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STUDIES ON ORGANOGENESIS FROM NODAL EXPLANT OF *RUTA GRAVEOLENS* L.

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ABSTRACT
Ruta graveolens L., commonly known as Rue is widely exploited for its active principles which are of high pharmaceutical value. *In vitro* techniques were employed to multiply and conserve this traditional medicinal plant. Nodal explants were cultured on Murashige and Skoog's medium supplemented with various growth regulators. Direct and indirect regeneration from the cultures were observed depending on the media composition. MS + 2, 4-D (2.26μM) + Kin(9.29μM) + GA_3 (1.44μM) was found to be best for direct regeneration, while MS + NAA(10.74μM) + BAP(13.20μM) + L-glutamine (1.36mM) + GA_3 (2.88μM) for indirect regeneration from the callus raised on NAA supplemented medium. Rooting of the regenerated shoots was obtained on ½ MS + NAA (0.01μM) + L-glutamine (1.36mM). Acclimatized plants are maintained in Botanical garden of the department. About 95% survivals were recorded.

INTRODUCTION

Ruta graveolens L., a member of Rutaceae, is well known for its wide utilities such as ornamental, aromatic and culinary in addition to medicinal properties. Medicinal value of this taxon is attributed to the accumulation of flavonoids, furanocoumarins, acridine alkaloids, furanoquinolins and also essential oils which led to its recognition as one of the sought after traditional medicinal plants by pharmaceuticals. Further, *Ruta graveolens* has been identified as one of the potential sources of allelochemicals (Feo *et al.,* 2002). Large scale destruction of natural habitats due to population pressure and over exploitation has become a major threat to important bioresources of medicinal plants. Hence there is a need to conserve this taxon. Conventional propagation through seeds is not sufficient enough to produce required number of plants as there is a poor viability of seeds. Establishment of *in-vitro* gene banks is one of the promising ex-situ approaches for conservation of elite germplasms.

Attempts have been made previously to develop protocols for the micropropagation of *Ruta graveolens* (Castro and Barros, 1997; Faisal *et al.,* 2005, 2006; Bohidhar *et al.,* 2008). The present studies have been carried out to establish *in vitro* gene bank of *Ruta graveolens* through direct and indirect organogenesis from nodal explants.

MATERIALS AND METHODS

Potted healthy plants were procured from University of Agricultural Sciences, Bangalore. They were maintained in the Botanical garden, Department of Botany, Bangalore University, Bangalore. Nodes of about 1cm were excised from healthy plants and used as explants.

Surface sterilization: The explants were washed with Tween-20 for 5-10min. After thorough washing under running water for 30 min they were, treated with Bevastin, a fungicide (0.1%) for 5 min. Further surface sterilization was carried out by treating the explants with saturated chlorine water followed by mercuric chloride (0.1%) for 2 min each. After each treatment the explants were washed thoroughly with double distilled water.

Culture medium: The surface sterilized explants were inoculated on to Murashige and Skoog's medium (MS-1962) supplemented with various auxins – IAA, IBA, NAA 2,4-D, cytokinins - BAP, Kin, TDZ, Zea and GA_3 either alone or in combinations at various concentrations. L-glutamine was also used as supplement along with auxins and cytokinins. Sucrose (3%) and bacteriological grade agar agar (0.8%) were used as carbon source and gelling agent respectively. pH of the medium was adjusted to 5.6 and autoclaved for 15min at 109kpa.

Culture conditions: The cultures were incubated at $25 \pm 2^{o}C$ under fluorescent tube lights with 16:8hr light and dark regime at light intensity of 25$\mu molm^{-2}s^{-1}$. All the experiments were atleast repeated thrice. The cultures were regularly subcultured either to the same composition or to other combinations of growth regulators depending on the requirements of the experiment.

Data analysis: The data has been subjected to one-way analysis

of variance. The significance of mean difference of shoots was carried out using Tukey Post-hoc test at 5% level of significance (Rosner, 2000).

RESULTS AND DISCUSSION

The presence of morphogenetic response of the explants depends on their position in the source plants and also season at which they were excised. Second and third node from the apex showed high percent of response in terms of induction of number of multiple shoots and time taken for initiation. This may be due to the differential expression of the genes in cells within the plant during its growth and development that has resulted in distinct patterns of morphogenesis in various parts of the plant body. The factors that influence the gene expression include tissue or organ selected for explant, season or growing conditions and the ontogeny of the source plants (Edwin *et al.,* 2008). In the present study, the explants collected from June to December responded better to the culture conditions than other seasons. Similar season dependent morphogenetic response in *in vitro* conditions was also reported in other medicinal plants (Sahoo and Chand, 1998; Ahuja *et al.,* 1982; Patnaik and Chand, 1996; Ramaswamy *et al.,* 2004). Apart from these facts, the morphogenetic potential of the explants largely depends on the critical balance between the endogenous and exogenously supplied growth regulators. When the explants were grown on basal MS medium, there was no response even after several subcultures to the fresh media. However, direct and indirect organogenesis from the explants was observed when MS medium was supplemented with various growth regulators.

Table 1: Effect of growth regulators on multiple shoot regeneration from the cultures of *R.graveolens* L.

MS + Growth regulators	Mean ± SD	95%CL
MS + Kin (9.29 μM)	14.00 ± 1.29	13.50 - 14.48
MS + 2, 4-D (2.26 μM) + Kin (9.29 μM) + GA_3 (1.44 μM)	18.00 ± 1.05(**)	17.60 - 18.39
MS + 2, 4-D (2.26 μM) + Kin (13.95 μM) + GA_3 (1.44 μM)	15.10 ± 0.55(**)	14.90 - 15.3
MS + 2, 4-D (2.26 μM) + Kin (18.60 μM) + GA_3 (2.88 μM)	12.00 ± 0.59	11.80 - 12.22
MS + 2, 4-D (2.26 μM) + Kin (23.25 μM) + GA_3 (2.88 μM)	9.00 ± 0.59	8.80 - 9.22
MS + BAP (13.20 μM)	10.00 ± 0.53	9.80 - 10.2
MS + 2, 4-D (2.26 μM) + BAP (8.87 μM) + GA_3 (1.44 μM)	11.03 ± 0.41	10.90 - 11.19
MS + 2, 4-D (2.26 μM) + BAP (13.20 μM) + GA_3 (2.88 μM)	12.00 ± 0.45	11.80 - 12.17
MS + 2, 4-D (2.26 μM) + BAP (17.74 μM) + GA_3 (2.88 μM)	9.00 ± 1.02	8.60 - 9.38
MS + 2, 4-D (2.26 μM) + BAP (22.20 μM) + GA_3 (2.88 μM)	8.00 ± 0.64	7.80 - 8.24
MS + IAA (11.42 μM) + BAP (4.44 μM) + GA_3 (1.44 μM)	5.00 ± 0.59	4.80 - 5.22
MS + IAA (11.42 μM) + BAP (8.87 μM) + GA_3 (1.44 μM)	8.00 ± 0.91	7.70 - 8.34
MS + IAA (11.42 μM) + BAP (13.20 μM) + GA_3 (2.88 μM)	12.13 ± 0.90	11.80 - 12.47
MS + IAA (11.42 μM) + BAP (17.74 μM) + GA_3 (2.88 μM)	10.00 ± 1.97	9.30 - 10.73
MS + IAA (11.42 μM) + BAP (22.20 μM) + GA_3 (2.88 μM)	11.07 ± 0.78	10.80- 11.36
Significance	F = 370.952; p < 0.001**	

NOTE: Results are represented as Mean ± SD. Data within a column followed by the symbol (**) represent 5% level of significance compared to all treatments by Tukey post-hoc test

Table 2: Effect of growth regulators and amino acids on multiple shoot regeneration from the cultures of *R.graveolens* L.

MS + Growth regulators + Amino acids	Mean ± SD	95%CL
MS + NAA (0.27 μM) + Kin(9.29 μM) + GA_3(1.44 μM)	6.93 ± 0.58	6.7 - 7.15
MS + NAA(5.37 μM) + Kin(9.29 μM) + GA_3 (2.88 μM)	7.87 ± 0.57	7.7 - 8.08
MS + NAA (5.37 μM) + Kin(13.95 μM) + GA_3(2.88 μM)	9.00 ± 0.69	8.7 - 9.26
MS + NAA (10.74 μM) + Kin (13.95 μM) + GA_3(2.88 μM)	12.00 ± 1.11(**)	11.6 - 12.42
MS + NAA (10.74 μM) + Kin(18.60 μM) + GA_3 (2.88 μM)	9.00 ± 0.45	8.8 - 9.17
MS + NAA (10.74 μM) + BAP(13.20 μM) + Glutamine (0.68 mM) + GA_3 (2.88μM)	8.00 ± 0.91	7.7 - 8.34
MS + NAA (10.74 μM) + BAP (13.20 μM) + Glutamine (1.36 mM) + GA_3 (2.88 μM)	20.00 ± 1.05(**)	19.6 - 20.39
MS + NAA (10.74 μM) + BAP (13.20 μM) + Glutamine (2.05 mM) + GA_3(2.88 μM)	10.00 ± 1.05	9.6 - 10.39
MS + NAA (10.74 μM) + BAP (13.20 μM) + Glutamine (2.73 mM) + GA_3(2.88 μM)	6.00 ± 1.39	5.5 - 6.52
MS + NAA (10.74 μM) + BAP (13.20 μM) + Phenylalanine(0.60 mM) + GA_3(2.88 μM)	8.00 ± 1.05	7.6 - 8.39
MS + NAA (10.74 μM) + BAP (13.20 μM) + Phenylalanine(1.2 mM) + GA_3 (2.88 μM)	10.00 ± 0.87	9.7 - 10.33
MS + NAA (10.74 μM) + BAP (13.20 μM) + Phenylalanine(1.81 mM) + GA_3(2.88 μM)	8.00 ± 0.91	7.7 - 8.34
MS + NAA (10.74 μM) + BAP (13.20 μM) + Phenylalanine(2.42 mM) + GA_3 (2.88 μM)	6.13 ± 1.96	5.4 - 6.87
MS + NAA (10.74 μM) + BAP (13.20 μM) + Glycine (1.33 mM) + GA_3 (2.88 μM)	4.00 ± 0.95	3.6 - 4.35
MS + NAA (10.74 μM) + BAP (13.20 μM) + Glycine (2.66 mM) + GA_3 (2.88 μM)	6.00 ± 0.69	5.7 - 6.26
MS + NAA (10.74 μM) + BAP (13.20 μM) + Glycine (3.99 mM) + GA_3 (2.88 μM)	3.00 ± 0.53	2.8 - 3.2
MS + NAA (10.74 μM) + BAP (13.20 μM) + Glycine (5.32 mM) + GA_3 (2.88 μM)	2.00 ± 0.69	1.7 - 2.26
Significance	F = 509.780; p < 0.001**	

NOTE: Results are represented as Mean ± SD. Data within a column followed by the symbol (**) represent 5% level of significance compared to all treatments by Tukey post-hoc test

Direct organogenesis

When nodal explants inoculated on to MS supplemented with various growth regulators, the first response exhibited was the basal swelling of explants within a week after inoculation. The axillary bud grows into a single shoot on MS + IAA/IBA without any shoot proliferation. Addition of cytokinin stimulates multiple shoot induction from the explant. Presence of BAP (13.20μM)/ Kin (9.29μM) in the MS medium promoted the proliferation of shoots as many as 10 ± 0.53 and 14 ± 1.29 shoots respectively (Fig.1). The percent of shoot emergence enhanced linearly upto the optimum level of kin (9.29μM) and BAP (13.20μM) and then declined as the concentration was increased (Table 1).

Although a small quantity of cytokinin may be synthesized by shoots *in vitro* (Kodo and Okozawa, 1980), roots are the

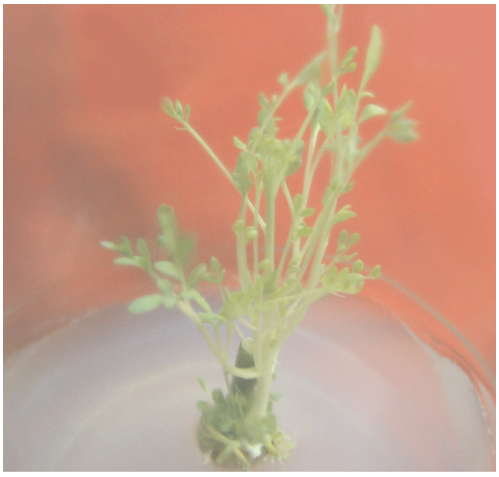

Figure 1: Multiple shoots from nodal explants on MS + Kin (9.29μM)

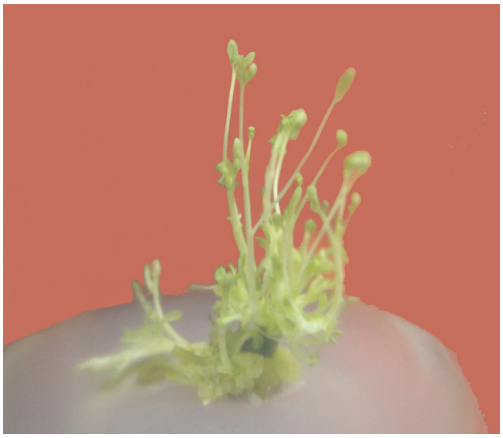

Figure 2: Multiple shoots from nodal explants on MS + 2, 4D (2.26 μM) + Kin (9.29 μM) + GA_3(1.44 μM)

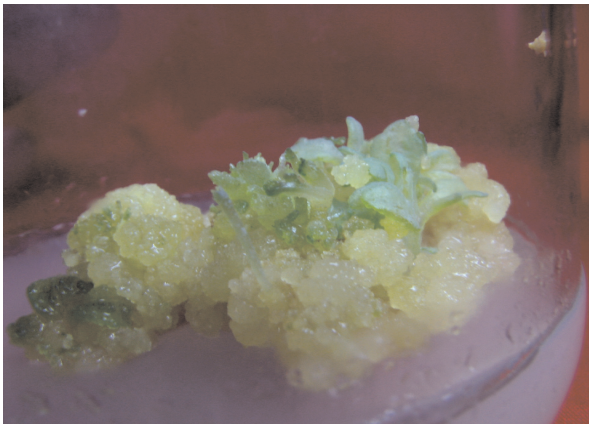

Figure 3: Multiple shoots from nodal callus culture on MS + NAA (10.74 μM) + Kin (13.95μM) + GA_3(2.88μM)

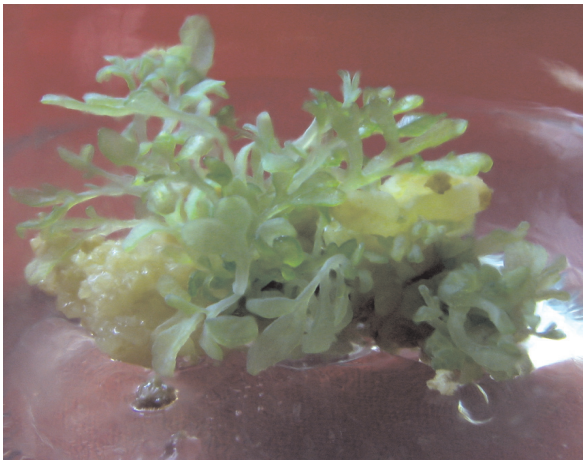

Figure 4: Multiple shoots from nodal callus culture on MS + NAA(10.74μM) + BAP (13.20μM) + L-Glutamine(1.36mM) + GA_3(1.44 μM)

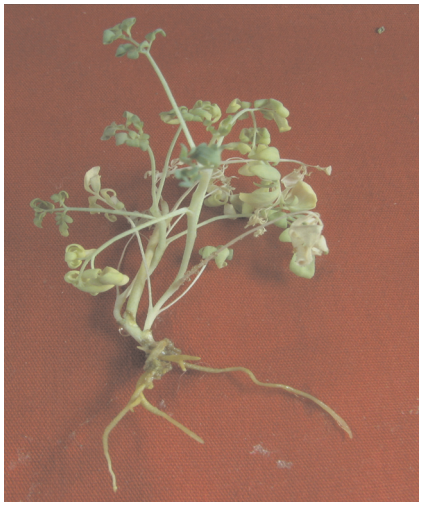

Figure 5: Plant with roots

Figure 6: Regenerated plant

principle sites of cytokinin biosynthesis. Hence, exogenous supply of cytokinin is needed to bring out the morphogenetic potential of the nodal explants. Multiple shoot induction was also recorded when BAP (13.20μM)/ kin (9.29μM) was combined with 2, 4-D (2.26μM) + GA_3 (1.44μM). The best combination for the proliferation of multiple shoots was found to be MS + 2, 4-D (2.26μM) + Kin (9.29μM) + GA_3 (1.44μM)

with 18 ± 1.05 shoots per culture (Fig. 2). When these new shoots grew into 5 to 6 cm in length having 4 to 5 nodes were cut into segments containing a single node and subcultured to the same combinations, resulted in the formation of 18 ± 1.05 shoots per culture. Thus from a single explant it was possible to obtain nearly 1000 shoots within a period of 10 to 12 weeks. Thus obtained shoots were healthy with elongated internodes. BAP is considered as a potential hormone to induce multiple shoots in several taxa (George and Sherrington, 1984). Tejavathi *et al.,* (2009) have observed the induction of maximum number of multiple shoots/explant from the nodal explants of mulberry when cultured on BAP supplemented medium compared to other cytokinins such as Kin, 2-ip and Zea. However in the present studies, kinetin either alone or with 2, 4-D and GA_3 was found to be better than BAP combinations. This is in conformity with the observations made by Ramaswamy *et al.,* (2004) in *Solanum surattense*. While Benneth and Davies (1986) and Bhat *et al.,* (1995) have reported that kin is less effective than BAP in *Quercus shumardii* and *Piper* spp. cultures respectively.

When BAP and kin concentrations were increased beyond 13.20μM and 9.29μM respectively, the rate of shoot multiplication was reduced in the present studies. Similar observations were made by Bohidhar *et al.,* (2008) in the same taxon and Ramaswamy *et al.,* (2004) in *S. surattense*. Cytokinin concentration has been several times reported to be decisive for shoot proliferation. A low auxin concentration in combination with a high concentration of cytokinin as was observed in the present studies is the most suitable combination for the proliferation of shoots (Tejavathi and Gayathramma, 2005). Presence of GA_3 along with auxin and cytokinin in the present studies promoted the elongation of shoots.

Indirect regeneration

Callus formation was observed from the swollen basal regions of the explants, when they were inoculated on MS + NAA (10.74μM) within four weeks of culture. The callus was compact and yellow in colour and later it became greenish yellow when it was subcultured to NAA (10.74μM) + BAP (13.20 μM). A segment of the greenish yellow callus was subcultured to shoot induction media containing BAP, kin, GA_3 and amino acids either alone or with NAA (Table 2). Shoot differentiation from the callus was observed within 15 days of incubation on MS + NAA (10.74μM) + Kin (13.95μM) + GA_3 (2.88μM) (Fig.3). However, higher frequency of shoot induction from the callus was recorded on MS + NAA (10.74μM) + BAP (13.20μM) + L-glutamine(1.36mM) + GA_3(1.44μM) (Fig. 4). L-glutamine is a potent amino acid and is being frequently used in several systems to enhance the morphogenetic potential of the explants (George and Sherrington, 1984). Mohamed (1996) is of the opinion that cultured cells are normally capable of synthesizing all the required amino acids, yet the addition of amino acid/s may be used to stimulate cell growth and facilitate plant regeneration. L-glutamine can also serve as a source of nitrogen which can be absorbed by the tissues more rapidly than inorganic nitrogen (Thom *et al.,* 1981). However, Faisal *et al.,* (2006) has reported that in *Ruta graveolens,* NAA showed the synergistic effect with BAP and enhanced the induction of shoot buds from callus and increased the morphogenetic potential than other cytokinin and auxin combinations. Callus mediated shoot morphogenesis has been accomplished in several medicinal plants (Agarwal and Sardar, 2006). High frequency callus mediated shoot regeneration can be further exploited by biotechnological approaches to develop the elite clones with high content of active principles.

Rooting and acclimatization

Plantlets with well developed roots in *in vitro* is essential for the successful establishment of regenerated plants in field. Well developed shoots thus obtained from both direct and indirect regeneration were transferred to various strengths of media containing different auxins. Presence of IAA, NAA and 2, 4-D in the medium promoted the formation of basal callus without any root induction as was observed by Bohidhar *et al.,* (2008) in the same taxon. Faisal *et al.,* (2005, 2006) had recorded rooting of microshoots of *Ruta graveolens* on ½ MS + IBA (0.5μM). Bohidhar *et al.,* (2008) are also of the opinion that IBA is a potential hormone for root induction than other auxins in the same taxon. IBA, as root inducing hormone is well established in several taxa (Santos *et al.,* 2003; Cheepala *et al.,* 2004). However the ½ MS with NAA (0.0054μM) and L-glutamine (1.36 mM) proved to be the best for induction of roots from the basal parts of the regenerated shoots in the present investigation (Fig. 5). Presence of L-glutamine in the medium promoted root induction of microshoots in *Helianthus annus* and inter specific hybrids of *Helianthus* (Witrzens *et al.,* 1988).

The plantlets were then first transferred to the plastic pots containing soilrite and watered regularly. After four weeks under lab conditions (25 ± 2ºC), they were transferred to earthen pots containing pot mixture of sand: soil: farmyard manure in the ratio of 1:1:2 and maintained in polyhouse (Fig. 6). Thus hardened plants showed 95% survival.

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