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IN VITRO STUDIES ON *RAUWOLFIA* FOR MASS PROPAGATION AND ALKALOID SYNTHESIS

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Rauwolfia serpentina is a tropical plant of woody nature (family Apocynaceae). The plant is of great medicinal importance because of it alkaloidol used for the treatment of high blood pressure and as a tranquillizer. Propagation of *Rauwolfia* by seed is not satisfactory due to highly variable germination percentage (10-75 %). The plant can be propagated vegetatively.

For this purpose various organs such as root, leaf and stem were used to induce *callus* formation and then organogenesis. Plantlet regeneration has been achieved in root and stem calli by a combination of different growth hormones. For this different media viz. White, Murashige and Skoog, Abou-Mandour were utilized. Hormonal requirement differed with the explant source. A large number of plantlets can now be produced *in vitro* for further establishment under the natural conditions. Root, stem and leaf calli were analysed for *Rauwolfia* alkaloids. Ajmaline was the major alkaloid produced by cultures. Alkaloids in such plants were higher in leaf and stem cultures than the parent plant.

Key words: Rauwolfia, Micropropagation, Ajmaline.

INTRODUCTION

Rauwolfia serpentina Benth have attracted worldwide attention and has been used for centuries in the ayurvedic and unani systems of medicines in South Asia. However, only during the last three decades, the importance of this medicinal plant and its therapeutically most important alkaloid, reserpine was isolated [1]. It is recognised for treatment of hypertension and as a sedative or tranquilizing agent in the allopathy. Its roots are used as raw material for the extraction of alkaloids like reserpine, rescinnamine, ajmaline and ajmalicine etc.

The roots used in our study were collected from moist deciduous forests ranging from sea level to 1200 m in India, Bangladesh, Sri Lanka, Burma, Malaya, Thailand and Java.

Propagation of plant by means of seed is unsatisfactory, due to unfavourable climatic conditions, like varying temperature and humidity prevailing during the months of July and August which cause shrivelling and desiccation of the endosperm. This hinders the development embryos and causes the formation of non-viable seeds in large number. The germination percentage of viable seeds is also highly variable from 10 to 75 [2].

The culture techniques are now used as an alternative mechanism for the asexual propagation of various plants [3-11]. Moreover the development of plant tissue culture techniques have allowed the cultivation [12-15] and exploration of their biosynthetic capabilities [16-24].

In the present report, the results of *in vitro* propagation of *R. serpentina* and potential of cultures for *Rauwolfia* alkaloids production are discussed.

EXPERIMENTAL

Plant material for these studies was obtained from *Rauwolfia* plants growing at PCSIR Labs Peshawar.

Culture media used were Murashige & Skoog [25], White's Root Culture medium [26] and Abou-Mandour [27].

Nutritional additives were coconut milk, casein hydrolysate and yeast extract. Coconut milk was obtained from unripe coconuts and processed according to Gamborg and Wetter [28]. These nutrients and growth hormones viz. NAA, IAA, IBA, BAP, K, AS, were added to the culture media before autoclaving. The pH of the media was adjusted to 5.6 with 1N NaOH.

Chemical analyses were carried out on freeze dried callus samples, extracted with equal quantity of methanol and chloroform. Acetomethanol-acetic acid (70:25:5) was used as solvent system for TLC.

Calculations were done on Scanner-Zeiss Chromatogram calculating apparatus. The wave length used was

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Abbreviations.

NAA = Naphathalene acetic acid; IBA = Indole butyric acid; K = Kinetin; IAA = Indole acetic acid; BAP = Benzyl amino purine; AS = Adenine sulphate.

290, 306 and 280 nm respectively for ajmaline, serpentine and raubasine.

Cultural conditions were set at 28 ± 1° and 1000 lux, 16 h photoperiod. Light intensity for plantlets was set between 2500 to 3000 lux. Knop's solution [29] was employed for irrigating the potted plantlets.

RESULTS AND DISCUSSION

A. *Propagation.* Propagation of *Rauwolfia serpentina* by seed is difficult due to the formation of a large number of non-viable seeds [2]. Propagation by vegetative methods is reported by root cuttings, root stumps and stem cuttings. The high percentage of successful plants obtained by root cuttings makes it preferable to propagation by the other two methods [30]. The growers naturally do not wish to divert roots for propagation as they are demanded for their contents.

Rooting of buds, was tried with little success [31] with only 10 percent rooting was obtained. However multiplication of cultured buds was noticed *in vitro* with 1 mg/l IAA and 10 mg/l BAP. This caused to expand our studies and the method was further improved by regeneration of buds in callus cultures, their multiplication [32] and rooting of individual component buds of each multiple aggregate.

Regeneration of buds for *R. serpentina* in leaf callus is known [33]. We obtained bud regeneration in stem [34] and root callus [35]. Regeneration of buds was 20 % for stem and 30 % for root callus. Stem callus regenerated buds gave spontaneous rooting while root callus buds were induced to root with 3 mg/l IAA and IBA. Success in root induction in root callus buds varied between 20 to 50 %. Shoot development in stem callus buds was 100 % while in root callus buds it was observed to be 42.85 %. After the development of root and shoot, the buds developed into autotrophic plantlets.

Plantlets were "thereafter" transferred to soil and produced flowers and fruits. These seeds produced normal seedlings when subjected to favourable germination conditions. Germination was 25 % for seeds obtained from plantlets regenerated in stem callus as compared to 10 % with cultivated plants (Table 1).

The basic guidelines indicated by these methods of propagation could be utilized. The results obtained with static cultures are a starting point for suspensions, leading to somatic embryogenesis with hormonal manipulations, which will in turn provide a required bulk of soil transferable plants of *R. serpentina* alongwith larger quantity of gel-enclosed embryos or "synthetic seeds" with 99 %

Table 1. Seed viability of seeds obtained from seed raised and stem callus differentiated plants of *Rauwolfia serpentina*, under similar growth conditions.

	Germination (%)		Average size of seedling (epicotyl) (mm)	
Period (days)	Seed raised	Callus differentiated	Seed raised	Callus differentiated
11	10	20	nc	nc
18	10	25	17	27
20	10	25	29	63
25	10	25	47	75

nc – not calculated.

viability and without loss of viability during storage, normally met with old seeds of *R. serpentina* under agricultural practice.

B. *Biosynthesis of alkaloids.* Gallus tissue of *R. serpentina* from leaf, stem and root were subjected to screening for 5 important alkaloids. Reserpine and raupine were found in traces only. Serpentine, ajmaline and raubasine were ascertained quantitatively on a dry weight basis.

The major alkaloid found was ajmaline. It was estimated at 0.0410, 0.0585 and 0.0573 % for leaf, stem and root callus respectively. The percentage of ajmaline for stem callus was 277.4 more* than that found in stems of cultivated plants, whereas in leaves of cultivated plants ajmaline was found in traces. Contrarily for root callus the quantity of ajmaline observed in cultures was a bit lower than in cultivated plants.

A comparative picture of the presence of alkaloids in various tissue is shown in Table 2. The results obtained are very encouraging. In tissue cultures, the known alkaloids are reported as altogether absent or when present are in traces [18]. The behaviour is well reasoned and documen-

Table 2. Presence of alkaloids in plant and leaf, stem and root cultures of *Rauwolfia serpentina.* Age of calli at the time of analyses was 12 months.

Alkaloids (%)	Leaf		Stem		Root	
	Plant	Callus	Plant	Callus	Plant	Callus
1. Ajmaline	+	0.0410	0.0155	0.0585	0.0642	0.0546
2. Serpentine	+	0.00735	ne	0.00777	0.1252	0.0121
3. Raubasine	–	0.0132	0.01419	0.00908	0.0536	0.0175
4. Reserpine	ne	–	–	+	+	–
5. Raupine	ne	–	+	+	++	+

ne = not evaluated; – = nil; + = negligible; ++ = better than +.

ted by various researchers [36-39]. Although secondary metabolites may be produced in large quantities by cultures but the type of compound produced is often unexpected or even novel and specific compounds of the species might not be produced at all [40]. Stockigt *et al.* were able to screen 10 previously not reported alkaloids from cell cultures of *R. serpentina.* The yield vomilenine was 51 times higher in cultures than that found in differentiated plants.

This does not necessarily imply that best known plant products or particular secondary metabolites can only be produced by organised cell system but suggests that cells growing *in vitro* under arbitrary conditions do not always produce a characteristic secondary metabolites, particularly when reverted to a more basic mode of existence of replicative growth with conditions approaching favourable for growth of microorganisms.

However, now techniques are available to modify and Improve the biosynthetic potential of cultured plant cells. In some plant species, cultures contain higher contents of secondary metabolites than intact plants e.g. pseudophedrine from *Ephedra foliata* is much higher (2.25 %) in suspension than that in stem [41]. Production of diosgenin in *Trigonella foenum-graecum* cultures was found at 1.82 % by Khanna and Jain [42] as compared to seeds containing 1.6 %. By the use of 50 mg/100 ml cholesterol in culture media they were able to increase diosgenin content to 3.54 %. Solasodine was increased upto a maximum of 680 ug/g dry weight with 70 mg/100 ml cholesterol in cultures of *Solanum xanthocarpum* [43]. Feeding of ascorbic acid to cultures of *Tagetes erecta* increased pyrethrin contents [44]. Similarly, Khanna *et al.* [45] were able to increase the *in vitro* production of major opium alkaloids with 12.5 mg/100 ml tyrosine.

In *R. serpentina* feeding experiments are not known except nutritional additives as coconut milk [46] or yeast extract [47]. In our results, the activity of casein hydrolysate (CH) was observed in root callus cultures. Nevertheless, the cultures produced 96.23 % more ajmaline in dark when compared to light grown cultures [48]. Therefore we believe sereening of new alkaloids in *R. serpentina* cultures. Our presumption is well supported by the work of Stockigt *et al.* [40] and Schubel and Stockigt [49].

Recently, a rather new technique, using enzyme systems for regulating biosynthetic potentials in *Rauwolfia* cultures is given by Stockigt [50] and Pfitzner *et al.* [51].

With these techniques and improvements, plant tissue and cell culture systems could plays an important role in the manufacture of biological compounds of therapeutic value.

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