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DEVELOPMENT OF SUSPENSION CULTURES AND EX-VITRO ROOTING IN *RAUWOLFIA SERPENTINA* FOR RAPID AND LARGE SCALE MULTIPLICATION

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ABSTRACT

Rauwolfia serpentina belonging to the family Apocynaceae is native to the Indian subcontinent and East Asia. It is used medicinally, both in conventional western medicine and in Ayurveda, Unani, and folk medicine due to the presence of a number of bioactive chemicals, including yohimbine, reserpine, ajmaline, deserpidine, rescinnamine and serpentinine. It possesses antimicrobial, antifungal, anti-inflammatory, antiproliferative, antidiuretic, anticholinergic activities, reduces blood pressure, depresses the activity of the central nervous system and also exhibits activity against drug-resistant tumor cells. We have used liquid MS media with 10 different concentration of growth hormones combinations such as IBA, BAP, KN, NAA, 2,4-D and TDZ for establishing an axenic culture in suspension. Five different media combinations having different concentrations of IBA, BAP, KN, NAA, 2, 4-D and TDZ were used for shoot proliferation from shoot apex. The result showed that liquid MS media supplemented with IBA(3mg/l) and KN (1mg/l) was found best for rapid shoots growth, whereas, liquid MS media supplemented with BAP(2mg/l) + IBA(2mg/l) + KN(1mg/l) was found best for increasing the number of shoots in 8-10 days under optimized tissue culture conditions. Ex-vitro rooting was carried out from in-vitro grown shoots in different potting mixtures under glass house conditions within 10 days of plantation with 95% of survival rate. This technique can be further utilized for carrying out rapid multiplication of this endangered medicinally important plant so it could prospect for medicinally important secondary metabolites.

Keywords: Ex-vitro rooting, Micropropagation, Rauwolfia serpentina, Sarpgangha, Suspension culture.

I. INTRODUCTION

Belonging to Apocynaceae family, *Rauwolfia serpentina* is an evergreen, short, woody, glabrous, and perennial shrub, bearing white or pinkish flowers. It has long, tuberous, tapering, crooked roots, with pale brown cork and irregular with zig-zag nodes which contain most of the alkaloids [1-2]. It is an important endangered medicinal plant that originated in South Asia (basically in the habitats of tropical and sub-tropical regions) and can attain a

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maximum height up to 60cm [3]. The plant possesses tuberous root with pale brown cork and elliptic to lanceolate or obovate leaves in whorls of three [4].It is commonly called as Black Indian snakeroot or devil pepper. According to the common folklores about this plant, it can serve as an antidote for snake poison. It has been reported to be used in different parts of India, Bangladesh, Nepal and Bhutan [5].

In India, this plant is distributed from the east of Punjab to Sikkim, and Assam, in the lower hills of the Gangetic plains, some parts of central India, Eastern Ghats and Western Ghats and in the Andaman Islands, It is also found in the foothills of Himalayan range, up to the elevation of 1300-1400 m [6].The natural reserves of this plant are declining, especially after reports of its medicinal properties appeared in the literature. International Union for the Conservation of Nature and Natural Resources (IUCN) has kept this plant under endangered status [7].

R. serpentina is known for its antifungal, anti-inflammatory, antioxidant, antiproliferative, anticancerous, antidiuretic, anti fibrillar, antiarrhythmic, anticholinergic, antidysenteric, antidiarrhoeal antihypotensive, anticontractile, antidiuretic, sympathomimetic, and tranquilizing agent[8-13].The roots, leaves and juice are of medicinal importance and have attracted the attention of practitioners of the indigenous system of medicine, as it contains a large number of secondary metabolites localized mainly in the roots and rhizomes[14]. The major alkaloids are ajmaline, ajmalicine, ajmalimine, deserpidine, indobine, indobinine, reserpine, reserpiline, rescinnamine, rescinnamidine, serpentine, serpentinine and yohimbine[15,16].Reserpine is the most prominent of all alkaloids and used mainly as a natural tranquillizer [17,18]. The antihypertensive actions of reserpine are due to its depressant action on central nervous system (CNS) and peripheral nervous system [19-21]. These substances are mostly involved in controlling heart rate, cardiac contraction and peripheral resistance. It also helps in sedation and lowering of blood pressure, especially in cases of hypertension exacerbated by stress and sympathetic nervous system activity.

In Ayurvedic medicines, the roots of *R. serpentina* is used as a remedy for curing hypertension, insomnia, mental agitation, gastrointestinal disorders, excitement, epilepsy, traumas, anxiety, excitement, schizophrenia, sedative insomnia and insanity[22].In Siddha medicine, *R. serpentina* roots are used for curing hypertension-associated headache, dizziness, amenorrhea, oligomenorrhea and dysmenorrhea like abnormalities.

Rauwolfia sp. is in threatened status in India due to over exploitation for commercial purposes to meet the requirements of pharmaceutical industry. A high diversity within population and high genetic differentiation among them based on RAPDs were revealed caused both by habitat fragmentation of the low size of most populations and the low level of gene flow among them [23].Seed germination in *Rauwolfia sp.* is highly variable, and it is reported to vary from 5 to 30 percent even when only heavy seeds are chosen for sowing purpose. Poor seed viability, low seed germination rate, and enormous genetic variability are the major constraints for the commercial cultivation of *R. serpentina* through conventional modewhichis forcing the farmers to use cuttings for propagation. The high genetic diversity observed in this otherwise self-pollinated plant as reported by (Padmesh Pandaram Pillai, 2011) entails the need for evolving effective *ex-situ* strategy for long-term conservation of this medicinal herb [24].As the plant takes a long time to grow via conventional methods, the supply of the alkaloids does not meet its demand .So to eradicate this problem, production of alkaloids must be coupled with some modern technique of micropropagation. In this study we developed suspension cultures for large scale shoot multiplication and ex-vitro rooting for rapid production of plantlets.

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II. MATERIAL AND METHODS

2. 1. Collection of Plant materials

Field grown plants of *Rauwolfia serpentina* was collected from Himalayan Forest Research Institute (HFRI), Panthaghati, Shimla and maintained in glasshouse of the Department of Biotechnology and Bioinformatics, JUIT, Waknaghat.

2. 2. Media preparation and culture conditions

Murashige and Skoog liquid media, with 10 different combinations and concentrations of plant growth hormones including IBA, BAP, KN and NAA were prepared for establishment of axenic cultures from shoot apices and in-vitro multiplication of micro shoots (Table 1).Sucrose (30 g/l) was added to liquid MS media containing plant growth hormone and pH was set between 5.6-5.8. Liquid media were autoclaved for 20 minutes at 121°C and 15 psi. Cultures were incubated in a plant growth chamber maintained at 25 ± 1°C under a16 h day/8 h night photoperiod with illumination of 3 klux intensity of white light, with 60-70% of relative humidity.

Table 1: Composition and concentrations of different media used.

S.No.	Medium Name	Media with Growth Hormones supplemented
1	RS1	MS
2	RS2	MS + IBA (3 mg/l)+ KN (1 mg/l)
3	RS3	MS + IBA(1 mg/l)+ KN (3 mg/l)
4	RS4	MS + IBA (2 mg/l) + KN (1 mg/l) + BAP(2 mg/l)
5	RS5	MS +l IBA (3 mg/) + KN (2 mg/l) + BAP(2 mg/l)
6	RS6	MS + IBA (1 mg/l) + BAP (2 mg/l)
7	RS7	0.5 MS + IBA (2 mg/l) + TDZ (1 mg/l)
8	RS8	0.5 MS + IBA (3 mg/l) + BAP (1 mg/l) + KN (1mg/l)
9	RS9	0.5 MS + IBA (2 mg/l) + NAA (0.5 mg/l)
10	RS10	0.5 MS + 2,4-D (0.5mg/l) + IBA (1 mg/l)

2. 3. Selection and culture of explants

Shoot apices from the mother plant were used as explant and were washed under running tap water by using few drops of Labolene. These were further surface sterilized by using fungicide Bavistin (0.5%) and mercuric chloride (0.1%) followed by 4 - 5 washings in autoclaved water under aseptic conditions. Shoot apices were used to establish axenic cultures using liquid MS media with different combinations of plant growth hormones including IBA, BAP, KN and NAA (Table 1.) under above mentioned culture conditions.

2. 4. Shoot multiplication

Micro shoots were cut and cultured on different liquid media combinations (Table 1) under above mentioned conditions. The data on days for shooting, average shoot numbers and shoot length (cm) were recorded. These experiments were performed in triplicates and repeated thrice.

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2. 5. Ex vitro rooting and hardening

Multiplicated shoots were transferred to various potting mixtures containing different combinations of soil: sand: vermiculite while maintaining 80 – 90% humidity by using glass jars/perforated polythene under natural light source in the greenhouse (Table 2). The plantlets were transferred to pots and were covered with glass jars/perforated polyethene bags for 10–15 days to avoid desiccation. The glass jars were taken off every day for 1–2 h so as to acclimatize the plantlets to the external environment. The data on days for rooting and percentage of survival rate were recorded.

Table 2: Composition of different potting mixtures used.

S.No.	Potting mixtures name	Composition of potting mixtures
1	PM1	Soil
2	PM2	Soil: Sand (1:1)
3	PM3	Vermiculite
4	PM4	Soil: Vermiculite (1:1)
5	PM5	Soil: Sand: Vermiculite (1:1:1)

III. RESULTS

3. 1. Shoot multiplication

The micro shoots multiplicated within 8 to 16 days of incubation in all the mentioned liquid media (Table 1). Maximum number of shoots occurred on liquid MS medium supplemented with IBA (2 mg/L) + BAP (2 mg/L) + KN (1 mg/ml) within 10 to 12 days with 13.8 shoot length while liquid MS medium supplemented with IBA (3 mg/L) + KN (1mg/L) was found best for rapid growth with increased internodal length and leaf size within 9-10 of incubation in liquid medium with 13.8 shoot length (Table 3).

Table 3: Effect of different media used on shoot multiplication of *R. serpentina.*

S.No.	Medium Name	Days for Shooting	Number of shoots	Shoot length (cm)
1	RS1	25-26	5-6	2.3
2	RS2	8-10	20-21	13.8
3	RS3	10-12	18-20	11.2
4	RS4	10-12	22-24	13.8
5	RS5	12-16	15-18	10.2
6	RS6	11-12	7-8	7.6
7	RS7	12-14	5-6	6.5
8	RS8	14-16	4-5	2
9	RS9	12-14	4-5	4.5
10	RS10	18-20	3-4	2.4

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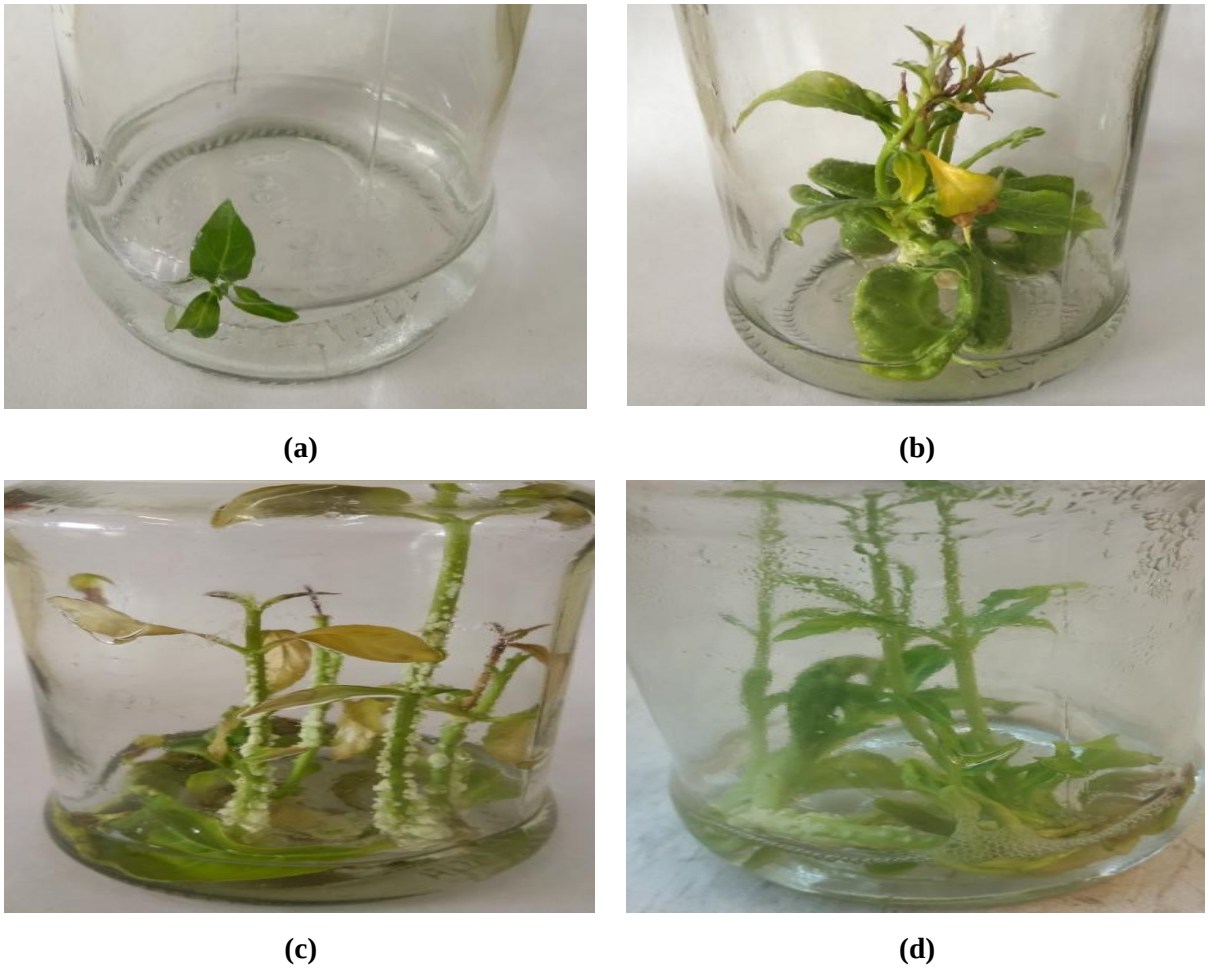

(a) **(b)**

(c) **(d)**

Figure 1: Effect of various growth hormone combination and concentration on shoot growth and multiplication *R. Serpentine.* (a) A micro shoot cultured (b) Shoot multiplication (5-6 days) (c) Shoot multiplication (9-10 days) (d) Shoot multiplication (13-15 days)

3. 2. Ex vitro rooting and hardening

Multiplicated shoots transferred to various potting mixtures developed roots in 10-20 days with a survival rate of 74 – 98% in different combinations. Potting mixture containing Soil: Sand: Vermiculite (1:1:1) developed roots in 10 days with a survival rate of 98% (Table 4).

Table 4: Effect of different potting mixtures used for ex-vitro rooting of *R. serpentina*

S.No.	Potting mixtures name	Composition of Potting mixtures	Days for rooting	Survival rate (%)
1	PM1	Soil	20	74
2	PM2	Soil: Sand (1:1)	15	86
3	PM3	Vermiculite	18	81
4	PM4	Soil: Vermiculite (1:1)	14	92
5	PM5	Soil: Sand: Vermiculite (1:1:1)	10	98

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(a)

(b)

(c)

Figure 2: Effect of different media on plant growth (a) Hardening of in vitro grown shoots in greenhouse conditions. (b) Ex-vitro rooting after one week during hardening (c) Ex-vitro rooting after two weeks during hardening.

4. Discussion

This study was conducted to develop a protocol for multiplication of *Rauwolfia Serpentina* shoots in suspension cultures and its ex-vitro rooting. We have tested liquid MS media with 10 different combinations of plant growth hormones to carry out clonal propagation of the plant. MS medium supplemented with IBA (3 mg/L) + KN (1mg/L) was found to be superlative for rapid shoot growth with 13.8 cm of shoot height in 8-10 days. Similar media, IBA (3 mg/L) + KN (1mg/L) was found best for the rapid growth of *Jatropha curcas* [25]. MS medium supplemented with IBA (2 mg/L) + BAP (2 mg/L) + KN (1 mg/ml) was found best for multiple shooting within 10 to 12 days with 13.8 shoot length. Similarly, MS medium supplimented with BAP and KN leads to shoot multiplication in *Rhodiola imbricata* [26]. The use of BAP for multiple shooting in *R. serpentina* was reported by earlier [15] with auxin to cytokinin ratio of 10:1 in their media with MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA.

Ex-vitro rooting of *R. serpentina* was earlier reported in which rooting was accomplished by supplementing the potting mixture with IBA (100 mg/l) [27] whereas, in this study, ex-vitro rooting is achieved without

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supplementing the potting mixture with any plant growth regulators along with 98% of survival rate in potting mixture containing Soil: Sand: Vermiculite (1:1:1) during hardening.

As reported earlier different combination of auxins were tested for rooting in different time intervals and after that spending 4-5 weeks for hardening. Through this study we not only save media, growth hormones but also time period of 4-6 weeks which could be of high significance of mass multiplication of this endangered herb.

V. CONCLUSION

The current study describes the rapid in-vitro mass-production protocol for *Rauwolfia serpentine* in liquid media which could be of high practical importance for meeting the demand of various pharmaceutical companies in a very cost effective and short time interval. The optimized in vitro conditions for plantlet development can provide a base for further genetic transformation studies for increased phytopharmaceuticals production and its sustainable conservation.

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