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Efficient Rooting and Biological Hardening of In Vitro Plantlets of Ruta Graveolens L.

KEYWORDS: Rooting, acclimatization, Ruta graveolens

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ABSTRACT *In the present paper, an efficient protocol for rooting and acclimatization of invitro plantlets raised from direct and indirect organonogenesis cultures of Ruta graveolens L.is described.The regenerated shoots from direct and indirect organogenesis plants were excised and transferred to half strength MS medium with different concentrations of IBA and NAA.The better response of rooting was observed in plants cultured with IBA. At the concentration IBA (2.46µM) frequency of root formation was better than other concentration of IBA. 90% shoots were developed on an average 4.5 ± 0.50 roots.The rootedplantlets were grown in pots containing different hardening media.The hardened plants were analyzed in terms of survival rate, length of shoot and morphology The hardening media soil: sand: compost 1: 1: 1 showed 90% survival rate with 12-13cm shoot length having erect stem. The hardened plantlets were successfully acclimatized in the greenhouse and transferred to open field conditions*

Introduction

In vitro production of plantlets with profuse rooting is important for successful establishment in regenerated plants in soil **(Tejawathi *et al.*; 2010).** Micro propagated plants require a fully developed root system prior to transplanting *ex vitro* to endure external environmental stress. The ratio between auxin and cytokinin in the medium may determine the type of morphogenetic response occurring *in vitro.* Usually elevated auxin levels are required to promote rooting

Poor development of adventitious roots is a major obstacle in micro propagation **(De Klerk, 2002).** Therefore micro propagated shoots are often transferred on rooting medium to induce rooting which will be helpful in successful acclimatization while hardening under green house conditions. Now a day's micro propagation has been extensively used for rapid multiplication of plant species however it's wider use is restricted by high percentage of plant loss or damage during hardening. This is because the regenerates have to adjust many abnormalities in *ex vitro* conditions like high level of irradiance, low humidity, temperature, etc. The greenhouse and fields have substantially lower relative humidity, higher light level and septic environment that are stressful to micro propagated plants compared to *in vitro* conditions.The benefit of any micro propagation system can, however, only be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found *ex vitro.* Most species grown *in vitro* require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil.The process of acclimatization of micro propagated plants to the soil environment has to be standardized for particular plant species. Consequently, the transplantation stage continues to be a major bottleneck in the micro propagation of many plants.

Profuse rooting of *in vitro* grown shoots is an essential step for the establishment of these shoots in the field, similarly hardening is vital process prior to transplantation of rooted plants to the soil. Plantlets or shoots that have grown *in vitro* have been continuously exposed to a unique microenvironment that has been selected to provide minimal stress and optimum conditions for plant multiplication. Plantlets developed *in vitro* are under low level of light, aseptic conditions, high level of humidity,on a medium containing ample sugar and nutrients facilitates heterotrophic growth in the *in vitro* plantlets. The plants produced by *in vitro* propagation are very soft to face ambient environmental conditions. **(Bhojwani and Razdan, 1992)**. Therefore hardening of tissue cultured plants are the most crucial step in micro propagation.

After *ex vitro* transfer, the plantlets need some time to correct *in vitro*-induced abnormalities and acclimate to autotrophic conditions, low air humidity, high irradiance, etc. Few weeks of growth under a shade and gradually lowering air humidity are usually prerequisite for successful establishment of vigorous plants. In some plant species, the leaves formed in vitro are unable to develop further under *ex vitro* conditions and they are replaced by newly formed leaves **(Preece and Sutter, 1991; Dietrich *et al.*; 1992).** The main problem during *ex vitro* transfer is the high rate of water loss from shoots of plantlets taken out of the cultivation vessels. Even if the water potential of the substrate (soil or sand with nutrient solution) is higher than the water potential of media with sucrose, the plantlets may quickly wilt **(Pospíšilová *et al.*; 1988).** The cause is unrestricted rate of transpiration due to the retardation in development of cuticle, epicuticular waxes and High concentrations of sucrose in the medium can retard development of photosynthetic apparatus but low concentrations not only stimulated plantlet growth but often also their vigor. Functional stomatal apparatus

Ruta graveolens L., commonly known as Rue is member of Rutaceae has known for its rich aromatic and medicinal properties., widely exploited for its active principles which are of high pharmaceutical value,. The medicinal properties of this plant have been attributed in the presence of rich bioactive principles. More than 120 natural compounds have been identified **(De feo *et al.*; 2002).**

The plant is prescribed in Indian systems of medicine for the treatment of dropsy, neuralgia, rheumatism, menstrual and other bleeding disorders. Unani medicines of India

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recommends *Rue* to treat various physical conditions and to improve mental clarity and as an aphrodisiac. In Latin America, *Rue* is well known as cold, menstrual cramp remedy, Arabs add *Rue* to suspect water to counteract any ill effects in South Africa. Women use to promote menstruation **(Pino *et al.*; 1997)**. *Ruta* was also used as traditional remedy for gynaecological complaints by South African women (Steenkemp, 2003). The plant is also reported to treat internal infections, inflammations, eczema and external ulcers (Wink *et al.*; 1998).

Ruta graveolen L. is propagated by seeds or through vegetative methods, but these methods of propagation cannot meet the requirement,Propagation through seed is hampered by a low germination rate and low viability **(Bohidar et al.; 2008,)** and On the other hand propagation through *in vitro* methods offers a scope to propagate plants with desirable traits in larger quantities. Attempts have been made previously to develop protocols for the micro propagation of *Ruta graveolens* **(Castro and Barros, 1997; Faisal et al., 2005, 2006; Bohidhar et al., 2008).**

Materials and Methods

A. Rooting:

Plantlet with well developed roots *in vitro* is essential for the successful establishment of regenerated plants in field. Well developed shoots having more than 2cm in length obtained from both direct and indirect regeneration were dissected and inoculated to root induction medium for root induction studies.

Media preparation: ½ strength MS basal medium supplemented with IBA (2.46µM, 4.90µM, 7.36µM, 9.80µM) and NAA (2.69µM, 5.37µM, 7.06µM, 10.74µM). The sucrose 3% and bacteriological agar 0.8% were used as carbon source and gelling medium respectively. The pH of the medium was adjusted to 5.7 before gelling with agar.

Culture conditions: the cultures were incubated at 25 ± 2^0C under fluorescent tube light with 16:8 light and dark periods.

Data recording: 20 tubes of each concentration were inoculated and each experiment was repeated thrice. Observations after 30 days for mean number of roots per shoot , length of root, time taken to initiate rooting, and percentage of shoots to which root is initiated has been calculated.

B.Hardening and Acclimatization:

In vitro cultured plantlets with well developed roots and shoot were removed carefully from the solid medium. They were washed with sterilized .distilled water to remove the adherent agar medium. The harvested plantlets were then treated with 1% Bavistin solution to protect them from fungal attack. The treated plants are then transferred to plastic pots containing hardening medium. The various hardening media evaluated were soil: sand: compost 1: 1: 1, soil: sand: compost 1: 2: 1, coco peat: soil: sand 2: 1: 1. The potted plants were then covered with polythin bags to maintain humidity, temperature and light control. The bagged plants were then incubated in hardening room at 28 ± 1^0C with 56% relative humidity for a period of 2 weeks. The perforations were the introduced to plastic bags and plants were then transferred to green house for further maintenance. After 15 days of growth in green house condition, plants were then shifted to shade house for one week. Such acclimatized plants were plotted in earthen pots (30cm dia.). The survival rate was recorded after 6 weeks of transfer to the pots.

Result and Discussion:

The regenerated shoots from direct and indirect organogenesis plants were excised and transferred to half strength MS medium with different concentrations of IBA and NAA(Table 1and 2). The better response of rooting was observed in plants cultured with IBA.At the concentration IBA (2.46µM) frequency of root formation was better than other concentration of IBA. 90% shoots were developed on an average 4.5 ± 0.50 roots (Fig.1A,B) and time taken for root initiation was 12-14 days with an average length 5.7 ± 0.50cm. The roots were healthier and white in color which later turns green in the period of further growth. After 4 weeks of incubation of culture the root length increased rapidly **.** Further it was also noticed that plantlet when incubated for six weeks in same media the plantlets increased in hight and plantlets were healthier (Fig. 1E, F). The results obtained in the present study are in agreement with **Bohidar *et al.*; (2008)** and **Faizal *et al.*; (2005)**. They recorded the optimum concentration for root induction in *Ruta graveloens, was* IBA (4.90µM) with average root length 3.0 ± 0.74cm and number of roots /shoot was 3.2 ± 0.43. Effect of IBA on root formation has been reported in many workers. **Barik *et al.*; (2007)** induced roots from shoots grown on half-strength MS medium with 0.25mg/l (1.47µM) IBA in *Clitoria ternata*. **Sahoo *et al.*; (1997)** successfully induced in vitro rooting in *Ocimum basilicum by using* half-strength MS medium with 1.0mg/l(5.0µM) IBA

The effect of IBA on rooting also recorded by **Saini *et al.*; (2011)** in *Boerhavia diffusa* L, they achieved rooting on half strength MS medium fortified with IBA (2.46µM) with 7.250 ± 0.31cm of length. **Mishra *et al.*; (2010)** demonstrated effect of different concentration of IBA on *in vitro* rooting of *Tinospora cordifolia*. They observed 2.46µM IBA was most effective in terms of percentage of rooting and maximum root length. They found that half strength MS supplemented with 2.46µM IBA, 100% shoots were rooted after 20 days on medium containing 0.5µM IBA with a maximum of 6.75cm root length. **Wang *et al.*; (2007)** demonstrated that, at the conc. of 2.46µM IBA the root induction frequency recorded was 98±0.8 and the highest mean length of root obtained 5.9±0.53 in *Camptotheca acuminate***.** They also demonstrated that half strength MS media was better than full strength MS and WPM medium with 3% sugar. **Tiwari *et al.*; (2000),** observed highest rate of rooting (90%) for *Bacopa monneiri* on full-strength MS medium containing MS +2.46µM IBA.In our studies 70% roots formation was also observed in MS + 4.90µM IBA in same days with 4 ± 0.00 mean number of roots / shoot recorded with an average length 4.4 ± 0.10cm. At highest concentration of IBA i.e. 9.80µM, number of days required to induce rooting was increased up to 16-18 with comparatively same number of roots/ shoot but having reduced length 1.45 ± 0.05cm. In case of NAA, the number of days required to induce root were found to be significantly more than with IBA (Table.2**).** Also though at higher concentration of NAA i.e. 7.06µM and 10.74µM the root number increased the length of roots reduced as compared to IBA. At lower concentration of NAA the root number and root length was found to be significantly lower (Fig 1C).At higher concentration of NAA the plantlets showed formation of callus on roots (Fig.1D).

Acclimatization: The shoots rooted on ½ MS supplemented with IBA 2.46µM IBA were grown in pots containing different hardening media like soil: sand: compost 1:

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1: 1, soil: sand: compost 1: 2: 1, coco peat: soil: sand 2: 1: 1.the hardened plants were analyzed in terms of survival rate, length of shoot and morphology, In first four days of transfer the plantlets were weak (Fig.2A).After week of transfer the plantlet started acclimatized in hardening room and started growing. Then gradually further acclimatization had done by exposing the plantlets to green house, shade house and in open light. The hardened plants then started for increase in height and biomass (Fig.C). In soil: sand: compost 1: 2: 1 media, survival rate was 70% with height of plant 10-11cm with erect green stem having green leaves. In coco peat: soil: sand: 2: 1: 1 the survival rate was 60% with length of shoot were 10-14cm with slightly weaker stem. Morphologically the leaves were slightly smaller than other two. The maximum survival rate was observed in soil: sand: compost 1: 2: 1 media with 90% survival rate with maximum length of shoot 13 cm.The six month hardened plants were with many branches and healthy (Fig2.D). Thus the efficient *in vitro* rooting and hardening protocols developed in the present study, thus, can be effectively utilized for commercial cultivation of the valuable medicinal plant *Ruta graveolens*.

Table. 1- Effect of IBA on root induction

r. No.	Concentration of IBA	% of shoots to which root initiated	Time taken to initiate root (days)	*Mean no. of roots/ shoot	*Mean root length (in cm)
1	0.00µM	0	30	-	-
2	2.46 µM	90	12-14	4.5 ± 0.50	5.7 ± 0.50
3	4.90 µM	70	12-14	4 ± 0.00	4.4 ± 0.10
4	7.36 µM	80	15-17	2.5 ± 0.50	1.55 ± 0.15
5	9.80 µM	75	16-18	5 ± 0.00	1.45 ± 0.05

*Values represent mean ± standard error of 20 replicates per treatment in three repeate d Experiments

Table.2- Effect of NAA on root induction

Sr. No.	Concentration of NAA	% of shoots to which root initiated	Time taken to initiate root (days)	*Mean no. of roots/ shoot	*Mean root length (in cm)
1	0.00µM	0	30	-	-
2	2.69 µM	58	18-20	2.5 ± 0.50	3.3 ± 0.10
3	5.37 µM	65	18-20	3.5 ± 0.50	3.05 ± 0. 05
4	7.06 µM	60	20-22	7.5 ± 0.50	1.50 ± 0.50
5	10.74 µM	70	20-22	8.0 ± 0.00	1.00 ± 0.00

*Values represent mean ± standard error of 20 replicates per treatment in three repeated experiments

Table. 3.3 Effect of various hardening medium of plant survival

Sr. No.	Hardening media	% survival	Shoot length
1	soil: sand: compost 1: 1: 1	90	12-13cm
2	soil: sand: compost 1: 2: 1	70	10-11cm
3	coco peat: soil: sand 2: 1: 1	60	10-14cm

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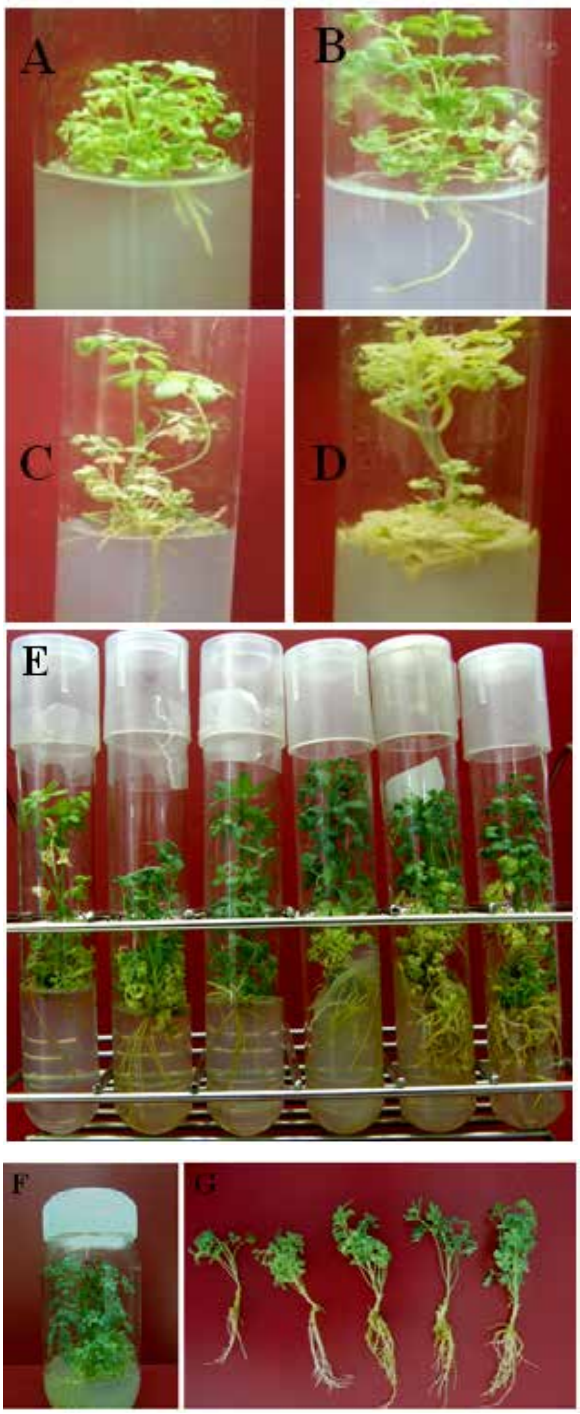

Figure: 1Effect of Auxins on root induction
A and B-Induction of Roots on MS+ 2.46µM. IBA
C-MS+2.69 µM. NAA.
D-At higher concentration of NAA (10.74) µM the plantlets showed formation of callus on roots.
E, F- six weeks in the plantlets with increased in hight and healthier plantlets .
G- Plantlets with different stages of root development

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Figure: 2 Different stages of Hardening.
A - First day of transfer in hardening media.
B - First week of transfer covered with polythene bags.
C - Two months hardened plants.
D - Six months hardened plants.
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