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GROWTH AND QUATERNARY ALKALOID PRODUCTION IN DIFFERENTIATING AND NON-DIFFERENTIATING STRAINS OF *RUTA GRAVEOLENS*

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Key Word Index—*Ruta graveolens*; Rutaceae; callus cultures; quaternary alkaloids; differentiating strains; precursors.

Abstract—Cultures of *Ruta graveolens* have been grown and maintained on Murashige and Skoog's medium supplemented with α-naphthalene acetic acid (1 mg/l.) and kinetin (0.1 mg/l.) or 2,4-dichlorophenoxyacetic acid (1 mg/l.), and glucose (30 g/l.). The production of dihydrofuroquinoline alkaloids has been investigated in these cultures. The present study showed that: (a) cultures of *Ruta* produced both differentiating and non-differentiating strains; (b) quaternary alkaloids (platydesminium, ribalinium and rutalinium) were present in all the strains and maximum contents were detected at 3–4 weeks growth; (c) high platydesminium and rutalinium content was associated with differentiation and callus formation, respectively; (d) changed concentrations of quaternary alkaloids were recorded in all the five strains grown on media supplemented with tryptophan and precursors (anthranilic acid, 5-methylanthranilic acid and 2,4-dihydroxyquinoline).

INTRODUCTION

Callus and cell suspension cultures of *Ruta graveolens* have been shown to produce diverse types of metabolites, viz. acridone alkaloids [1], antimicrobial compounds [2], coumarins and furoquinoline alkaloids [3] and volatile oils [4, 5]. Callus tissues have also been raised from leaf, stem and root explants to test the regenerative potentialities [6]. The callus derived from stem explants, when grown on various media in darkness and light produced roots and shoots, respectively.

Variability in plant tissue cultures has been discussed at length [7, 8] and differential response of clones has been established [9–11]. Precursors and amino acids have been frequently used to enhance the secondary products [11, 12]. Various amino acids [13–15], anthranilic acid [1, 16] and 2,4-dihydroxyquinoline [17] were used to synthesize or to optimize the desired plant product *in vitro*.

Three quaternary alkaloids were isolated from plant material of *Ruta graveolens* in this laboratory [18]. Subsequently, cultures were raised from stem explants obtained from one plant, grown in the botanical garden of the Faculty [19]. In this paper we report the characterization of various strains established *in vitro* in relation to growth, morphology and quaternary alkaloid production. Fluorometric scanning of quaternary alkaloids on thin-layer chromatography (TLC) plates, isolation of alkaloids from callus tissues, and the effects of tryptophan and precursors on growth and alkaloids are also described.

RESULTS

Establishment of strains

Tissues of *Ruta graveolens* grew well on Murashige and Skoog (MS) medium [20]. Four strains (R1, R3, R7 and R10) were maintained on MS medium supplemented with kinetin (0.1 mg/l.) and α-naphthalene acetic acid (NAA) (1 mg/l.). Strain R7D was obtained from strain R7 by subculturing the tissues on a medium containing NAA (1 mg/l.) and 2,4-dichlorophenoxyacetic acid (2,4-D) (1 mg/l.), without any cytokinin. The morphological characteristics of all the five strains established during the course of culture on MS medium in the past 3 years are presented in Table 1. It is clear from the table that two strains are differentiating and three are non-differentiating (calli). Strain R1 can be described as a highly differentiating callus mass producing numerous small, thin shoots, whereas strain R10 has clumps of well-developed shoots and produces little callus by dedifferentiation of stems.

Characterization of alkaloids

Three quaternary alkaloids were isolated from the strains as described in the Experimental. They were identified as platydesminium, rutalinium and ribalinium by their UV and mass spectra, and co-chromatography with pure and authentic samples obtained from plant material in this laboratory. The total quaternary alkaloid yield of the strains is given in Table 1.

Time-course study of growth and alkaloids

The results obtained with five strains harvested each week for their growth and quaternary alkaloids are

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Table 1. Morphological characteristics of five strains of *Ruta graveolens* cultured on MS medium*, after 3 weeks growth in light (2000 lx) at 25°

Strains	Colour, texture and state of differentiation	Total quaternary alkaloids† (mg/g dry weight)
R1	Green, small, thin shoots (1–3 cm), profusely differentiating callus, leaves small, translucent, number of shoots ∝, callus soft	9.44
R3	Green, globular, compact, hard, and non-differentiating callus	3.12
R7	Greenish-yellow, amorphous, non-differentiating soft callus	6.30
R10	Green, vigorously growing shoots (4–8 cm), number of shoots 15–25/explant, little, compact and hard callus at the base of shoots	8.55
R7D	Yellow with very little green cells, amorphous, soft, non-differentiating callus	3.55

*See text for the composition of the medium for the strains.
†The results are the average of several passages.

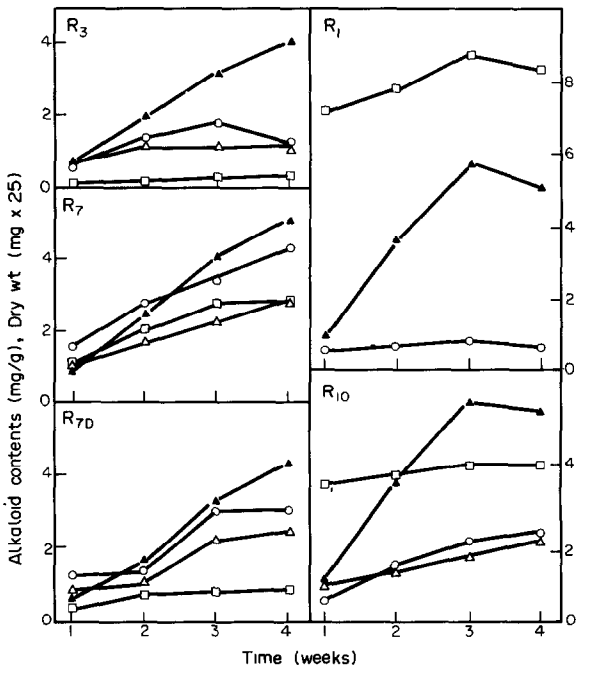

Fig. 1. Growth (dry weight) and quaternary alkaloid contents of five strains of *Ruta graveolens* (R3, R7, R7D: non-differentiating strains; R1, R10: differentiating strains). Tissues were grown in light (2000 lx) at 25° and harvested each week. All the readings are the average of at least three separate samples. (▲) Dry weight; (□) platydesminium; (O) rutalinium; (Δ) ribalinium. The same symbols have been used in all the figures.

presented in Fig. 1. It is evident from the results that differentiating strains were fast-growing as compared to non-differentiating strains. The maximum dry weight was recorded in R10 whereas R7 and R7D attained minimum dry weights.

High platydesminium contents were recorded in the differentiating strains at 3 weeks growth, R1 followed by R10. Compact and hard callus of strain R3 yielded a minimum amount of alkaloids.

Effect of tryptophan (Trp)

Tryptophan increased the callus growth in non-differentiating strains moderately. The increase in growth values in differentiating strains (R1, R10) was due to an increase in callus ratio in some treatments. The increase in callus has been directly related to a decrease in platydesminium contents in strains R1 and R10. The results obtained with tissues grown on Trp-supplemented medium are presented in Fig. 2. It is evident from the figures that increase in Trp concentration in the medium was associated with a decrease in the quaternary alkaloids in all the strains.

Effect of anthranilic acid (AA)

Mixed results were obtained with the tissues grown on the medium with AA in terms of growth, differentiation and contents of alkaloids. The results are presented in Fig. 3. A slight increase in platydesminium was recorded in differentiating strains (R1, R10). The contents of rutalinium, ribalinium and platydesminium in these strains can be directly correlated with the increase or decrease in callus in various treatments. In the non-organogenetic strains (R3, R7, R7D) the alkaloid contents

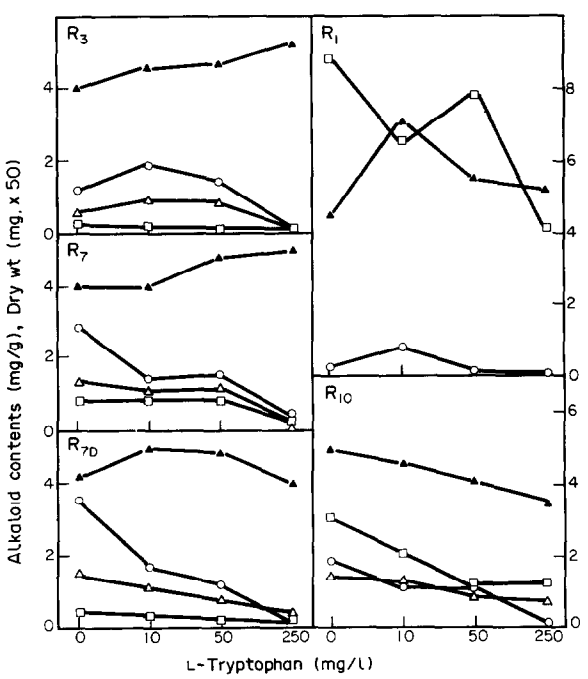

Fig. 2. Effect of L-tryptophan on growth and quaternary alkaloids. In this and the following figures, the tissues were harvested at 4 weeks growth in light.

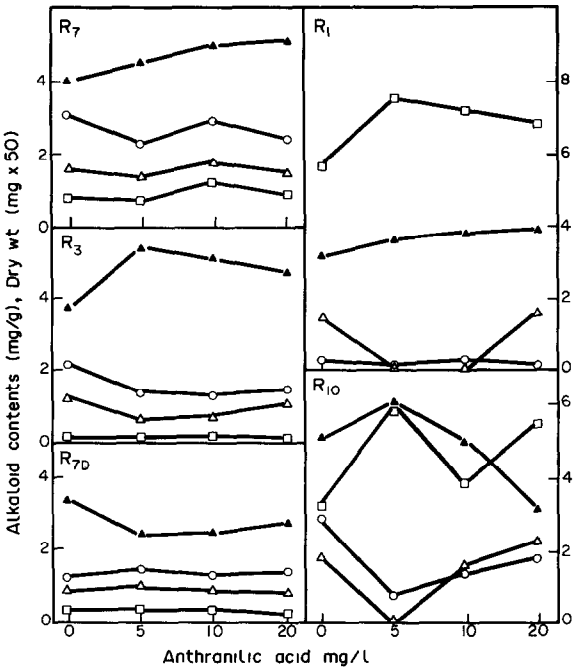

Fig. 3. Effect of anthranilic acid on growth and quaternary alkaloids.

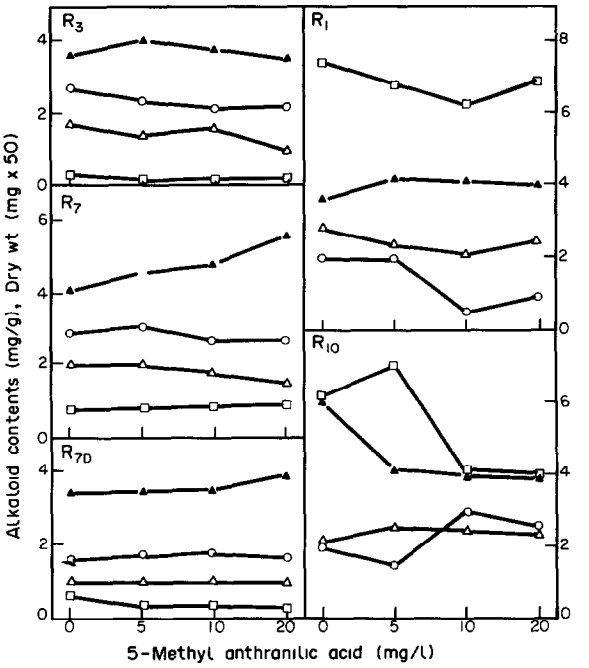

Fig. 4 Effect of 5-methylanthranilic acid on growth and quaternary alkaloids.

remained more or less the same or were decreased. Slight browning was observed in the callus tissues grown on medium supplemented with 20 mg/l. AA. Thus, it is difficult to draw a general conclusion about the effect of AA on growth, differentiation and quaternary alkaloid contents of *Ruta* tissues grown in culture.

Effect of 5-methyl anthranilic acid (MA)

The results obtained with organogenetic and non-organogenetic strains of rue grown on media supplemented with MA are shown in Fig. 4. A slight increase in growth was recorded in non-differentiating strains, whereas an increase in growth in R1 was due to an increase in callus ratio. Growth of strain R10 was reduced in all treatments. Tissues became granular, hard and dry, i.e. attained less fresh weight (results not presented) and accumulated more dry matter. The least affected tissues with respect to growth, colour, texture and metabolites were those of strain R7D. In general, alkaloid contents were decreased in all strains, more severely in organogenetic strains, except R7D.

Effect of 2,4-dihydroxyquinoline (DHQ)

A decreased dry weight was recorded in all the strains grown on media supplemented with DHQ (50–1250 mg/l.). With maximal concentration, tissues showed slight browning and dryness. The results obtained with DHQ are presented in Fig. 5. All the strains showed different responses to DHQ with respect to quaternary alkaloids. An increase in platydesminium in R1, platydesminium and/or rutalinium in R10, and rutalinium in R7 and R7D was recorded with an increase of DHQ in the medium. In R3, the alkaloid contents decreased with an increase of DHQ in the medium. It has been observed that DHQ increased the total alkaloid contents of some of the strains irrespective of whether they were organogenetic or non-organogenetic. It is also evident from the figures that an increase in one alkaloid content in the tissues is at the expense of other quaternary alkaloids.

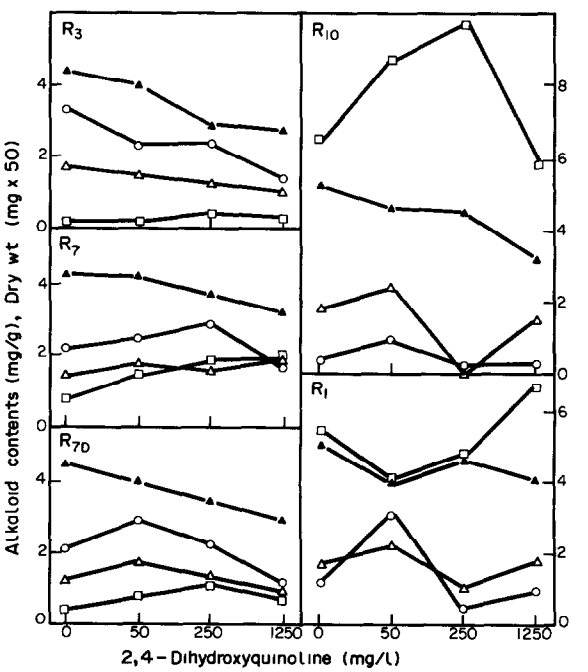

Fig. 5. Effect of 2,4-dihydroxyquinoline on growth and quaternary alkaloids.

DISCUSSION

Callus culture of *Ruta graveolens*, developed from stem explants taken from one plant, resulted in the establishment of differentiating and non-differentiating (callus) strains. These strains retain their growth and morphological characteristics after 3 years of growth on MS medium. The strains were in successive stages of differentiation, from fragile callus (R7) → hard callus (R3) → organogenetic callus (R1) to well-developed shoots producing strain R10. Therefore, the development of organogenetic and callus strains of the same genotypic origin growing on the same medium provides an excellent system for the study of variability and inter-relationship of differentiation and metabolism.

As far as our information goes, no report is available on quaternary alkaloid production in *Ruta*. We have isolated three quaternary alkaloids from differentiating and non-differentiating strains of *R. graveolens*. Boulanger *et al.* [21] obtained edulinine from cultures of *Ruta* in the basic phase. Platydesminium may form edulinine by opening of the furan ring in a basic solution.

Growth phase affected markedly the yield of quaternary alkaloids in the callus strains (R3, R7 and R7D), but growth phase affected the alkaloid contents in differentiating strains (R1 and R10) very little, perhaps due to the fact that these strains were always in a state of differentiation. Therefore, a high platydesminium content was recorded at all stages of growth in the differentiating strains. In callus strains, large amounts of alkaloid were detected after 3 weeks growth. These results were in accordance with the results obtained with *Solanum nigrum* [22] callus cultures. Organogenetic strains produced larger amounts of alkaloids as compared to callus strains. Hard callus strain (R3) yielded alkaloids in low amounts.

It has been reported that 2,4-D and kinetin strongly inhibited and promoted the nicotine synthesis in tobacco cells, respectively [23]. Similarly in *Lithospermum* cultures, formation of shikonin was completely inhibited by synthetic auxin 2,4-D or NAA [24]. In *R. graveolens*, strain R7D maintained only on 2,4-D- and NAA-supplemented medium for the past 3 years produced quaternary alkaloids, albeit to a lesser extent.

All the strains grew well on MS medium but it was difficult to obtain uniform inoculum from differentiating strains and care was taken in this respect. The variable response observed in some treatments may be attributed to the heterogeneous nature of the inoculum in these strains. DHQ formed a suspension in the medium and at maximal concentration little sedimentation was observed. This was also observed by earlier workers with DHQ [17]. The effect of various precursor amino acids in increasing the yield of metabolites has been reported in several cultured tissues [11]. Phenylalanine increased the alkaloid content of *Datura* [13] and *Ephedra* [15] tissues. Simultaneously, failure of the precursors to increase the yield of metabolites has also been reported [12]. In the present investigation, increase, decrease or no change in the alkaloid content have been recorded in differentiating and non-differentiating strains grown on media supplemented with Trp, AA, MA and DHQ. The ineffectiveness of Trp, AA and MA in enhancing the quaternary alkaloids can be postulated on two grounds: (1) that these compounds increase auxin production instead of alkaloid, or the enzymes responsible for AA → Trp → Indole were more active than enzymes of alkaloid biosynthesis; and (2) cultured tissues produced optimal levels of alkaloid, and therefore further enhancement was not possible. These conclusions are supported by the observations that the effect of Trp, AA and MA was similar to that produced by auxins: enhanced callusing, retardation of shoot formation, and decrease in alkaloid contents [22]. Secondly, most of the strains produced quaternary alkaloid equivalent to the *in vivo* system (*ca* 6 mg/g total quaternary alkaloids). DHQ has been shown to be a precursor of quaternary alkaloids in *Skimmia japonica* [25]. This compound has moderately enhanced the quaternary alkaloids of *R. graveolens* grown in culture at low concentrations. Poor solubility of the compound in the medium may be the reason for the poor uptake by the cells and the resultant low enhancement. Callus strains of *R. graveolens* produced small amounts of platydesminium during the growth phase or in various treatments of Trp, AA, MA and DHQ, whereas high platydesminium contents were recorded in *Choisya ternata* cell cultures after 20 days of growth [26]. The total alkaloid contents of a fragile callus strain (R7) were recorded in fair amounts in control and treated tissues. It was observed, generally, that fast-growing, disorganized tissues produce a low level of secondary products [22]. However, R7 produced alkaloid at *ca* 60% that of differentiating strains. It was found that different nutrient media did not influence the composition of the volatile oil in *R. graveolens* [4], but light affected the composition of volatile oils in *R. graveolens* cultures markedly [5]. The same authors showed that tissues grown in light yield volatile oils comparable to the *in vivo* system. In the present study also, tissues were grown in light and some of the strains yielded quaternary alkaloids comparable to leaf tissues. A slight variation was observed in the amounts of platydesminium, ribalinium or rutalinium in the tissues kept as control in different experiments, but this did not influence overall the quantitative alkaloid contents.

It may be concluded from the results obtained with differentiating and non-differentiating strains of rue that: (1) callus and differentiating strains can be maintained on the same medium; (2) they produce some quaternary alkaloid after prolonged culture; the site of quaternary alkaloid synthesis is the aerial parts; (3) maximum alkaloid contents were recorded at the end of the exponential phase (3–4 weeks growth); (4) high platydesminium and rutalinium contents were associated with differentiation and callusing, respectively; (5) high dry weight and alkaloid contents were recorded in differentiating strains; (6) strain R7D, grown on 2,4-D and NAA (without kinetin), produced alkaloid after prolonged culture; (7) increase in one quaternary alkaloid at the expense of other alkaloids was more frequent in organogenetic strains as compared to non-organogenetic strains.

Suspension cultures have been obtained from R7 and R7D. Further studies are in progress to characterize these strains ultrastructurally and to draw some conclusions about the inter-relationship of organogenesis and metabolite production.

EXPERIMENTAL

Tissue culture. Cultures were obtained from surface-sterilized stem explants obtained from a single plant of *Ruta graveolens* L. Explants grew well and calli were obtained in subsequent subcultures of 3 weeks each. Tissues were maintained on Murashige and Skoog's medium (pH 5.8–6.0) [20] supplemented with vitamins of Murashige and Tucker's medium [27]: NAA

(1 mg/l.) and kinetin (0.1 mg/l.) or 2,4-D (1 mg/l.). During the course of subsequent subcultures [19], 5 different strains were established as described in the Results. The stock cultures were grown in 250 ml flasks containing 100 ml medium. Tissues (6 explants, *ca* 200 mg) were grown in pre-sterilized plastic Petri plates (9.0 cm) containing 20 ml medium for growth phase studies. Callus tissues were inoculated (*ca* 6 g) in 1 l. flasks containing 200 ml MS medium for alkaloid isolation and characterization. In further experiments, tissues were grown on MS media supplemented with L-tryptophan, anthranilic acid, 5-methylanthranilic acid and 2,4-dihydroxyquinoline In all the experiments, tissues were grown in continuous light (2000 lx) at 25° and harvested at the end of 4 weeks growth except in the experiments on growth phase. All the chemicals were of the highest purity available and incorporated before autoclaving (110°, 20 min)

TLC of alkaloids. Calli were freeze-dried and the dry wt was determined Three samples from each treatment (30 mg each) were extracted in MeOH (3 ml, 6 hr) at room temp. After centrifugation (1500 *g* × 10 min), 2 ml supernatant was evapd and the residue was dissolved in 1 ml bromothymol blue (BTB, 10^{-3} M in NaPi buffer, pH 6, 0.2 M). The alkaloid–BTB complex was extracted with CH_2Cl_2 (1 ml × 2) [18]. The organic phase after evapn, was diluted suitably in CH_2Cl_2 and applied to TLC plates (0.2 mm Kieselgel-60, precoated on Al foil, Merck). Various solvent systems described [2, 19] for rue metabolites were tried and EtOAc–HCOOH–H_2O (40:5:4) was found to be the most suitable and was used in all studies. Ribalinium and rutalinium are highly fluorescent in UV light. The plates were read on a high speed TLC scanner (Shimadzu) in the fluorescence mode, emission filter-1, stray light cut off filter 'position in', Hg lamp at 313 nm. The alkaloids were compared with authentic samples obtained from the plant [18]. The R_f values of platydesminium, ribalinium and rutalinium with the above-mentioned solvent were 0.22, 0.27 and 0.33, respectively. The results are presented as mg alkaloid per g dry wt All of the results are the average of at least 3 samples.

Isolation of quaternary alkaloids. Lyophilized tissues (31 g) were extracted in MeOH (1 l. × 2) at room temp. The alkaloid–BTB complex from the organic phase, as described above, was loaded on the top of a silica gel H (60 mesh) column (30 × 3 cm) and eluted with the TLC solvent system. The movement of alkaloids was monitored by UV, and separated alkaloid fractions were monitored by TLC, pooled and evapd. The alkaloids were passed separately from glass columns packed with Amberlite resin (IRA 400, Cl^-) using MeOH. The products were analysed by UV absorption and mass spectroscopy for confirmation.

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