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(III)

EFFECT OF SUBSTRATES ON THE GROWTH OF *OROXYLUM INDICUM* (V.)

Oroxylum indicum Vent. is a medicinally important deciduous forest tree belonging to family Bignoniaceae (Anonymous, 2001) and most of the plant parts are used in preparation of various herbal medicines. A well known ayurvedic formulation 'Dasamoola', is used as anti-inflammatory, digestive, carminative etc. which contains root extracts of this plant.

O. indicum is now under the threatened category (Tiwari *et al*., 2007) as it faces difficulty in its natural propagation due to poor seed set with low seed viability. Another reason is the indiscriminate collection of tree parts for medicinal purposes. To overcome this problem the present study was conducted to optimize a suitable substrate for germination and development of healthy seedlings using the current year seed stock. The seedlings were grown in root trainers as these are now commonly used for planting stocks of forest tree species (Mohanan and Sharma, 2005).

Seeds of *O.indicum* (Vent.) of plus trees of the current year were collected from Forest Division, Rajpipla.

The seeds were soaked overnight in distil water and then inoculated in three different substrates like cocopeat, cocopeat:soil (1:1) and cocopeat: sand (1:1). Cocopeat which was a common substrate had to be soaked overnight in distil water prior to autoclaving. All the substrates were autoclaved with 15 psi at 121^{0}C temperature for 40 minutes and then filled into root trainer (with a capacity of 98 wells) under sterile conditions. Seeds were surface sterilized with $HgCl_2$ (0.1%) and then inoculated individually in each well of the root trainers containing the respective substrate. Approximately 5 ml of bavistin solution (200 mg/l) was also added to the wells to reduce contamination. The root trainer's were kept under a glass case to maintain humidity. Five seedlings were randomly removed at weekly interval and observations were recorded for per cent germination and different growth parameters like collar diameter, biomass (fresh weight and dry weight) and length.

There are several reports stating about the influence of growing media on seedling growth of forest tree species (Gharib, 1976; Lackey and Alm ,1982) . As cocopeat is one of the popular substrate; commonly used in horticulture practices for growing seedlings (Yau and Murphy, 2000) the same was utilized individually and in combination with soil or sand for growing *O. indicum* seeds.

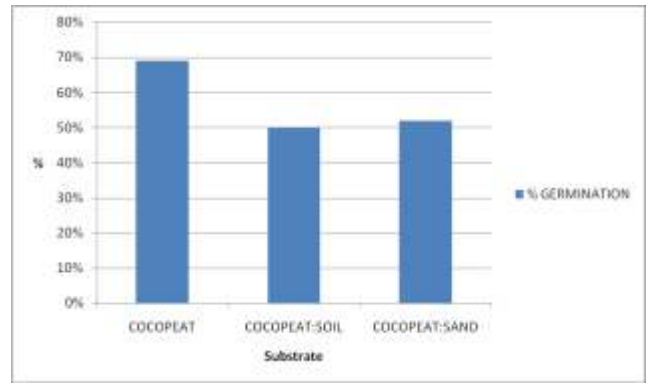

Fig. 1: Effect of different substrates on % Germination

The observations of the present study revealed that per cent germination varied for different substrates (Fig.1), maximum being in individual cocopeat (69 %) followed by cocopeat:sand (52%) and the least was recorded in cocopeat:soil (50%).The other parameter for assessment of growth was collar diameter which did not show much variation in all the three substrates (Fig.2). It remained more or less same in cocopeat (1.22 ± 0.052 cms) and cocopeat:sand (1.2 ± 0.048 cms) followed by

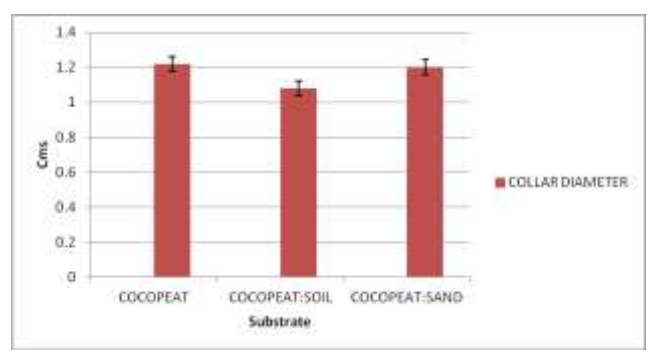

Fig. 2: Effect of different substrates on collar diameter after 4 weeks

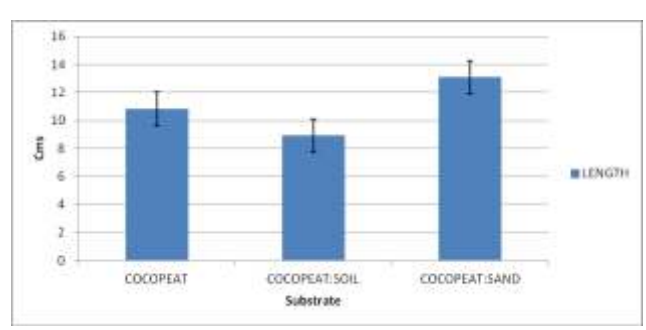

Fig. 3: Effect of different substrates on seedling length of *O.indicum* after 4 week.

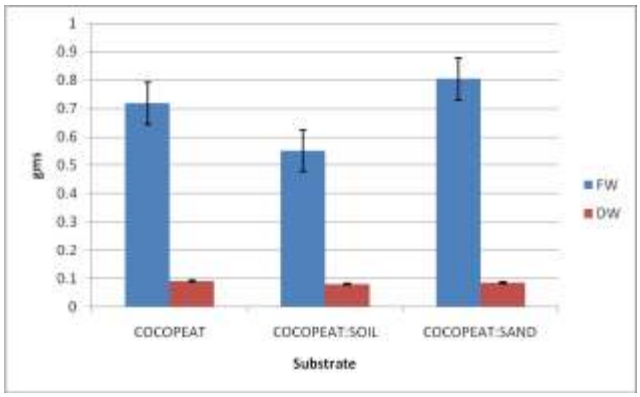

Fig. 4: Effect of different substrates on biomass of *O. indicum* seedling after 4 weeks

cocopeat:soil (1.08 ± 0.095 cms) by the end of four weeks. The growth of the seedlings when recorded in terms of the length was found to be optimum in cocopeat: sand (Fig.3) in comparison to cocopeat and cocopeat: soil. Similarly the biomass values were also found to be optimum in cocopeat:sand mixture as fresh weight reached to 0.8043 ± 0.08gms and dry weight to 0.0869 ± 0.011 gms by the end of four weeks. In cocopeat the values of fresh weight (0.7186 ± 0.052 gms) and dry weight (0.0918 ± 0.009 gms) decreased and it became least in cocopeat:soil (Fig. 4).

The present study revealed that cocopeat: sand mixture (1:1) proved beneficial for the healthy growth of *O. indicum* seedlings. It played a synergistic role in the overall growth of seedlings as cocopeat helped in retaining moisture, providing organic nutrients while sand improved the texture in terms of porosity and aeration. In sandalwood also the presence of sand along with other substrates in a potting medium helped to improve seedling growth (Fox *et al*., 1990). Similarly in tree species like *Pterocarpus macrocarpus* (Kijkar, 1991), *Eucalyptus terticornis* (Kumar and Marimuthu, 1997) and *Swietenia macrophylla* (Woods *et al*., 1998) individual cocopeat was found to improve the growth of seedlings. For *O.indicum* a combination of cocopeat and sand in equal proportions can be considered as an ideal substrate for producing good quality seedlings. It can be one of the methods for conservation of this medicinally important tree species which has now been placed in the vulnerable category of threatened plants.

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Darshini Trivedi and Aruna G. Joshi

Department of Botany
Faculty of Science
The Maharaja Sayajirao University of Baroda
Vadodara -390002 (Gujarat)

Studies on seed germination of *Stereospermum suaveolens* with respect to different parameters

Darshini R. Trivedi, Aruna G. Joshi*

The Maharaja Sayajirao University of Baroda, Faculty of Science, Department of Botany, Vadodara 390002, India

*Corresponding author, E-mail: arunajoshimsu@gmail.com

Abstract

Stereospermum suaveolens DC. is a valuable medicinal tree species, now placed in the threatened category due to its overexploitation. This species is usually propagated through seeds but has a very poor rate of germination. The right choice of planting substrates and pretreatments given to seeds are important factors influencing germination and growth of the seedlings. The present study was conducted to determine suitable planting substrate(s) and an effective pretreatment for germinating the seeds. The moisture content of one year old seeds was high (15 ± 1.3) and that of the current year (fresh) seeds were low (8.1 ± 0.3). Fresh seeds were pre-soaked overnight in water and then grown in various substrates like coco peat, soil, sand, coco peat / sand (1:1) and coco peat / soil (1:1), filter paper, Murashige and Skoog's medium (MS) and Woody Plant Medium. Results revealed that the optimum seed germination (68%) was recorded in coco peat substrate followed by MS medium (66 %) and lowest rate was in soil (6%). The other germination parameters studied were mean daily germination, which was same in coco peat as in MS medium (2.2), followed by filter paper (2.0). The germination rate was faster in coco peat (5.4) when compared to that on other substrates, while germination index was highest in MS medium (9.9). Coco peat was suitable in terms of germination. The effect of different pretreatments on germination were therefore studied for this substrate. Seeds soaked in distilled water showed maximum percent of germination compared to other pretreatments.

Key words: coco peat; germination; *Stereospermum suaveolens*; substrate.
Abbreviations: IAA, indole-3-acetic acid; MDG, mean daily germination; MS, Murashige and Skoog's medium.

Introduction

Many tree species are propagated by seeds, but reports on seed germination are limited due to insufficient research (Mng'omba et al. 2007). AOSA (1970) defines germination as the emergence and development from the seed of those essential structures which are indicative of the ability to produce a normal plant under favourable conditions. In the germination test rules, two germination counts are mentioned: the 'first day count' which is the day when approximately two thirds of the germinable seeds are expected to germinate and the 'final count' is the end of the test period (Bedell 1998). In tree species seed germination is difficult due to hard seed coats and dormant seed embryos (Jaiswal, Chaudhary 2005) and they often fail to germinate even under favourable moisture, oxygen and soil conditions (Urgenc, Cepel 2001). Moisture content of seeds is one of the factors influencing germination, as it is a test in retention of viability of seeds (McDonald, Copeland 1999) and it also depicts the seed quality and storagibilty of seeds. Pretreatments like mechanical scarification ie. soaking in cold or hot water, acid scarification with sulphuric or hydrochloric acid are known to overcome dormancy of seeds (Bedell 1998). Pre-soaking of seeds in growth regulator like gibberellic acid and indoleacetic acid (IAA) can also enhance seed germination percentage (Chauhan et al 2009). The type of planting substrate is another factor that influences germination and can be species-specific; therefore in nurseries importance is given to substrate for raising seedlings (Jaiswal, Chaudhary 2005). A suitable substrate is the one which contain numerous air spaces, provides adequate aeration (Thomson 1992), non toxic, free from moulds and microorganisms, cost effective and is easy to handle (Agrawal 1997).

Stereospermum suaveolens DC. is an important medicinal tree belonging to the family Bignoniaceae (Anonymous 1998). It contains lapachol and lapachonone, which act against dermatitis (Anonymous 1998). Its roots are used in preparation of the ayurvedic formulations Dasmoolarisht and Chywanprash (Yashoda et al. 2004). Destructive harvesting practices have seriously reduced seed production and caused gradual erosion of its natural populations. Now this species is placed under the threatened category. The species is mainly propagated through seeds and collecting them becomes a laborious process as their pericarps are winged. Another difficulty it faces is poor germination rate and thus propagation through seeds in the wild is limited (Baul 2006). Hence, steps have to be taken to conserve this tree of great economic value by finding suitable methods for its large scale propagation.

The present study was planned with an objective to find a suitable planting substrate and an effective pretreatment for germination of *S. suaveolens* seeds.

Materials and methods

Seed source and substrates for germination

The seeds of *Stereospermum suaveolens* DC. were collected from the Rajpipla Forest Division.

Different substrates used for seed germination were pure coco peat, soil, sand, a mixture of coco peat / sand (1:1) and coco peat / soil (1:1), a Petri dish containing filter paper (single layer), Murashige and Skoog's (MS) and Woody Plant Medium (WPM). Coco peat and its mixtures were filled in plastic root trainers (98 wells) while soil was filled in pots and flasks were used for the medium.

To prepare coco peat substrate, 150 g of cocopeat (dry mass) was soaked in distilled water (four times dry mass) overnight. Coco peat and a mixture of coco peat with soil / sand (1:1) were prepared and autoclaved at 121 °C and 103.4 kPa for 45 min.

MS and WPM (one litre) basal medium was prepared with all macronutrients, micronutrients and vitamins. The media and petridishes containing filter paper were sterilized by autoclaving at 121 °C and 103.4 kPa for 25 min.

Seed germination

The winged pericarp was removed from the seeds and then they were soaked for 24 h in distilled water before placing in the different substrates.

For coco peat-containing substrates, seeds were treated with 0.1% $HgCl_2$ for 2 min and inoculated singly in wells of the root trainer containing a specific type of the substrate. Bavistin (200 mg L^{-1}) was added to the substrate to avoid contamination and then the root trainers were housed in a culture room at 25 ± 2 °C. All the manipulations were carried out in a Laminar Air Flow Cabinet.

Garden soil was filled in pots and the seeds (five per pot) were placed in it. All the pots were housed in the M.S.University Botanical Garden.

Sand was filled in polyethylene bags and one seed per bag was placed in them. All the polybags were maintained in the Botanical Garden.

For germination of filter paper, seeds were treated with 0.1% $HgCl_2$ for 2 min, washed three times with sterile water, and then five seeds were placed in each Petri dish containing 5 to 10 mL of sterile water.

For germination in MS and WPM seeds were treated with 0.1% $HgCl_2$ for 2 min, washed three times with sterile water and five seeds were inoculated in each flask.

There were two replicates with 50 seeds in each. The substrates coco peat and its combination with sand and soil in root trainers were irrigated every alternate day and sprayed with Bavistin fungicide with five days interval to avoid fungal contamination. Sand and soil substrate kept outside were also watered every day.

Observations were made every day for development of seedlings for a period up to 30 days.

Germination parameters

The first count, as well as final count of germinating seedlings, was done according to germination test rules (AOSA 1981). The first count was made after one week (10 days) and final count by the end of the 4th week (30 days).

Moisture content (%) of seeds was determined on fresh mass basis after drying samples at 103 ± 2 °C for 17 h (ISTA 1993) by using the following formula:

$$\text{Moisture content} = FM - DM / FM \times 100,$$

where *FM* is fresh mass of seeds and *DM* is dry mass.

Final germination percentage (FG) and mean daily germination (MDG) were calculated as follows (Panwar, Bhardwaj 2005):

$$\text{FG} = n / N \times 100,$$

$$\text{MDG} = FG / D,$$

where *n* is the number of germinated seeds, *N* is the total number of seeds and *D* is the number of days to final germination.

The germination rate (GR) as well as germination index (GI) were calculated as follows (Panwar, Bhardwaj 2005):

$$GR = \sum_{i=1}^{n} n_i / t_i,$$

$$GI = \sum_{i=1}^{n} n_i t_i / N,$$

where n_i is the number of germinated seeds in day t_i.

Pretreatments before germinating in coco peat

For treatment with plant hormones, seeds were soaked for 24 h in gibberellic acid (GA_3; 15 μM) or indole-3-acetic acid (IAA; 5 μM) before germinating in coco peat substrate. Seeds were scarified with concentrated and diluted sulphuric acid (20%) for 10 min and 1 h, respectively, later washed thoroughly with water and then placed in the substrate. For hot water treatment, seeds were soaked in hot water (100 °C) for 15 min, then transferred to distilled water at room temperature and thereafter placed in substrate for germination.

Observations were recorded only for final germination in different treatments and data were statistically analyzed using ANOVA.

Results

Before seed germinaton experiments were carried out, moisture content of old and fresh seeds of *S. suaveolens* were determined. It was observed that old seeds retained high percent moisture (15 ± 1.3) whereas fresh seeds had low moisture content (8.1 ± 0.3).

Observations on seed germination experiments

revealed that of the different planting substrates that were used, coco peat gave higher percent germination (68%) of *S. suaveolens* seeds, followed by MS medium (66%), filter paper (60 %), least in soil (6%), and failure to germinate in WPM medium (Table 1). The maximum first count percent was recorded in coco peat, which indicates this substrate is quick and effective for seed germination, as compared to other substrates (Table 1).

Germination rate of seeds was highest in coco peat (5.4) as seed emergence occurred within 10 days and all seedlings developed by the end of third week. In other substrates the germination rate was poor and the growth of seedling was also slow, as it took more than 15 days for seed emergence and seedlings developed after a month (Fig. 1).

The mean daily germination (MDG) had the same value (2.2) for both coco peat and MS medium, followed by filter paper and sand. The other substrates, like soil, mixture of coco peat with sand and soil had lower MDG (Fig. 2).

The germination index was greater in MS medium (9.9) compared to coco peat (8.9), and lowest in soil (Fig. 3).

The results show that coco peat was the most suitable substrate for germination of *S.suaveolens* seeds; therefore, further study on effect of different pretreatments on seed germination were carried out only in this substrate. The test showed that pretreatments of seeds, excepting distilled water, were not effective and that they did not have any additional effect on germination. Soaking seeds in distilled water induced the highest germination when compared to other pretreatments, like dilute sulphuric acid, gibberellic acid, IAA and hot water, where percent germination was poor, and no germination was observed in concentrated sulphuric acid (Fig.4). ANOVA showed that the seed germination significantly differed among the six different pretreatments ($p < 0.05$).

Discussion

Moisture content of seeds was calculated before carrying out seed germination. In our studies on *S. suaveolens* the fresh seeds had a low moisture content, indicating high permeability of the seed coat which was lower in old seeds. An ideal planting substrate should be sufficiently porous, and be able to retain moisture, which plays an important role in seed germination (Richard et al. 1964) and growth of seedlings (Jaiswal, Chaudhary 2005). There are several reports on effect of substrates on germination on plants like *Jatropha curcas* (Gairola et al. 2011), *Gonystylus bancanus*

Table 1. Percentage of germination at first count and final count of seeds of *Stereospermum suaveolens* soaked in distilled water for 24 h and germinated in different substrates

Substrate	Temperature (°C)	First count (%)	Final count (%)
Sand	38	20	35
Soil	40	0	6
Coco peat	25	56	68
Coco peat / sand	25	8	11
Coco peat / soil	25	6	16
Filter paper	25	44	60
MS medium	25	23	66
WPM	25	0	0

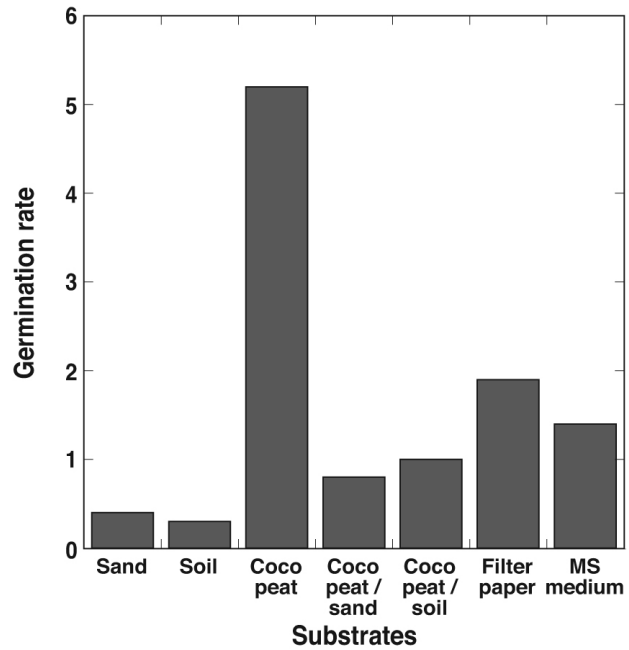

Fig. 1. Germination rate of *Stereospermum suaveolens* seeds in different planting substrates.

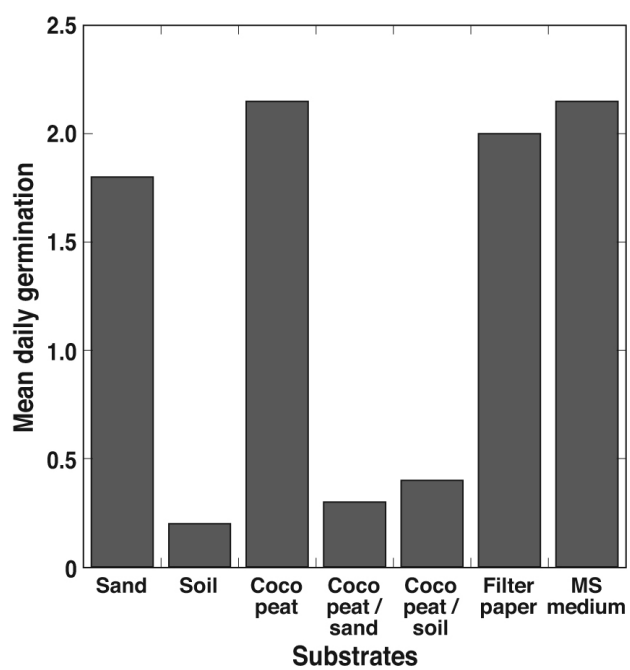

Fig. 2. Mean daily germination of *Stereospermum suaveolens* seeds in different planting substrates.

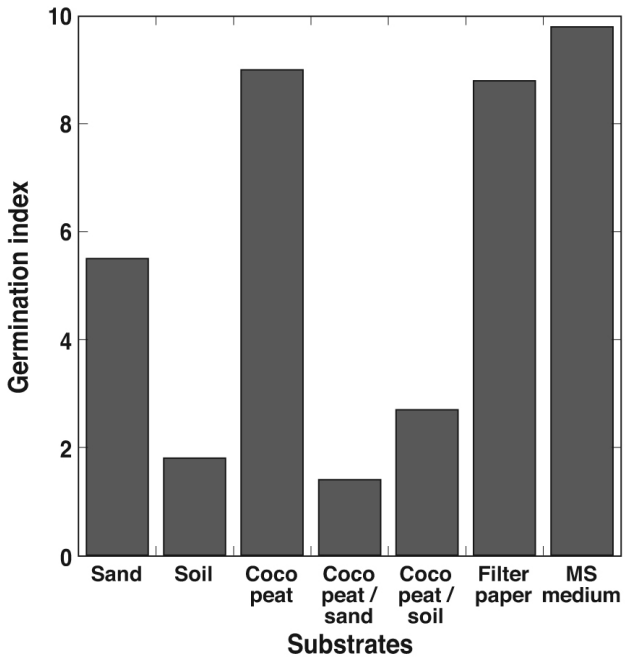

Fig. 3. Germination index of *Stereospermum suaveolens* seeds in different planting substrates.

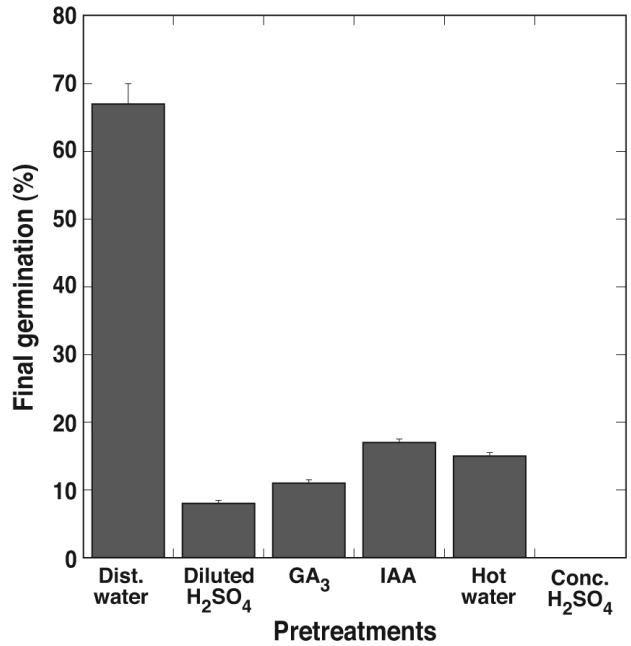

Fig. 4. Final germination of *Stereospermum suaveolens* seeds in coco peat with different pretreatments.

(Utami et al. 2006) and *Calendula officinalis* (Ming et al. 1999).

Coco peat is a commonly used substrate in horticultural practices for growing seedlings (Yau, Murphy 2000). This substrate is beneficial for germinating seeds, as it is 100% organic with high water holding capacity, good aeration, has nutrient absorption qualities and is non toxic. Improved growth of seedlings using coco peat has been reported in a number of species like *Pterocarpus macrocarpus* (Kijkar 1991), *Eucalyptus tereticornis* (Kumar, Marimuthu 1997) and Swietenia macrophylla (Woods et al. 1998). The present work also showed coco peat as a suitable substrate for germination, as a maximum number of seedlings can be generated within three weeks. Similar observations were recorded for *Gonystylus bancanus* (Utami et al 2006), and *Oroxylum indicum* (Trivedi, Joshi 2012) which had the highest percent of seed germination in coco peat and least in soil.

Filter paper can hold sufficient amount of water and dissipate water evenly all over the surface quickly and thus has effect on germination of seeds (Jaiswal, Chaudhary 2005). In our experiments a number of seeds germinated in petridish containing filter paper but the rate of germination was less and growth of seedlings was also uneven. Other substrates like sand and soil did not favour germination of seeds, as sand has high permeability and soil has a very high tendency of compression, resulting in poor rate of germination (Ming et al. 1999).

MS and WPM medium have different mineral salt concentrations which greatly affects *in vitro* germination. It has been successful for seeds of *Senna macranthera* (Faria et al. 2012), while MS medium in *Pterocarpus marsupium* (Mishra et al. 2013) and *Salvia sclarea* (Ghanbari et al. 2012). Different substrates are species specific. For *S. suaveolens* MS medium was equally effective but the rate of germination was slow, while WPM medium was ineffective.

Different pretreatments are also essential for effective germination and are species specific. Acid pretreatment is advantageous for seeds with a very hard seed coat (Hosseini et al. 2013). Soaking seeds in hot water significantly improved seed germination percentage in *Bobgannia malagascarionsis* (Thokozani et al. 2011). In contrast to this, acid and hot water treatment proved to be least effective in terms of germination in our experiments. Similar observations were recorded for *Flacourtia indica* and *Parinari curatellifolia* seeds, where different pretreatments did not enhance the percent germination (Prins, Maghembe 1994). Acid and mechanical treatments have failed in germinating seeds of *Arceuthobium abietinum* (Scharpf 1970). Treatment of *Prunus mahaleb* seeds with gibberellic acid has been reported to overcome dormancy and ensure uniform germination (Cetinbas, Koyunko 2006; Al-Absi 2010). Seeds of horse gram and black gram when treated with IAA and gibberellic acid showed enhanced percent germination (Chauhan et al. 2009), whereas *S. suaveolens* seeds did not respond to these hormones.

The studied species had maximum seed germination when soaked in distilled water, as similar for seeds of Strychnous spinosa (Prins, Maghembe 1994). Soaking in water prior to sowing is also known to enhance germination percent and rate in species like *Cedrus deodara, Hardwickia binata, Pongamia pinnata, Cinnamomum camphora, Melia*

azadarach, Terminalia chebula and *Terminalia tomentosa* (Bedell 1998).

Conclusions

In order to generate a large number of seedlings of *S. suaveolens* in a short period, studies on seed germination in different substrates along with pre-treatment's were carried out. From our studies it can be concluded that coco peat was effective for germination as the rate of germination was faster in comparison to filter paper and MS medium. The seeds were simply soaked in distilled water without any special pretreatments before placing in the substrate. A large number of seeds germinated into healthy seedlings in this substrate, more than in the other substrates tested and hence coco peat was suitable for germinating seeds of this species.

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In vitro Shoot Regeneration of *Stereospermum suaveolens* DC. using Cotyledonary Node and Nodal Explants

Darshini R. Trivedi and Aruna G. Joshi*

Department of Botany, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara-390002, India

Key words: Nodal explants, Woody plant medium, *Stereospermum suaveolens*

Abstract

The cotyledonary node and nodal explants *Stereospermum suaveolens* DC. were cultured on Woody Plant medium (WPM) supplemented with individual BAP 2 - 25 μM), Kn (2 - 25 μM) and TDZ (0.1 - 2 μM. Observations revealed that in both cotyledonary node and nodal explants**,** lower concentrations of cytokinins (BAP/Kn/TDZ) were effective in inducing proliferation response. The axillary bud of both the explants proliferated into single shoots which were further subcultured on fresh medium with respective concentrations for shoot multiplication. It was observed that multiple shoots developed only in presence of Kn (8 μM) in both the explants but the shoots developed from nodal explants were strong and healthy with average shoot number reaching to 3.8 ± 0.6 and length 5.4 ± 0.4 cm by the end of third passage. Rooting (100%) was achieved in microshoots when transferred to half strength WPM liquid medium supplemented with IBA (2 μM). The plantlets were transferred to different substrates for acclimatization.

Introduction

Forest trees have been exploited over the years as they are the renewable sources of food, fodder, fuel wood, timber and medicinal properties. Due to rapid growth of population there has been a tremendous reduction in forest cover from the earth (Giri et al. 2004) and number of plants in the wild has progressively declined pushing them into endangered category. Hence, steps have to be taken towards conservation and sustainable utilization of important forest trees. To propagate trees on large scale, vegetative methods become difficult and time consuming, an alternative method like *in vitro* propagation could be the solution

*Author for correspondence: <arunajoshimsu@gmail.com>.

for this and several other woody species (Thorpe et al. 1991, Ahuja 1993, Vinocur et al. 2000). *In vitro* techniques can produce clonal planting stock for afforestation, woody biomass production and conservation of elite and rare germplasm (Rout et al. 2008).

Stereospermum suaveolens DC. is a valuable tree species belonging to family Bignoniaceae (Anon. 1998). Roots of the plant are the source of active components, used in the preparation of ayurvedic formulations like Dashmoolarisht and Chywanprash where the whole plant is uprooted resulting in total destruction of the species (Yashoda et al. 2004). Conventionally the plant is propagated through seeds, however it is a tedious process as the seeds are winged creating difficulty in its collection and they also have a poor germination rate (Baul et al. 2009). Hence there is a need to conserve this important tree species which can be achieved through *in vitro* techniques. There are many reports documenting successful establishment of cultures utilizing cotyledonary nodes in tree species like *Sterculia urens* (Purohit and Dave 1996), *Murraya koenigii* (Bhuyan et al. 1997), *Dalbergia sissoo* (Pradhan et al. 1998), *Quercus floribunda* (Purohit et al. 2002), *Cassia sophera* (Parveen and Shahzad 2010). Whereas plants like *Psidium gujava* L. (Amin and Jaiswal 1987), *Sterculia urens* Roxb. (Devi et al. 2011), *Balanites aegyptiaca* L. (Siddique and Anis 2009), *Vitex negundo* L. (Ahmad and Anis 2007) have been regenerated utilizing nodal explants. Therefore, the present work was aimed to study the *in vitro* regeneration potential of *S. suaveolens* from cotyledonary node and nodal explants in WPM medium fortified with different individual cytokinins.

Materials and Methods

The present year seeds of *S. suaveolens* DC. were collected from Rajpila Forest Division and germinated in cocopeat for obtaining a large number of seedling explants (Trivedi and Joshi 2014). Healthy seeds were presoaked overnight, next day kept under running water and then washed with Teepol for 5-10 min. A treatment of bavistin (200 mg/l) was given for 2 min and thereafter the seeds were surface sterilized with $HgCl_2$ (0.1%) for 2 min. They were thoroughly washed three times with sterile distiled water and germinated singly in each well of root trainer which was filled with sterile cocopeat. The root trainers were kept in culture room at 25 ± 2°C.

Twenty day old seedlings were selected and kept under running water for 2 hrs, washed with Teepol for 5 - 10 min. They were treated with PVP (100 mg/l) for 3 min, followed by bavistin (200 mg/l) treatment for 3 min. and then surface sterilized with $HgCl_2$ (0.1%) for 3 min, finally rinsed 3 - 4 times with sterile distil water. Cotyledonary node (1 - 1.5 cm) was excised aseptically from these

seedlings. Nodal segments (1 - 2 cm) collected from 1 - 2 year old plants were treated similarly with PVP (100 mg/l) for 3 min, followed by bavistin (200 mg/l) treated for 5 min, then by an additional treatment of streptomycin (100 mg/l) for 5 min, lastly surface sterilization with $HgCl_2$ (0.1%) for 5 min was given and finally rinsed (3 - 4 times) with sterile distiled water.

Cotyledonary nodes and nodal explants were placed on WPM medium supplemented individually with BAP/Kn (2, 4, 8, 16, 20, 25 μM) and TDZ (0.1, 0.2, 0.25, 0.5, 1, 2 μM). One set of both the explants were cultured on basal medium which served as a control. The pH of all the media was adjusted to 5.8 and solidified with 0.8% agar before autoclaving and sterilization was done at 121°C with 15 psi for 25 min. All the cultures were incubated at 25 ± 2°C and the data for shoot proliferation were recorded after four weeks in all the explants.

The *in vitro* shoots were cut into single nodal segments and transferred to fresh medium of the same concentration for multiplication after four weeks interval. The data for per cent response, number of shoots, shoot length were recorded after eight weeks.

The elongated *in vitro* shoots (4 - 5 cms) were transferred to induce roots in basal (liquid and static) full and half strength WPM medium and fortified with different concentrations of IBA (1, 2, 2.5, 5μM). The shoots were washed with water to remove agar attached to it, then dipped in bavistin for one min to avoid fungal contamination and thereafter transferred to rooting media**.** In case of liquid medium the microshoot was inserted in filter paper bridge which served as a support. The per cent response, number of roots and root length were recorded after four weeks.

All the experiments were repeated twice with five replicates. The data were analysed using ANOVA and mean separation were carried out using DMRT at 5% level of significance ($p = 0.05$).

Results and Discussion

In *Stereospermum suaveolens* studies were carried out for regeneration of shoots from nodal and cotyledonary node explants in WPM medium fortified with different individual cytokinins with various concentrations. Shoot cultures were successfully established from both the explants but the optimal media requirements varied.

WPM basal medium evoked a proliferation response in cotyledonary node in the form of single shoot but it failed to survive after third week. Thus the medium was fortified with individual cytokinin to overcome this problem. The presence of BAP/Kn/TDZ in the medium allowed the axillary bud to proliferate

and develop into shoot where morphogenic response was high in lower concentration as compared to high concentrations. It has been reported that percentage of shoot emergence decreases with increasing concentration of cytokinins (Reddy and Saritha 2013). In medium supplemented with BAP the concentrations (2 - 20 μM) stimulated axillary bud to proliferate and at 25 μM it failed to respond. The lower concentrations (4, 8 μM) evoked 100% response with only 1.2 ± 0.2 shoots per node which decreased to single shoot in 2, 16 and 20 μM (Table 1). Similar findings were observed in *Salix tetrasperma* (Khan et al. 2011) where BAP at lower concentration was effective in shoot proliferation.

When Kn was added in the medium, all the concentrations evoked a proliferation response but lower concentrations (2, 4 μM) induced an optimum percent response (100), In comparison to 4 μM, shoot emergence and its development was fast at 2 μM resulting in highest number of nodes 3.2 ± 0.3 (Table 1). At concentrations 8, 16 and 20 μM also single shoot formation was observed. Both BAP and Kn resulted into similar morphogenic response but shoots were longer in Kn as compared to BAP. Similar findings are reported in *Salvadora persica* (Kumari and Singh 2012) where Kn had a profound effect on length of shoots as compared to BAP.

TDZ was also tried, as it is known to be potent in inducing the axillary bud to proliferate and form multiple shoots in woody species (Huetteman and Preece 1993, Murthy et al. 1998). TDZ at all concentrations was able to stimulate the bud to proliferate and develop into a shoot, but lower concentration (0.1 μM) evoked an optimum response (83%) in terms of shoot formation. Observations revealed that in the presence of this TDZ (0.1- 2 μM) there was formation of profuse callus at the base of explant which hindered the growth of shoot. Similar results are reported in *Murraya koinegii* (Bhuyan et al. 1997) and *Oroxylum indicum* (Dalal and Rai 2004) where addition of TDZ resulted in heavy callus formation.

In cotyledonary node explant among all the cytokinins BAP and Kn were effective in terms of shoot induction as compared to TDZ. Cytokinins are known to play a key role in cell division and its presence in the medium is required for multiple shoot induction, but the type and its optimal concentration varies with the system (Park et al. 2008).

Nodal explants are, with vegetative buds, have a potential for rapid shoot regeneration and therefore in this species it was utilized to establish cultures. These explants were cultured in basal medium but resulted in poor proliferation response which improved when cytokinins were added to the medium. In BAP fortified medium (2 - 25 μM) an optimum per cent response (75) was observed at 16 μM (Table 2).

In Kn supplemented medium, all the concentrations were equally effective in shoot formation in which 2 μM proved to be optimum per cent response (80) and long healthy shoot with 2.8 ± 0.8 nodes were formed with well developed leaves. Observations revealed that presence of Kn in the medium induced an early response in terms of shoot formation when compared with other cytokinin. There are reports which state that Kn promotes elongation of buds in *Vigna radiata* (Chandra and Pal 1995).

Table 1. Effect of individual cytokinin on shoot bud initiation from cotyledonary node and nodal explants after four weeks in WPM Medium.

Cytokinins (μM)	Cotyledonary node explant			Nodal explant		
	% response	No. of shoots	No. of *in vitro* nodes	% response	No. of shoots	No. of *in vitro* nodes
0	50	0.5 ± 0.3^{abc}	0.5 ± 0.3^{a}	20	0.2 ± 0.1^{a}	0.2 ± 0.1^{a}
BAP						
2	90	0.8 ± 0.1^{bc}	2.7 ± 0.7^{b}	80	1.2 ± 0.4^{a}	1.7 ± 0.5^{a}
4	100	1.2 ± 0.2^{c}	2.3 ±0.4^{b}	40	0.6 ± 0.4^{a}	1.1 ±0.7^{a}
8	100	1.2 ± 0.2^{c}	2.4 ± 0.4^{b}	80	1.4 ± 0.5^{a}	1.5 ± 0.6^{a}
16	71	0.4 ± 0.2^{ab}	1.3 ± 0.6^{ab}	75	1.0 ± 0.4^{a}	1.8± 0.6^{a}
20	50	0.6 ± 0.4^{abc}	0.5 ± 0.2^{a}	50	0.5 ± 0.3^{a}	1.5 ± 0.9^{a}
25	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
Kn						
2	100	1.0 ± 0.0^{bc}	3.2 ± 0.3^{c}	80	0.8 ± 0.2^{a}	2.8 ± 0.8^{b}
4	100	1.0 ± 0.0^{bc}	2.4 ± 0.4^{bc}	60	0.6 ± 0.2^{a}	1.8 ± 0.7^{b}
8	80	0.8 ± 0.2^{ab}	2.0 ± 0.5^{c}	50	0.5± 0.1^{a}	0.5 ± 0.2^{a}
16	50	0.5 ± 0.1^{a}	1.5 ± 0.5^{ab}	66	0.7 ± 0.3^{a}	1.7± 0.9^{ab}
20	83	0.8 ± 0.1^{ab}	2.7 ± 0.7^{bc}	75	0.8 ± 0.3^{a}	1.8 ± 0.8^{ab}
25	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
TDZ						
0.1	83	0.7 ± 0.2^{a}	2.2 ± 0.7^{b}	80	1.0 ± 0.3^{b}	2.3 ± 0.7^{b}
0.2	50	0.5 ± 0.2^{a}	1.5 ± 0.7^{ab}	100	1.8 ± 0.8^{c}	3.6 ± 0.7^{c}
0.25	66	0.7 ± 0.2^{a}	2.3 ± 0.8^{b}	40	0.4 ± 0.2^{a}	1.8 ± 1.2^{b}
0.5	40	0.4 ± 0.2^{a}	0.6 ± 0.3^{ab}	10	0.1 ± 0.1^{a}	0.1± 0.1^{a}
1	40	0.4 ± 0.2^{a}	0.5 ± 0.2^{a}	10	0.1± 0.1^{a}	0.1± 0.1^{a}
2	50	0.5 ± 0.2^{a}	0.5 ± 0.2^{a}	20	0.2 ± 0.1^{a}	0.3± 0.2^{a}

Values represent mean ± SE of each experiment consist of five replicates conducted twice. Means values followed by different superscript letters within a column are significantly different at p = 0.05 according to DMRT.

The effect of TDZ was also evaluated and observations revealed that at 0.2 μM a 100% response was obtained which decreased with increasing concentration (0.25 - 2.0 μM). The reduction in the regeneration potential may be due to detrimental effect of high concentration on the cells predetermined to form vegetative buds (Khan et al. 2011). Presence of TDZ in the medium resulted in profuse callusing at the base of explants. The shoots which developed in 0.2 μM were with high number of nodes 3.6 ± 0.7 (Table 2) among all the three cytokinins

but remained stunted in growth. One of the reasons for suppressed growth of shoots may be due to high cytokinin activity of TDZ (Huetteman and Preece 1993). In the nodal explants the cytokinins induced similar morphogenic response, but the shoots developed were healthy as compared to cotyledonary node.

Table 2. Effect of IBA on root induction in WPM medium.

WPM		% response		No. of roots		Root length (cm)	
	IBA (μM)	Liquid	Static	Liquid	Static	Liquid	Static
Full strength	0	50	33	0.75 ± 0.5^{a}	1.0 ± 1.0^{a}	1.4 ± 0.8^{ab}	0.5 ± 0.5^{b}
	1	80	83	2.0 ± 0.5^{ab}	1.7 ± 0.3^{ab}	0.7 ± 0.3^{a}	0.3 ± 0.1^{ab}
	2	75	100	1.5 ± 0.9^{a}	3.7 ± 0.9^{b}	1.3 ± 0.4^{ab}	1.1 ± 0.3^{b}
	2.5	40	0	2.2 ± 1.4^{b}	0.0 ± 0.0^{a}	0.5 ± 0.4^{a}	0.0 ± 0.0^{a}
	5	40	40	1.6 ± 1.0^{ab}	3.8 ± 3.1^{b}	1.2 ± 0.8^{b}	0.1 ± 0.1^{a}
Half strength	0	50	66	0.75 ± 0.5^{a}	0.7 ± 0.3^{a}	4.9 ± 3.2^{b}	1.8 ± 1.4^{b}
	1	50	20	1.75 ± 0.5^{a}	0.2 ± 0.2^{a}	0.9 ± 0.8^{ab}	0.02 ± 0.02^{a}
	2	100	66	8.5 ± 3.1^{b}	0.7 ± 0.7^{a}	1.3 ± 0.8^{ab}	0.1 ± 0.1^{a}
	2.5	50	60	1.7 ± 0.8^{a}	1.2 ± 1.0^{a}	1.6 ± 0.8^{ab}	0.5 ± 0.4^{ab}
	5	75	75	2.0 ± 1.4^{a}	1.3 ± 1.3^{a}	0.2 ± 0.1^{a}	0.2 ± 0.2^{a}

Values represent mean ± SE of each experiment consist of five replicates conducted twice. Mean values followed by different superscript letters within a column are significantly different at α=0.05 according to DMRT.

Single *in vitro* nodes of both the explants were utilized as propagules for further multiplication of shoots by transferring them to fresh media with respective concentrations. Observations after eight weeks revealed that in both the explants there was slight increase in number of shoots in the medium fortified with only lower concentrations of BAP/Kn with increased shoot length whereas TDZ induced single shoots only.

The cotyledonary node explants failed to respond in cultures supplemented with high levels of BAP (16 and 20 μM) whereas at lower levels (2, 4 and 8 μM) only one to two shoots developed which considerably varied in their length from 4.7 ± 1.6, 3.5 ± 1.0 and 3.2 ± 1.5 cms, respectively. Presence of BAP in the medium could not enhance the number of shoots in further passages. Replacing BAP with Kn in the medium could induce morphogenic response at lower levels (2, 4 and 8 μM) whereas the axillary bud failed to respond at higher levels (16, 20 μM). At lower levels i.e. 2 μM the number of shoots reached 1.4 ± 0.2 with a length of 2.3 ± 0.3 cm (Fig. 1,a), whereas at 4 μM only single shoot with a length of 3.1 ±1.1 cm was formed and at 8 μM the number reached 1.5 ±0.4 with 2.0 ± 0.5 cm shoot length (Fig.1,b). TDZ at all concentrations failed in terms of shoot multiplication.

Therefore, Kn was effective for shoot multiplication in this explant and similar observations are reported for *Vigna* and *Gerbera jamesonii* (Sen and

Mukherjee 1998, Tyagi and Kothari 2004) but in *S. suaveolens* the shoots obtained were weak.

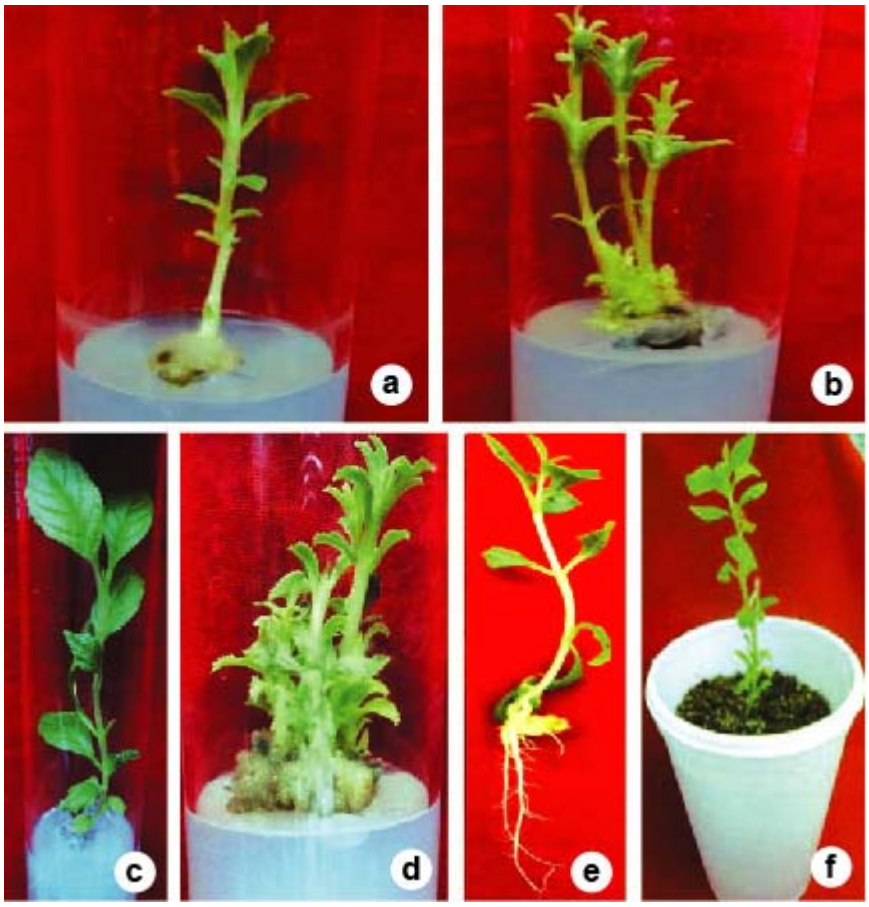

Fig. 1a-f: a. Shoot induction in WPM + Kn (2 μM) through cotyledonary node explant after eight weeks. b. Proliferation of multiple shoots in WPM + Kn (8 μM) through cotyledonary node explant after eight weeks. c. Single healthy shoot in WPM +Kn (2 μM) nodal explants after eight weeks. d. Multiple shoots induced from nodal explant in WPM + Kn (8 μM) after eight weeks. e. Root induction in liquid half WPM + IBA (2 μM). f. Plantlets acclimatized in cocopeat: sand : soil (1 : 1 : 1).

When the morphogenic response of nodal explants was evaluated, observations after eight weeks revealed that BAP and TDZ failed to respond in terms of shoot multiplication as in their presence only single shoot was produced. In Kn supplemented medium higher concentrations (16 and 20 μM) failed to form multiples shoot formation. This cytokinin with lower concentrations (2, 4 μM) developed one or two shoots only but the shoots were long (4.6 ± 0.4 and 4.7 ± 0.6 cms) and healthy with large leaves (Fig. 1c) which on further transferring to new media failed to form multiples. At 8 μM the number

reached 1.7 ± 0.5 and length was 2.0 ± 0.3 cm (Fig. 1,d). TDZ fortified medium developed into single shoots only with slight increase in length of shoots and at 0.1 μM it reached 3.3 ± 1.2 cm which on further transfer failed to respond.

In subsequent passages it was observed that Kn 8 μM was optimum in inducing multiple shoot with average number reaching 3.1± 0.5 and shoot length up to 3.8 ± 0.6 cm by the end of 12 weeks in cotyledonary node explants. But the shoots obtained were long and thin with very minute leaves. Compared to cotyledonary node the average shoot number was higher in nodal explants with 3.8 ± 0.6 shoots and length reaching 5.4 ± 0.4 after 12 weeks (Fig. 2). The shoots obtained from this explants were strong and healthy with well developed leaves as compared to cotyledonary node. Explant type has been known to effect multiple shoot induction in number of tree species like *Dalbergia sisoo* (Pradhan et al. 1998), *Pterocarpus marsupium* (Anis et al. 2005) and *Albizia odoratissima* (Rajeswari and Paliwal 2006).

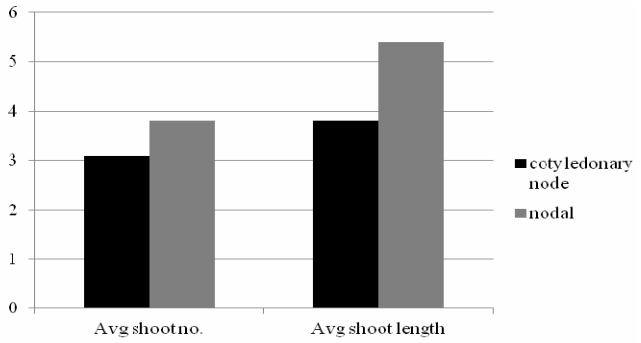

Fig. 2. Average shoot number and shoot length in cotyledonary node and nodal explants after 12 weeks in WPM + Kn (8 μM)

In the present studies the microshoots were transferred to full and half strength WPM (liquid and static) supplemented with IBA (0 - 5 μM). As it is a potent auxin for induction of roots (Mansor et al. 2003, Rajeswari and Paliwal 2008) and in woody trees usually low level of this growth regulator is effective for rooting of shoots (Rai et al. 2010). Root induction was observed in all the concentrations of both liquid and static media after four weeks, but half strength was superior to full strength WPM in terms of number of roots and their length. Similar results are reported in *Salix tetrasperma* also where half strength was superior to full strength WPM medium (Khan et al. 2011). After four weeks the root length was highest (4.9 ± 3.2 cm) in half strength basal liquid media but number of roots were less (Table 3) which were thin whitish in colour. Root induction in basal WPM has been reported earlier in *Salix* by Gebhardt (1992) and Park et al. (2008). The production of adventitious roots in medium without

auxin may be due to endogenous level of salicylic acid playing important role in plant growth development (Raskin 1992) and *in vitro* rooting (Khalafalla and Hattori 2000). Half strength liquid media with 2 μM concentration recorded optimum number of roots (8.5 ± 3.1) after four weeks (Fig. 1e). Same concentration was optimum in full strength static media with 3.7 ± 0.9 roots (Table 3). Zimmerman and Brome (1980) in blueberry noted that rooting can be obtained in solid and for better results in liquid media. In *S. suaveolens* also the liquid media obtained better results in terms of highest number of roots and root length compared to static media. Liquid media are known to be effective for rooting as the nutritive elements are easily available to explants (Hammerschlag 1982) and in static agar can create a critical pressure of turgescence which puts the cells in situation of stress.

The plantlets with well-developed roots were transferred to thermocol cups containing sterilized cocopeat, sand : soil (1 : 1), cocopeat: sand (1 : 1) and cocopeat : sand : soil (1 : 1 : 1) substrates and were covered with transparent perforated polybags. All the cups were kept in glass chamber placed in culture room at 25 ± 2°C. The plantlets were irrigated with MS basal medium and bavistin was sprayed on each substrate every alternate day to avoid fungal contamination. The mixture cocopeat : sand : soil (1 : 1 : 1) proved to be beneficial for the plantlets (Fig. 1f) as they started to grow and could survive even after four weeks while in others substrates the plantlets could not survive after second week. Further studies on hardening the plantlets in a suitable substrate are being carried out.

There are several factors like the type of medium, plant growth regulator and the explant which affects shoot regeneration as all are responsible in inducing an optimum morphogenic response. In our studies on *S. suaveolens* although both the explants were effective in establishing cultures the nodal explant was suitable in comparison to cotyledonary node for *in vitro* regeneration of shoots. Because nodal explants developed healthy plants compared to cotyledonary nodes. Out of the various combinations tried, WPM medium fortified with Kn 8 μM induced an optimum response for formation of multiple shoots and half strength liquid medium with IBA 2 μM was optimal for root induction in terms of number of roots. This protocol of regeneration through nodal explants is highly effective for clonal multiplication.

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