species (Husain *et al*., 2008, Perveen *et al*., 2011).

The survival percentage of plants was affected by the planting substrates used. Among the various hardening media used for the acclimatization of plantlets, 76.6 % of the plantlets survived in soilrite (Fig. 1e),

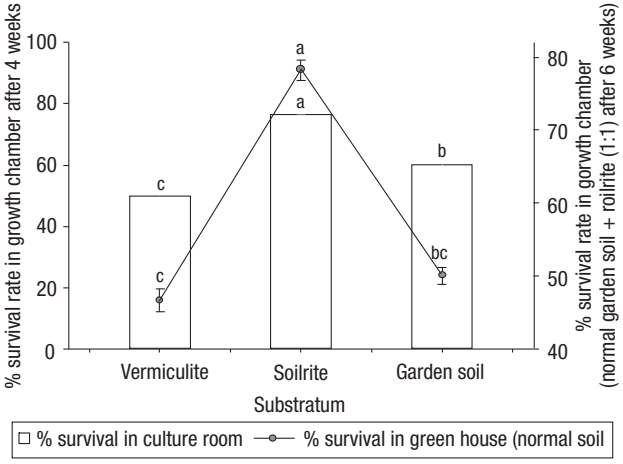

Figure 6. Influence of different planting substrates for hardening of *in vitro* raised plantlets of *A. lebbeck.* Values represent means ± SE. Means sharing the same letter within columns are not significantly different ($p = 0.05$) using Duncan's multiple range test.

whereas 60 % and 50 % plantlets survived in garden soil and vermiculite respectively after 4 weeks of transplantation (Fig. 6). Similarly, in *Tecomella undulata* (Varshney and Anis, 2012) plantlets acclimatized in soilrite pots showed 80 % survival rate. Soilrite being more porous substrate holds more water than vermiculite and garden soil, and thus promoted better growth of tender roots of tissue culture raised plants during hardening. Moreover, roots were easily penetrated in soilrite than in other planting substrates. The primary hardened plants on soilrite when transferred to normal garden soil + soilrite (1:1) in the growth chamber showed 78% survival rate after 6 weeks, whereas those on garden soil and vermiculite exhibited 50% and 46% survival respectively (Fig. 6). The micropropagated plants grew well without any detectable phenotypic variation.

In the present investigation, multiple shoot induction took place in a short period of time (ranging from 25 to 90 days) by using aseptic seeds as explants on TDZ containing medium. Hence, intact seedling explants produced significant shoot multiplication rate and shoot length per seedling stump. Apart from this, intact seedling method is superior to the other plant propagation methods in terms of reduced cost due to reduced steps in *in vitro* propagation without any adverse effect on plant regeneration efficiency. For the root induction in *in vitro* raised shootlets, *ex vitro* rooting method proved more suitable. Soilrite was the best growth substrate for *ex vitro* rooting and acclimatization of plantlets.

The results of the present investigation indicate that the protocol could be adopted for successful large scale plant production in short period of time especially in the fast growing tree species and can contribute to arrest deforestation, soil deterioration and supports application of biotechnology in agriculture and specifically in forestry sector.

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