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PROPAGATION OF *RUTA GRAVEOLENS* L. BY *IN VITRO* CULTURE OF NODAL EXPLANTS

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SUMMARY

A rapid and efficient *in vitro* propagation protocol for *Ruta graveolens* L. from nodal explants was developed. Multiple shoot formation was induced from nodal segments on Murashige and Skoog (MS) medium with 4.4-17.6 μM^{-1} 6-benzyl adenine (BA), 1.13-9.06 μM^{-1} thidiazuron (TDZ). Maximum number of shoots were induced (58.0±1.51) with 4.4 μM BA and 1.42 μM indole-3-acetic acid (IAA). Shoot differentiation occurred directly from the cut ends without any callus formation. *In vitro* regenerated shoots rooted best on half-strength MS medium containing 1.22 μM indole-3-butyric acid (IBA). Plants with a well developed root system could be successfully established under *in vivo* condition, where the survival rate was 90 per cent.

***Key words*: Growth regulators, nodal explants, *Ruta graveolens*, shoot regeneration**

INTRODUCTION

Ruta graveolens L. (Rutaceae), commonly known as 'Garden rue', is well known for its aromatic and medicinal uses. The essential oils, distributed mainly in the aerial parts, accumulate in the specific secretory cavities of the plant and represent 0.2-0.7% of the dry aerial parts. The major constituents of the oil are undecan-2-one, nonan-2-one, decan-2-one and tridecan-2-one (De Feo *et al*. 2002). The spasmolytic activity of the herb is attributed to the presence of coumarins, essential oil and an unidentified blue fluorescing agent. The constituents such as rutin, imperatorin, isoimperatorin, xanthotoxin and several alkaloids (Ivanova *et al*. 2005) are of pharmaceutical importance. Being an important medicinal plant, this species is being exploited by the local people and pharmaceutical industries to a large extent. As a result, natural reserves of this plant are declining. Unless measures are taken to conserve and domesticate this valuable species, there is going to be its severe shortage for future uses.

Although *R. graveolens* is conventionally propagated by seeds or through vegetative methods, conventional methods of propagation cannot meet the requirement as the number of plants produced through this method is limited. Moreover, propagation through seeds 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. To our knowledge, there is no significant information on the *in vitro* micropropagation of this species available. In this communication, an efficient procedure for adventitious shoot regeneration from nodal explants of *R. graveolens* is described.

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MATERIALS AND METHODS

The plants maintained in the experimental plot of the Institute of Minerals and Materials Technology (IMMT), Bhubaneswar, were used for the present work. Shoot twigs with 3-4 nodes were reared from a one year old plant and immersed in tap water to avoid drying of shoots. The twigs were washed thoroughly in a mild, non-phytotoxic liquid detergent (2% Labolene), stirred for about 10 min and then washed in tap water. Nodal explants were prepared from the shoot twigs, washed thoroughly with distilled water and used to analyze the regeneration potential. They were disinfected with 0.1% mercuric chloride solution for 3 min. Disinfection was performed under aseptic conditions in a laminar airflow cabinet. Finally, the explants were washed thoroughly with sterile distilled water before inoculation onto sterilized nutrient agar shoot induction medium in culture tubes.

The nutrient medium consisted of the salts and vitamins of Murashige and Skoog (1962) basal medium (MS) solidified with 0.8 % agar and supplemented with 3% sucrose and 4.4-17.6 μM 6-benzyl adenine (BA), 1.13-9.06 μM thidiazuron (TDZ) and 1.42-5.7 μM indole-3-acetic acid (IAA) individually and in various combinations. MS medium without any growth regulator served as control. After 6 weeks of culture, elongated shoots were transferred to the root induction medium. For root induction studies, half strength MS basal medium supplemented with 1.22-4.9 μM of indole-3-butyric acid (IBA) or 5.4-16.2 μM α-naphthalene acetic acid (NAA) were used. The pH of the medium was adjusted to 5.8 before gelling with agar. MS basal medium with or without growth hormones was dispensed either into 150 x 25 mm test tubes or in conical flasks of 250 ml capacity. The medium was autoclaved for 20 min at 121ºC. All the cultures were incubated at 25 ± 2 °C and 16 h photoperiod provided by cool fluorescent lamps with a photon flux density (PFD) of 50 μmol ml^{-2} s^{-1}. Each treatment consisted of 10 replicates and all experiments were repeated thrice.

The plantlets produced under *in vitro* conditions were washed with distilled water to remove adhering culture medium and were transferred to polypots (100 x 50mm) containing sterile vermiculite saturated with micronutrients. Potted plants were incubated in an acclimatization chamber at 28±1ºC. After 3 weeks of acclimatization, they were transferred to polybags containing soil + sand + farm yard manure (1:2:1) for a period of 2 weeks and then transferred to field. Data on percent response, total number of shoots and shoot length were determined 6 weeks after culture initiation. Similarly data on percent rooting, total root number and root length were determined 3 weeks after culture. Statistical analysis was carried out using analysis of variance (ANOVA) and least significant difference (LSD).

RESULTS AND DISSUCION

It was mandatory to augment the medium with a cytokinin in order to induce shoot organogenesis. Of the two cytokinins (BA and TDZ) tested, BA was more effective in inducing multiple shoots and further shoot growth (Table 1). Addition of BA in the culture medium has shown positive effects in a number of medicinal plants such as *Plumbago zeylanica* (Sahoo and Debata 1998), *Desmodium gangeticum* (Behera and Thirunavoukkarasu 2006), and *Rouwolfia serpentina* (Singh and Guru 2007)**.** The concentration of cytokinin used in the present work, significantly affected the percentage shoot regeneration, shoot numbers and shoot length. When BA was used at 4.4 μM concentration, the maximum number of shoots produced per explant was 13.6±0.47, whereas at 17.6 μM, the response was 27.8±1.05. There was synchronization between BA concentration and shoot numbers. A steady increase in shoot number production was observed with increasing concentration (Table 1). Although BA is effective in initiating multiple shoot proliferation, a combined effect of BA and IAA was more efficient in shoot bud initiation and subsequent proliferation. Shoot buds started appearing after 4 to 5 days of culture. These shoot buds soon elongated into slender shoots. Multiple shoots predominantly emerged from the cut end of the explant (Fig 1A), which continued till the 5^{th} week of culture. Of the various treatment tested, MS + BA (4.4 μM) + IAA (1.42 μM) elicited optimal response, in which an average of 58±1.51 shoots and a mean shoot length of 3.27±0.29 cm per explant were recorded (Fig 1 B). The addition of either IAA or NAA in the culture medium has earlier been reported to improve the response in a

Table 1. Effect of plant growth regulators in MS medium on multiple shoot induction on nodal segments of *Ruta graveolens*. Data scored after 6 weeks of culture.

Supplements (μM)			Days to bud break	% of responding cultures	Mean shoot numbers *± SE	Mean Shoot length (cm) *± SE
BA	**TDZ**	**IAA**				
4.4			3-4	53.3	13.6 ± 0.47	4.8 ± 0.55
8.8			3-4	63.3	16.6 ± 0.88	4.9 ± 0.18
13.2			4-5	73.3	17.8 ± 1.52	2.6 ± 0.32
17.6			4-5	86.6	27.8 ± 1.05	3.0 ± 0.31
F- Value (P < 0.001)					**6.51**	**35.8**
LSD (P < 0.05)					**13.6**	**2.74**
	1.13		4-5	56.6	2.4 ± 0.45	2.8 ± 0.18
	2.26		4-5	76.6	3.5 ± 1.47	2.4 ± 0.19
	4.53		4-5	66.6	5.2 ± 0.80	2.7 ± 0.13
	9.06		4-5	66.6	5.4 ± 0.74	2.8 ± 0.15
F- Value (P < 0.001)					**4.52**	**0.18**
LSD (P < 0.05)					**2.55**	**1.63**
4.4		1.42	4-5	76.6	58.0 ± 1.51	3.2 ± 0.29
4.4		2.85	4-5	70.0	43.0 ± 1.25	3.4 ± 0.32
4.4		5.7	4-5	63.3	33.5 ± 1.38	3.6 ± 0.09
F- Value (P < 0.001)					**0.66**	**0.25**
LSD (P < 0.05)					**29.64**	**1.02**

*± SE Mean standard error

number of species in terms of shoot growth (Bandhopadhya *et al*. 1999, Barik *et al*. 2006, Singh and Guru 2007). In the present study, addition of a low dosage of IAA (1.42 μM) was effective for obtaining maximum shoot numbers. As the concentration of IAA increased, the frequency of shoot development declined (Table 1). A similar response was reported in nodal cultures of *Desmodium gangeticum* (Behera and Thirunavoukkarasu 2006).

Thidiazuron (TDZ), reported to be the most effective diphenylurea, has earlier been used for inducing adventitious and axillary shoot proliferation in several plant species (Fasolo *et al*. 1989, Huetteman and Preece 1993, Kaneda *et al*. 1997, Collen and Jarl 1999). In the present work, however, TDZ was observed to be less effective than the adenine type cytokinins, like BA, in the nodal cultures of *R. graveolens*. This is in conformity with the results reported in grass pea (Barik *et al*. 2006). The shoots developed on TDZ–supplemented medium remained stunted and only an average of 5.4 ± 0.74 (Table 1) number of shoots were produced as the maximum response.

In vitro production of plantlets with profuse rooting is important for successful establishment of regenerated plants in soil (Ohyama 1970). Shoots cultured in the medium devoid of auxins failed to form roots. The auxins IBA and NAA were used singly to induce rooting from *in vitro* raised shoots. Of the two auxins tested, IBA proved to be better than NAA for the induction of a healthy root system in *R. graveolens*. Depending upon the auxin concentration, root initiation was noticed between 10-22 days after culture (Table 2), the earliest response being observed in the IBA–supplemented media, where root initiation was observed 10 days after inoculation. Explants responded 18 days after culture in medium supplemented with NAA. Root initiation proceeded with the formation of either callus or little bulging at the bottom from where roots were produced. Though mean root number was very high (26.8±1.2) in NAA (16.2 μM) supplemented medium, the roots were

Table 2. Effect of different concentrations of IBA and NAA in half-strength MS medium, on root induction in regenerated shoots.

Supplements (μM)		% 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	25	21-22	1.6 ± 0.6	1.4 ± 0.50
1.22		65	13-15	2.4 ± 0.6	3.3 ± 0.49
2.45		70	10-12	2.4 ± 0.6	3.0 ± 0.39
4.9		55	15-17	2.0 ± 0.4	2.2 ± 0.32
	5.4	65	18-20	14.0 ± 2.4	1.4 ± 0.31
	10.8	85	18-20	25.0 ± 3.4	1.6 ± 0.25
	16.2	80	18-20	26.8 ± 1.2	1.6 ± 0.24

*± SE Mean standard error

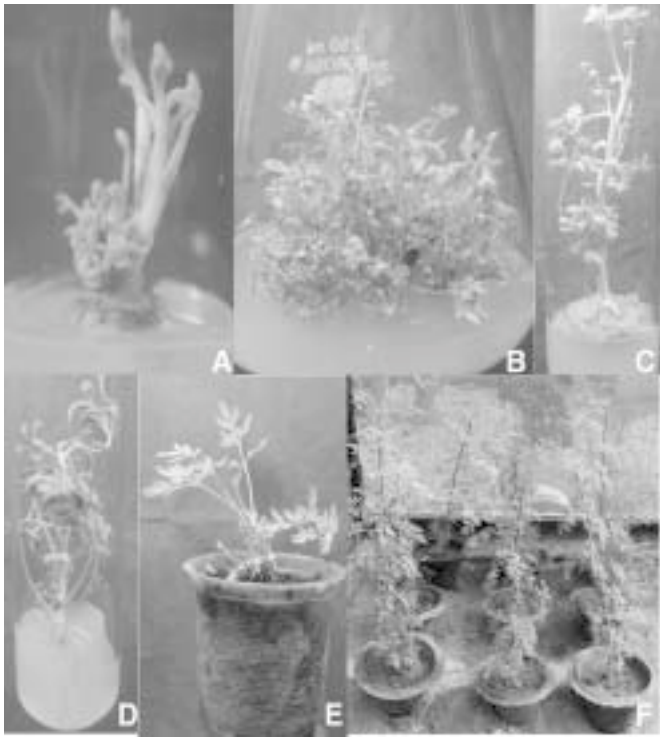

Fig. 1. Plant regeneration from nodal segments of *R. graveolens.* (A) Shoot bud sprouts (2 weeks old) from cultured nodal segment in the MS medium supplemented with BA (4.4 μM) + IAA (1.42 μM), (B) Multiple shoots from cultured nodal segment on the same medium (5 weeks old), (C) *In vitro* shoot rooted in the MS medium supplemented NAA (16.2 μM/l) showing poor root development, (D) *In vitro* shoot rooted in the MS medium supplemented with IBA (1.22 μM/l) showing a well developed root system, (E) Plantlet potted in vermiculite medium in mist chamber (2 weeks old), (F) Hardened plants potted in earthen pots showing luxurious growth (5 months old).

short and superficially spread on the surface of the medium (Fig 1 C) and did not show any further elongation. Such cultures when maintained for a prolonged period started wilting and no further root growth was observed. Root length was better when the cultures were maintained in medium with 1.22 μM IBA, where about 2.4±0.6 mean root numbers with an average length of 3.3±0.49 was recorded as the maximal response (Fig 1 D). Shoots cultured in an auxin free medium showed poor response. 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). Rooted plants when transferred to vermiculite medium (Fig 1E) showed 90 % survival rate in the acclimatization chamber. Further, they were transferred to polypots containing soil + sand + farm yard manure where the plants showed luxurious growth (Fig 1F) with 90 % survival. The *in vitro* propagation protocols developed in the present investigation, thus, can be effectively utilized for commercial cultivation and domestication of the valuable medicinal plant *Ruta graveolens.*

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