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Effect of Plant Growth Regulators On In Vitro Micropropagation of 'Garden Rue' (*Ruta Graveolens* L.)

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Abstract
In the present study we report a highly efficient and cost effective protocol for high frequency plantlet regeneration from nodal explants of *Ruta graveolens.* Nodal explants obtained from vegetative stem segments were grown on Murashige and Skoog (MS) medium enriched with different concentrations and combinations of cytokinins i.e. Benzyladenine (BAP), Kinetin (Kn) and Thidiazuron (TDZ) and auxin i.e. Indole -3-acetic acid (IAA) to evaluate the optimum conditions for multiple shoot proliferation. The highest number of shoot buds was obtained with media containing 1.0 mg l^{-1} BAP+0.25 mg l^{-1}IAA. Shoot multiplication rate was maintained by repeated sub culturing onto fresh media containing the same growth regulators. Elongated shoots were rooted on half strength MS media supplemented with one of the three auxins i.e. Indole -3-acetic acid (IAA), Indole-3-butyric acid (IBA), α Napthalene (NAA). The well-rooted plantlets were established on soil with 90% survival.

Keywords: Growth regulators, nodal explants, *in vitro* propagation, shoot regeneration, tissue culture.

INTRODUCTION

Ruta graveolens L. belongs to the family Rutaceae and is a native of the Mediternean regions. It is an herbaceous, ornamental perennial shrub with blue-green foliage and yellow flowers and is commonly known as "garden rue" or "herb of grace". From time immemorial rue has been known for its rich aromatic and medicinal properties. More than 120 compounds of different classes of natural products such as acridone alkaloids, coumarins, essential oils, flavonoids and furoquinolines have been isolated from *Ruta graveolens* (Kuzovkina *et al.*, 2004; Berdoncés, 1998; De feo *et al.*, 2002). The medicinal properties of this plant have been attributed to the presence of these biologically active principles.

The whole herb is abortifacient, anthelmintic, antispasmodic, carminative, emmenagogue, expectorant, haemostatic, ophthalmic and rubefacient (Browner, 1985, Ivanova *et al.*, 2005; Guarrera, 1999). It is also used to cure several skin diseases like psoriasis and vitiligo (Massot *et al.*, 2000). The plant is also being studied in context of neural disorders like multiple sclerosis (Koppenhöfer, 1995). The flavanoid content of the herb has been reported to possess antibacterial (Alzoreky and Nakahara, 2003) and cytotoxic activity (Trovato *et al.*, 1996) whereas acridone alkaloids have antiviral (Yamamoto *et al.*, 1989) and antiplasmodial (Queener *et al.*, 1991) properties. Rue extracts have been proposed as topical pharmaceutical fungicides (Ali-Shtayeh and Abu-Ghdeib, 1999; Trovato *et al.*, 2000). In homeopathy, rue is popularly employed in treating rheumatism and eyestrain induced headaches. Rutin, a flavonoid present in rue possesses nitric oxide scavenging activity (Van Acker *et al.*, 1995) thereby suggesting towards the alleged anti-inflammatory property of the plant.

Pharmaceutical industries collect plant materials primarily from wild stands resulting in indiscriminate large-scale exploitation of this natural resource. Moreover no significant endeavor is being made for the cultivation and replenishment of the wild stock. Therefore, now there is an urgency to conserve wild populations for future uses and at the same time, produce

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enough planting materials by adopting improved and efficient propagation approaches including mass cultivation of these species.

In nature *R. graveolens* is propagated by seeds or through vegetative methods, but conventional methods of propagation cannot meet the requirement, as the number of plants produced is limited. Propagation through seed is hampered by a low germination rate and low viability. On the other hand propagation through *in vitro* approaches offers a scope to propagate plants with desirable traits in larger quantities.

A number of workers have attempted to multiply *Ruta graveolens* using different explants. Kuzovkina *et al.*, (1980) obtained complete plants of *Ruta graveolens* from cultured stem explants. John *et al.*, (1997) and Castro and Barros (1997) developed protocols for micropropagation of the species from shoot tip, inter nodal and leaf segments and evaluated different culture media for optimum micropropagation.

The present communication demonstrates an effective high frequency regeneration method for producing a large number of plants from nodal explants of *Ruta graveolens.*

MATERIALS AND METHODS

Plant material

The study was undertaken at Institute of Minerals and Materials Technology (IMMT), Bhubaneswar (20°17′ 45″ N latitude and 85°49′15″ E longitudes at the altitude of 200 feet). Nodal segments used as explants in the present study were collected from six months old plants of *R. graveolens* maintained in the experimental garden of the Institute. Shoot twigs with 3-4 axillary buds were reared from the mother plants and were kept under tap water for 10 minutes and then immersed in a mild, non-phytotoxic liquid detergent (2% Labolene, Qualigens Fine Chemicals) for about 5 minutes and then washed in distilled water to make the explants free from detergent. They were disinfected with 0.1% (w/v) mercuric chloride solution for 5 minutes. Finally, the explants were washed thoroughly with sterile water before inoculation onto sterilized nutrient agar media pre-packed in culture tubes. Disinfection was performed under aseptic conditions in a laminar airflow cabinet.

Culture medium and Culture conditions

Nodal explants were inoculated onto semisolid MS medium (Murashige and Skoog, 1962) containing 3% w/v sucrose enriched with different concentrations of BAP, Kinetin, TDZ, Zeatin (0.0,1.0, 2.0, 3.0 mg l^{-1}) and BAP (1mg l^{-1}) with different concentrations of IAA (0.25, 0.5 and 1 mg l^{-1}). The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or 0.1% HCl before gelling with 0.8%agar (w/v) (Qualigen, India). Growth hormone BAP was procured from Sigma Chemical Co., USA and IAA, IBA, TDZ and NAA were procured from E-merck, (India) Ltd. 20ml of molten nutrient medium were dispensed into 150 x 25 mm test tubes that were plugged with pre-sterilized non absorbent cotton. Media were steam-sterilized at 121ºC and 1.1Kg cmÕ² pressure for 16 min. All the cultures were incubated in a culture room maintained at 25 ± 2°C, under a 16/ 8 h light dark cycle, 45 µmol mÕ² sÕ¹ irradiance level provided by cool white fluorescent tubes (Philips, India) with 50-80% relative humidity.

Induction of Rooting and acclimatization

Microshoots (1-3 cm long) were excised from the culture and transferred to half strength MS medium augmented with different concentrations of IAA, IBA or NAA (0.25, 0.5, 1.0 mg $l^{-1)}$ in and 2% (w/v) sucrose for root initiation. Microshoots were cultured in one tube each under the same culture conditions as described above. After 4 weeks, the percentage of shoots forming roots, number of roots per shoot and root length was assessed. *In vitro* cultured plantlets with well developed root and shoot systems were removed carefully, washed thoroughly with tap water and then with distilled water to remove adhering culture medium and transferred to poly pots (100 X 50mm) containing sterile vermiculite saturated with micronutrients. Potted plants were incubated in an acclimatization chamber at 28 ± 1ºC. After 3 week of acclimatization they were transferred to poly bags containing soil+sand +fym (1:2:1) for a period of 2 weeks and then transferred to field conditions. The survival rate was recorded 45 days after transfer to pots.

Statistical analysis

10 replicates were used in each treatment. All the experiments were repeated thrice. Data on percent response, Mean number of multiple shoots per explant and shoot length were determined after 30 days of incubation. Likewise, the percentage of shoots forming roots, the average number of roots per shoot and root lengths were recorded after 30 days. The data were analyzed statistically by using analysis of variance (ANOVA) and Least Significant Difference (LSD).

RESULTS AND DISCUSSION

High frequency shoot proliferation

When explants were grown on MS media devoid of any growth regulators no morphogenetic response was observed. Whereas sprouting and shoot proliferation was observed within one week of culture on MS medium enriched with different types and concentrations of cytokinins and auxin. Therefore it was mandatory to augment the medium with a cytokinin or a combination of cytokinin and auxin in order to induce shoot organogenesis.

Of the three cytokinins (BAP, Kinetin and TDZ) tested, BAP supplemented media was most effective in shoot induction, proliferation and elongation (Table 1 *[Supplementary data]*).

Within 3 days of incubation explant swelling was observed. First appearance of buds was observed within 3 to 5 days. Multiple shoots commenced to emerge from the cut ends in case of BAP and Kinetin supplemented media whereas in TDZ comprising media shoot buds emerged from the explant protuberances. TDZ is reported to increase the levels of endogenous cytokinins by, at least partly, inhibiting the action of cytokinin oxidase (Hare and Van Staden 1994). Therefore the reason for explant swelling incase of TDZ supplemented media may be attributed to the increased levels of endogenous cytokinins. In BAP and Kinetin enriched media the shoot buds soon elongated into slender shoots with well developed branching and leaves but in TDZ media poor shoot elongation was noticed. A maximum response of 96.6% was recorded in BAP 1mg l^{-1} where the number of shoots per explant was highest (35.3±2.5) with an average length of 2.3±0.1 cm. BAP an adenine type cytokinin has been reported to be the best cytokinin for multiple shoot proliferation for other Rutaceae members such as *Murraya koenigii* (Mathew *et al.*, 1999), *Aegle marmelos* (Nayak *et al.*, 2007;Varghese *et al.*, 1993; Arumugam *et al.*, 1996; Ajithkumar and Seeni 1998), *Dictamus albus* (Jones *et al.*, 1994) and *Feronia limonia* (Purohit and Tak 1992).

MS medium augmented with Kinetin showed comparatively less response. MS medium enriched with 2 mg l^{-1} Kinetin showed maximum response as compared the two other Kinetin concentrations (1 mg l^{-1} and 3 mg l^{-1}) assessed. Kinetin at 2 mg l^{-1}showed a maximum of 86.6% sprouting frequency and an average of 5.4±0.8 number of shoots per nodal segment. Shoot length was maximum (3.2±0.4 cm) in MS + Kinetin 1 mg l^{-1}. In case of TDZ supplemented medium, although shoot bud sprouting was in abundance, very few shoots exhibited healthy growth. Most of the shoots regenerated on TDZ comprising medium remained stunted with swelled basal ends and poorly developed leaves. TDZ at 1mg l^{-1} showed maximum morphogenetic response producing an average of 20.03±3.02 number of shoots (Fig.1A &B). Although a good number of shoots were regenerated in TDZ comprising media, most of the shoots were lost during sub culturing due to their poor elongation. Hyperhydricity was observed in shoots regenerated on TDZ fortified medium and the frequency were highest in media supplemented with TDZ at 3 $mgl^{-1.}$ Hyperhydricity causes translucent, thick and brittle explants (Ziv, 1991; Majada *et al.*, 2001). These hyperhydric explants when subcultured in fresh medium with the same composition gradually turned brown and perished thereby suggesting the strong adverse effects of hyperhydricity on cultured explants. Hyperhydricity has been reported to be influenced by cytokinin type (Kadota and Niimi 2003) and cytokinin concentration (Andrade *et al.*, 1999), which is in agreement with the present findings. It has been reported that TDZ is the most effective among the diphenylureas that have been used for inducing adventitious and axillary shoot proliferation in several plant species (Fasolo *et al.*, 1989; Huetteman and Preece, 1993; Kaneda, 1997; Collen *et al.*, 1999). However, in the present study, TDZ was less effective than the adenine type cytokinin BAP as reported in grass pea tissue culture (Barik *et al.*, 2006).

Figure 1: (A–F). In vitro regeneration and plant establishment of *Ruta graveolens* (A)Sprouting in MS+TDZ (1mg/l). (B)Numerous but poorly differentiated multiple shoots in MS+TDZ (2mg/l). (C)Induction of shoot buds on MS+BA(1mg/l)+IAA (0.25mg/l). (D)Four-week-old culture exhibiting numerous multiple shoots with well-developed leaves on the same media. (E)Microshoot rooted in ½ MS+IBA (0.25mg/l) showing well developed root system. (F)Microshoot rooted in ½ MS+NAA (0.25mg/l) exhibiting poorly developed root system. (G-H). Acclimatization of in vitro rooted plants. (G) Plantlet potted in vermiculite medium (2 weeks old). (H) Hardened plants potted in earthen pots showing luxurious growth (5 months old).

Massot *et al.*, (2000) in their effort to optimized culture conditions for the production of furanocoumarins by micropropagated shoots of *Ruta graveolens*, evaluated the influence of BAP, Kinetin and 2,4-D (2,4-dichlorophenoxyacetic

Table 2: Effect of different concentrations of IBA and NAA in half-strength MS medium on root induction in regenerated shoots (Ten tests tubes were inoculated for each concentration; experiment repeated twice)

Supplements (mg/l)		% of shoots to which root initiated	Time taken to initiate rooting (days)	Mean number of roots ± SE*	Mean root length (cm) ± SE*
IBA	NAA				
0	0	0	0	0	0
0.25		65	10-12	3.9± 0.6	4.5 ± 0.4
0.5		70	10-12	5.6±0.2	4.2 ± 0.3
1.0		55	15-17	5.3±0.7	3.6 ± 0.3
	0.25	65	18-20	8.4±0.2	1.4 ± 0.3
	0.5	85	18-20	11.2±1.5	1.6 ± 0.2
	1.0	80	18-20	10.7±1.5	1.6 ± 0.2

*± SE Mean standard error

acid) on *in vitro* shoot proliferation from leaf explants using Gamborg's (1968) basal medium. They reported that shoot number was significantly suppressed 2, 4-D supplemented medium whereas with both the cytokinins greater number of shoot were recorded.

The concentration of cytokinin used significantly affected the percentage shoot regeneration, shoot numbers and shoot length. At higher concentrations though more number of buds were observed, the growth of the shoots was stunted and basal callusing of the shoots was noticed. Cytokinin concentration has been several times reported to be decisive for shoot proliferation and elongation of many medicinal plant species (Saxena *et al.*, 1998; Rout *et al.*, 2000; Rout, 2004).

Although BAP is effective in initiating multiple shoot proliferation, a combined effect of BAP and IAA was more efficient in shoot bud initiation and subsequent proliferation. The proliferation rate, expressed as number of shoots per nodal explant was significantly highest in the media containing BAP1 mg l^{-1} + IAA 0.25 mg l^{-1} (Fig 1C &D). A low auxin concentration in combinations with a high cytokinin concentration promotes shoot proliferation (Sharma *et al.*, 1993, Shasany *et al.*, 1998). Of the combination tested, MS + BAP 1mg l^{-1} + IAA 0.25 mg l^{-1} elicited optimal response in which an average of 58 ± 1.51shoots (Fig 1B) with a mean shoot length of 3.2 ± 0.2 cm per explant was recorded. In the present study addition of a low level of IAA (0.25mg l^{-1}) was effective for obtaining maximum shoot numbers. As the concentration of IAA increased, the frequency of shoot development declined (Table 1). Similar results were reported in case of *Desmodium gangeticum* (Behera and Thirunavoukkarasu, 2006). Nayak *et al.*, (2007) reported that in *Aegle marmelos* highest regenerative response was observed on medium containing 6.6 µM BAP+1.14 µM IAA where approximately 86.6% of the cultures responded with an average shoot numbers of 487.5 per explant.

Subcuturing of explants was carried out after four weeks onto fresh medium with same composition. The number of shoots per explant increased with the increase in the number of subcultures. 5 to 7 additional shoots were produced in most of the explants after the third subculture (data not shown). Ajithkumar and Seeni (1998) established that by repeated subculturing of node and leaf explants of *Aegle marmelos* through five cycles continuous production of healthy callus-free shoots could be generated without any sign of decline. Amplified regeneration rates might be attributed to the adaptation of the explants to *in vitro* milieu. This is in accordance with previous reports (Upadhyay *et al.*, 1989, Rout *et al.*, 1999).

Rooting and Acclimatization

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama, 1970). Since shoot number as well as shoot length was best in media enriched with BAP1 mg l^{-1} + IAA 0.25 mg l^{-1}, micro shoots grown in the above mentioned media were subjected to rooting studies. Shoots greater than 2 cm in length were dissected and inoculated in rooting media containing half strength MS supplemented with varying concentrations of NAA, IBA or IAA.

Shoots cultured in the medium devoid of auxins failed to form roots. Rooting experiments with

three concentrations of IAA did not give any encouraging results. Most of the shoots inoculated in IAA fortified medium remained as such but did not give rise to healthy roots. This is in accordance with previous reports (Müller *et al.*, 2005). Epstein and Müller, (1993) summarized the reasons to explain the better performance of IBA versus IAA**:**(i) higher stability, (ii) differences in metabolism, (iii) differences in transport, and (iv) IBA is **a s**low release source of IAA. Hence only rooting data for IBA and NAA supplemented media were recorded.

Of the two auxins assessed, IBA proved to be better than NAA for the induction/development of a healthy root system in *R. graveolens*. Depending upon the auxin type and concentration, root initiation was noticed between 10-20 days after culture (Table 2), the earliest response being in the IBA supplemented media where root initiation was observed after 10 days of inoculation. Although number of roots per shoot in IBA enriched medium was few, the root system was very healthy and roots were longer than those in NAA supplemented medium. MS basal salts supplemented with 0.5 mg l^{-1} IBA gave rise to an average of 5.63±0.2 number of shoots with an average length of 4.2 ± 0.39 (Fig 1E).

Medium supplemented with NAA responded after 18 days of culture. Root initiation proceeded with the formation of either callus or little bulging at the bottom from where roots were produced. Though mean root numbers were very high (11.2±1.56) in NAA0.5 mg l^{-1} supplemented medium, the roots were short and superficially spread on the surface of the medium and did not show any further elongation (Fig 1F). Such cultures when maintained for prolonged periods started wilting and no further root growth was observed. The stimulatory effect of IBA on root formation has also been reported in many medicinal plants like *Murraya koenigii* (Bhuyan *et al.*, 1997), *Ocimum basilicum* (Sahoo *et al.*, 1997), and *Clitoria ternatea* (Barik *et al.*, 2007). Rooting response in *Aegle marmelos* with IBA than with a combination of NAA and IAA treatment was higher (Nayak *et al.*, 2007). In another member of the Rutaceae family i.e. *Citrus* sp. Rooting of regenerated shoots was achieved on three-fourth strength of MS medium with NAA 3 mg l^{-1} (Thirumalai and Thamburaj, 1996).

The acclimatization procedure described here resulted in a high survival rate. There was 90% survival rate of the plantlets that were rooted in IBA supplemented medium where the plants showed healthy growth (Fig 1G). After three weeks in vermiculite medium the plants were transferred to earthen pots (30 cm diameter) where the plants showed luxurious growth (Fig 1H), while shoots rooted in NAA supplemented medium did not survive. This may be due to improper development of root system in such cultures. The *in vitro* propagation protocols developed in the present study, thus, can be effectively utilized for commercial cultivation and domestication of the valuable medicinal plant *Ruta graveolens.*

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