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Tissue culture of *Rhazya stricta* Decaisne

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ABSTRACT

Seeds of *Rhazya stricta* Decaisne were germinated in nutrient medium enriched with gibberellic acid (GA_3). The auxin indoleacetic acid (IAA) stimulated profuse callus initiation in excised roots segments, and abundant root formation. Naphthalenacetic acid (NAA) promoted soft friable callus in stem and leaf segments, and 2,4-dichlorophenoxyacetic acid (2,4-D) in cotyledons as well as leaf segments. Different combinations of various growth-regulating substances promoted callus development. Plant regeneration was obtained from stem-derived callus grown in medium containing NAA. Preliminary investigation of calli extracts suggested the presence of alkaloids.

INTRODUCTION

Rhazya stricta Decaisne (Apocynaceae) is a small, erect shrub, about 0·9–1 m high that grows wild in the desert regions of Iraq, Saudi Arabia, Yemen, Pakistan and Iran (Omar 1988). The plant contains high amounts of certain indole alkaloids (Mukhopadhyay *et al.* 1981). According to Chatterjee *et al.* (1974), this plant has been used to cure fever, sore throat and chronic rheumatism. Recent pharmaceutical studies indicated that these indole alkaloids can be used as anticancer agents (Mariee 1983).

Tissue and cell culture techniques can provide alternative means to propagate plants that are difficult to propagate by traditional methods (Murashige 1974). Valuable medicinal substances of plant origin such as alkaloids, terpenoids, flavonoids and quinones have been isolated from cultured cells (Furuya 1982). In a previous study, we reported on the effect of growth regulators and gamma rays on the tissue culture of *R. stricta* (Omar *et al.* 1985). More detailed information has recently been published on different aspects of the tissue culture, somatic enbryogenesis and alkaloids extracted from this plant (Omar 1988). The objective of this investigation is to develop a tissue culture technique for clonal propagation of *R. stricta*, and to determine whether the cultured cells can retain their ability to produce alkaloids *in vitro*.

MATERIALS AND METHODS

I. Seed germination experiments:

Seeds of *R. stricta* were surface-sterilized by immersing for 10 min in 10-X diluted "FAS" solution (commercial bleach containing 0·6% active ingredient NaOCl), followed by 3 rinses in autoclaved deionized distilled water. Working within the confines of a laminar-air-flow hood, the seeds were aseptically transferred to culture tubes containing germination medium, at a rate of one seed per tube, and incubated in the dark at 27°C. Nutrient medium employed in this investigation consisted of Murashige & Skoog (1962) salts and the following (in mg/l): sucrose, 30,000; thiamine-HCl, 0·4; myoinositol, 100; and agar, 8,000. The pH of the medium was adjusted to 5·7 with 1N NaOH or HCl and dispensed into 25 × 150 mm culture tubes at a rate of 25 ml per tube. The tubes were capped with cotton plugs and sterilized by autoclaving for 15 min at 121°C and 1·04 kg/cm.

Preliminary results showed that seed germination rate was very low on this medium. Attempts were carried out to enhance seed germination. In one attempt the seed coat was removed from 50% of the cultures, and left intact in the remaining cultures. Similarly the effect of gibberellic acid (GA_3) on seed germination was tested at 0, 0·01, 0·1, 1 and 10 mg/l. GA_3 was added to the autoclaved media following cold sterilization through Nalgene funnel filters (0·45 μ pore size).

II. Callus initiation and organogenesis experiments:

In vitro-germinated seedlings were further employed to initiate callus tissue from different explants. Stem, leaf, root and cotyledonary segments were cultured on nutrient media supplemented with one of the auxins (indol-3-acefic acid) IAA or NAA (1-napthaleneacetic acid) (at concentrations of 0, 1, 3, 10, 30 and 100 mg/l), or 2,4-D (2,4-dichlorophenoxyacetic acid) (at 0, 0·01, 0.1, 1, 10 and 100 mg/l) levels. All cultures were incubated for 4 weeks in the dark at 27 ± 1°C.

Callus initiated from different explants was subcultured at 4 weeks interval on fresh media of the same composition and maintained as stock cultures. These cultures were further employed for manipulation of organ initiation. The effects of kinetin and benzyladenine (BA) in combination with either IAA (3 mg/l), 2,4-D (1 mg/l) or NAA (30 mg/l) were tested for their ability to induce organogenesis. In these experiments, 0·5 g of callus was used as initial inoculum Twenty cultures were initiated for each treatment, and all cultures were incubated at 16-h daily exposure to 1000 Lux Gro light and a constant temperature of 27 ± 1°C.

III. Thin layer chromatography of the alkaloids:

The alkaloidal contents of callus tissue, initiated from different explants and grown in different auxin-containing media, were extracted with 95% ethanol. The extract was filtered through filter paper and its volume was reduced to 5 ml in a flash evaporator. The extracts, were spotted on thin layer plates (20 × 20 cm) at a rate of 100 μl/spot, using disposable micropipette, Three solvent systems were used, namely chloroform: petroleum ether: methanol (70 : 10 : 20 v/v); butanol : acetic acid : water (63 : 1 : 27 v/v) and isopropanol : butanol : water (70 : 10 : 20 v/v). Migration was allowed to proceed for 15 cm from the base line. Initial identification

of the alkaloids was performed through observation of the spots under 530 and 254 nm UV light, followed by spraying with Dragendorff's reagent (Harborne 1973) to facilitate the location of the alkaloidal spots. Presence of alkaloids was further confirmed by Mayer's reagent (Martindale 1955).

RESULTS

I. Seed germination:

Seed germination was very low on minimal organics medium. Removal of the seed coat prior to culture enhanced the germination rate from 2·5% for those with intact seed coat to 37·5% for those with no seed coats. Similarly, inclusion of 10 mg/l GA_3 in the culture medium stimulated embryo germination under dark or light conditions (Table 1). Germination of such embryos was evident after 3 days, while it took 7–9 days in the absence of GA_3 or presence of the seed coat. These seedlings were employed to initiate callus from different explants.

II. Callus initiation and organogenesis:

Preliminary experiments indicated that IAA stimulated callus initiation in root segments, NAA in stem and leaf segments, and 2,4-D in cotyledons and leaf. The effect of IAA on callus initiation in root segments is shown in Fig. 1. A concentration of 1–3 mg/l IAA was optimal for callus initiation, where the callus had white creamy appearance. However, organogenic capability of this callus was restricted to root initiation, where different numbers of roots were initiated at various IAA concentrations (Table 2).

Profuse callus was initiated at the 30 mg/l NAA level in the excised stem, and at 10 mg/l level for leaf segments. Texture of such callus varied from friable at low

Table 1. Effect of GA_3 on embryo germination in *Rhazya stricta*. Twenty seeds per treatment. Data collected after 4 weeks in culture

GA_3 conc. (mg/l)	% germination	
	Light	Dark
0	2·5	10·0
0·01	22·5	22·5
0·1	10·0	10·0
1·0	22·5	28·5
10·0	30·0	37·5

Table 2. Effect of IAA on root initiation in *Rhazya stricta* root explants. Twenty cultures per treatment. Data collected after 4 weeks.

IAA conc. (mg/l)	No. root/culture (± SE)
0	3·9 ± 1·39
1	20·3 ± 3·32
3	8·8 ± 2·24
10	10·0 ± 2·99
30	4·0 ± 1·05
100	0·1 ± 0·1

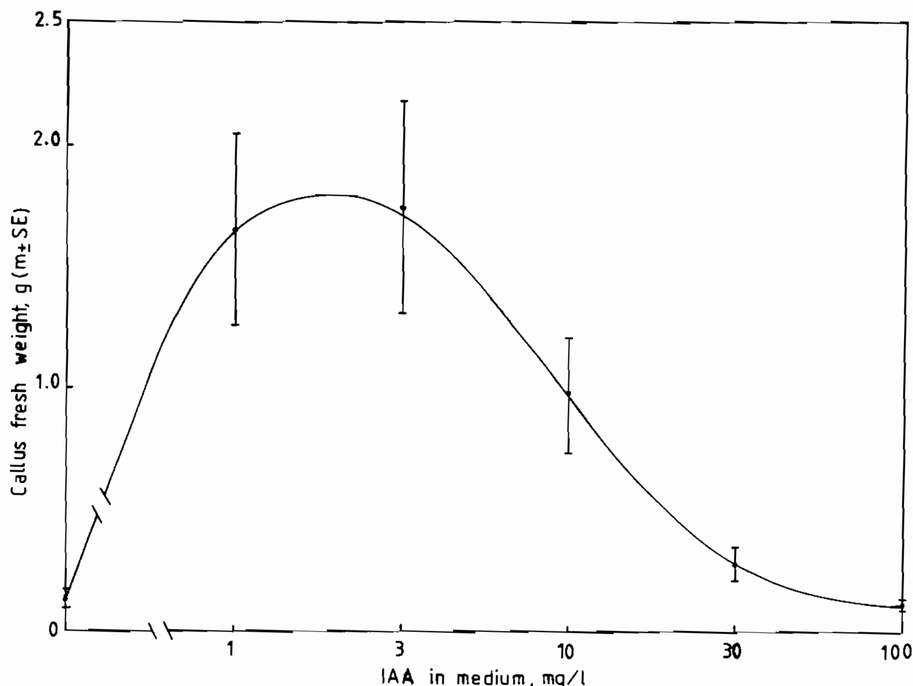

Fig. 1. Effect of indole-3-acetic acid on callus initiation in *Rhazya stricta* root segments.

auxin levels (1–10 mg/l) to firm at the higher concentrations. No organogenesis was observed in any culture. Callus initiation was also observed in the cotyledonary segments cultured on media supplemented with 0·1, 1 or 10 mg/l 2,4–D, with maximum callugenesis (callus initiation and development) at 0·1 mg/l level for cotyledonary segments, and 1 mg/l for leaf.

Presence of kinetin in the culture media was generally less effective than BA in inducing cell division as determined by fresh weight increase (Fig. 2A, B). Little, if any, change in response was observed when 2,4-D was included in the culture media instead of IAA. An increase in callus fresh weight seems to be obtained in the presence of 3 mg/l BA and the absence of NAA (Fig. 2A). Representative culture of stem-derived callus is shown in Fig. 3 which illustrates the intensity of callugenesis in *Rhazya.*

Plant regeneration was observed upon subculturing stem-derived callus, originally grown in a medium supplemented with 30 mg/l NAA, onto fresh medium of the same composition under dark condition. Such plants were initiated through adventitious bud formation in the cultured callus. Further plant propagation was carried out by culturing stem nodal segment on nutrient medium containing 2 mg/l kinetin and 0·1 mg/l NAA.

III. Thin layer chromatography of the alkaloids:

To determine whether cultured cells can retain their ability to synthesize the alkaloids, crude extracts of callus tissue were spotted on thin layer plates and chromato-

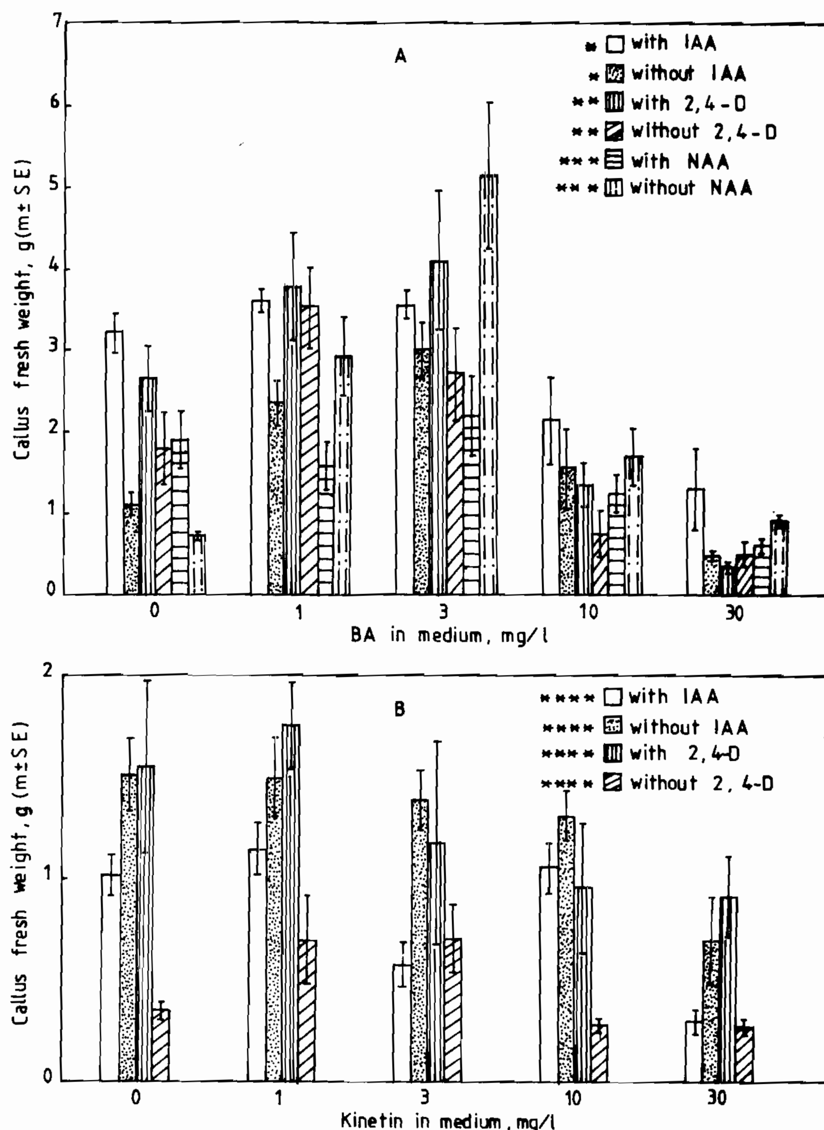

Fig. 2. Effect of different auxins and cytokinins on fresh weight of *R. stricta* callus: * root callus, *** stem callus, ** colyledonary callus, and **** leaf callus.

graphed in three different solvent systems. Colour of spots observed under both 530 nm and 254 nm UV, reaction to Dragendorff's reagent as well as Rf's of the detected spots are recorded in Table 3. Preliminary results suggested the presence of alkaloids as determined by Dragendorff's, ferric chloride-perchloric acid and Mayer's reagents. A thin layer chromatogram of stem, leaf, cotyledon and root calli is shown in Fig. 4. Alkaloidal contents of stem and leaf callus were nearly identical, although extra compounds were observed in leaf-derived callus. Cotyledonary callus showed the least alkaloidal content.

Fig. 3. Representative cultures of stem-derived callus.

Table 3. Colour reaction to Dragendorff's reagent and R values of *R. stricta* calli derived from different explants.

Compound No.	Colour under UV light		Response to Dragendorff's reagent				Rf
	530 nm	254 nm	stem callus	leaf callus	cotyledon callus	root callus	
1	light blue	bright blue	+	+	+	+	4·16
2	faint yellow	yellow	+	–	+	+ +	11·66
3	light blue	yellow	+	+	–	+ +	15·00
4	yellow	yellow	+	+	–	+ +	18·75
5	yellow	yellow	–	+	–	+ +	25·00
6	yellow	yellow	–	+	–	+ +	37·55
7	blue	bright blue	–	+	–	+	60·83
8	yellow	yellow	+	+	–	+	70·83
9	light blue	yellow	–	–	–	+ +	75·83
10	Blue	bright blue	–	–	–	+	81·66
11	yellow	yellow	–	–	–	+	91·66
12	yellow	yellow	–	–	–	+	98·33

\+ = Orange colour developed, + + = deep orange
– = No orange colour developed

DISCUSSION

In this investigation *R. stricta* seeds were aseptically germinated on nutrient media enriched with 10 mg/l GA_3. Seed coat removal also promoted embryo germination. This promotive effect may be discussed in relation to its content of alkaloids that might inhibit or reduce embryo viability. It should be noted that the alkaloidal

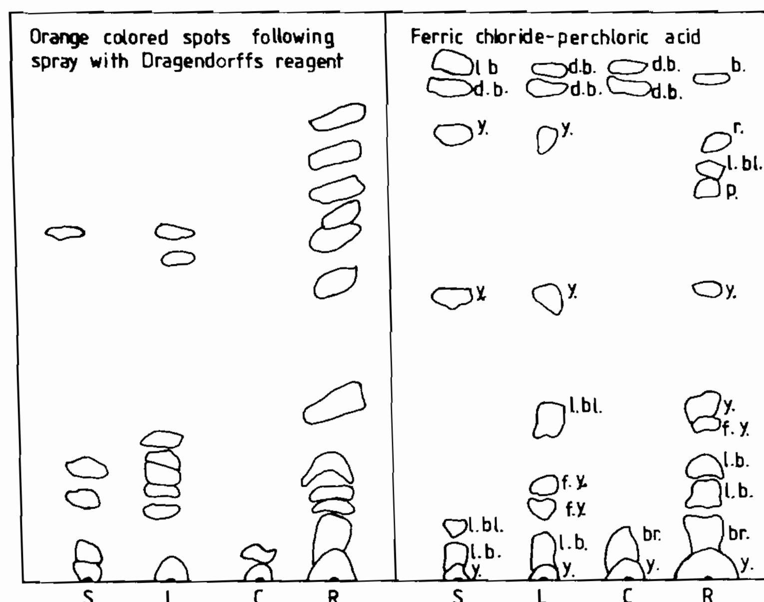

Fig. 4. Thin-layer chromatogram of extracts of *R. stricta* calli derived from various explants, on silica gel G in chloroform: petroleum ether : methanol (70 : 10 : 20 U/V) solvent system (S, stem; L, leaf; C, cotyledon; R, root); br.—brown; d.b.—dark brown; l.b.—light brown; f.y.—faint yellow; p.—purple; l.bl.—light blue; r.—red; y.—yellow.

content of this plant has been well documented (Chatterjee *et al.* 1974; Mukhopadhyay *et al.* 1981; Mariee 1983). Similarly, preliminary experiments in this investigation suggested the presence of alkaloids in calli derived from the cotyledons. Those alkaloids might accumulate in the vicinity of shoot and root apices, and prevent embryo germination.

Callus was initiated from leaf, stem, root and cotyledonary segments on nutrient media supplemented with specific auxins which are essential for callus initiation and development (Yeomann & Macleod 1978). Differences in fresh weight of callus may be explained by the fact that different auxins have different activities on callus induction (Murashige 1974).

High cytokinin to auxin ratio favours shoot formation, while root formation is usually regulated by low cytokinin to auxin ratio (Skoog & Miller 1957). Plant regeneration in medium containing NAA and cytokinin has been reported for *Brassica campestris* (Cheema & Sood 1984) and *Panax ginseng* (Kwang *et al.* 1982). In this research, inclusion of a cytokinin in the culture media was unnecessary for shoot formation, and shoots were initiated in a medium containing NAA alone. Multiplication of such shoots, however, required the inclusion of a cytokinin, i.e. 2 mg/l of kinetin, in the culture media.

Numerous plant extracts have been tested for their antitumour activity. Eight indole alkaloids have been isolated from *R. stricta* leaves and stems (Mariee 1983), of which 3 showed cytotoxic activity against KB carcinoma of nasopharynx *in vitro* (Mukhodhyay *et al.* 1981). In this investigation the presence of alkaloids in leaf and stem calli is suggested. Presence of alkaloids in the roots and cotyledon-derived callus has not been reported before.

This investigation has shown conclusively that the cells of *Rhazya stricta* are totipotent, and this has been expressed in their ability to initiate callus and plant regeneration. It was also disclosed that alkaloid biosynthesis has been retained by the cultured cells. Further investigation towards identification and testing the biological activity is in progress.

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زراعة أنسجة نبات اللويزة *Rhazya stricta* Decaisne

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خلاصة

تم استنبـات بـذور اللويـزة (*Rhazya stricta* Decaisne) في وسط غـذائي مجهـز بحـامض الجبريليك . لقد حفز الأوكسين إندول حامض الخليك (IAA) استحداث أنسجة الكالس في قِطَع الجذور المستأصلة إضافة إلى تكون الجذور بكميات كبيرة . اما النفثالين حامض الخليك (NAA) فقد شجع نشوء الكالس الطري في قِطَع الساق والأوراق ، في حين أدى الأوكسين ٢ و ٤ ثنائي الكلورفينوكسي حامض الخليك (2, 4-D) إلى نشوء الكالس في قِطَع الفلقـات والأوراق . وقد تكشفت أنسجة الكالس باستخدام تراكيز مختلفة لمنظمات نمو متعددة . اما إخلاف (تجدد) النبات فقد حدث في أنسجة الكالس المستحدثة من الساق النامية في وسط غذائي يحتوي على الـ NAA . كما دلت الدراسات الأولية على وجود القلويدات في مستخلص أنسجة الكالس .