International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Optimising Plantlet Regeneration by Stem Node Explant Culture in *Rauvolfia Micrantha* Hook. F, A Vital and Endangered Medicinal Plant

Rajesham P.[1], Rama Devi B.[2]

Department of Botany Osmania University, Hyderabad-50001
Corresponding Author Email: *rama.81379[at]gmail.com*

Abstract: ***Rauvolfia micrantha Hook. f. has garnered significant attention within the biomedical industry due to its efficacy in reducing blood pressure, primarily attributed to the presence of reserpine in the oleoresin extracted from its roots. However, the commercial cultivation of R. micrantha faces challenges such as poor seed viability, low germination rates, and considerable genetic variation. This study presents an optimized protocol for establishing aseptic cultures using a semisolid nutrient culture medium to produce plantlets. Stem node explants of R. micrantha were cultured on MS medium with varying concentrations of cytokinins TDZ/BAP/KN (0.5-8.0 mg/L). The highest proliferation of shoot buds was observed at 6.0 mg/L TDZ, with diminished efficacy at concentrations exceeding this threshold. Moreover, incorporating (2.0 mg/L) NAA in the MS medium enhanced root formation in the laboratory-grown shoots. Following successful cultivation, the plants derived from nodal cultures were transferred to pots with garden soil and placed in shaded outdoor conditions, mirroring suitable temperature and light levels. Remarkably, under glasshouse or field conditions, 90–95% of lab-grown plants exhibited survival, with 85% maintaining a consistent phenotype. After 8 weeks in their new environment, these plants blossomed, underscoring the robustness of the established protocol. The methodology outlined herein offers a rapid and reliable means to propagate true-to-type R. micrantha plants, addressing the challenges associated with traditional cultivation methods. This advancement holds promise for supporting the sustainable production of R. micrantha for its biomedical applications.***

Keywords: *Rauvolfia micrantha*, stem node Reserpine, Genetic variation, Aseptic cultures, Cytokinin, Shoot buds, and Root formation

1. Introduction

Rauvolfia micrantha Hook. f. (Apocynaceae) is a perennial woody shrub native to the Tinnevelly and Travancore hills of the Western Ghats in southern India, typically found at an elevation of 600 meters. The roots of this plant contain notable alkaloids, such as ajmalicine, reserpine, sarpagine, reserpine, and serpentine, known for their antihypertensive, hypertensive, and tranquilizer properties (Anonymous, 1969). Notably, *R. micrantha* has been recognized as an alternative to *Rauvolfia serpentina* roots in business lots supplied to American buyers (Youngken, 1954).

In traditional Indian medicine (Ayurveda), *R. micrantha* is employed as a substitute for R. serpentina in treating various nerve ailments, particularly in the Kerala region (Sahu, 1979). The plant is considered rare and unique to the southern woodlands of the Western Ghats (Sahu, 1979). Unfortunately, factors such as endemic habitats, limited range, small populations in accessible locations, and anthropogenic pressures on forestlands have led to the decline of *R. micrantha* in the wild. Conventional multiplication methods suffer from issues like poor seed viability, limited germination, and the slow development of roots in seedlings and vegetative cuttings.

Given the imperative need for conservation, in vitro multiplication techniques, specifically shoot tip and nodal explant cultures, have been explored (Sudha and Seeni, 1996). Plant tissue culture, a well-established biotechnological method, has proven instrumental for the rapid growth of medicinal plants for commercialization, conservation (Nadeem *et al.,* 2000), and cryopreservation (Kitto, 1997) (Decruse *et al.,* 1999). Clonal proliferation in medicinal plant species, including *Rauvolfia*, has been achieved through somatic embryogenesis and organogenesis (Gary and Brent, 1986). Notably, somatic embryogenesis has enhanced plant production in terms of both quantity and quality of secondary metabolites (Gastaldo *et al.,* 1994).

In vitro regeneration of *Rauvolfia* species has primarily been achieved through organogenesis (Sarker *et al.,* 1996; Sudha and Seeni, 1996; Patil and Jayanthi, 1997) and, rarely, via somatic embryogenesis in *Rauvolfia vomitoria* (Tremouillaux- Guiller and Chenieux, 1991) and *Rauvolfia caffra* (Upadhyay *et al.,* 1992). Embryogenesis typically utilizes plant tissues such as cotyledons, hypocotyls, leaves, or internodes, with roots being rarely employed (Vuorela *et al.,* 1993).

Root segment culture, involving organogenesis and somatic embryogenesis, is particularly valuable for genetic transformation experiments with *Agrobacterium rhizogenes*, and it serves as an experimental platform for studying cell differentiation and the regulatory mechanisms of totipotency in plant cells. Additionally, plants regenerated from root segments are considered genetically homogeneous (Sharma *et al.,* 1993). Chaturvedi *et al.* (1981) suggest that root culture can be employed for the germplasm preservation of various plant species, including *R. serpentiana*.

This recent research outlines a micropropagation method for *R. micrantha* involving the cultivation of stem node explants as the source for the direct generation of numerous shoots. This approach holds promise for efficiently propagating and conserving *R. micrantha* while addressing the challenges associated with traditional cultivation methods.

2. Materials and Methods

The present study introduces a novel approach to the micropropagation of *R. micrantha* by utilizing stem node segments (1.0 cm to 2.5 cm) with young branches. The significance lies in the meticulous sourcing of plant material from a healthy, one-year-old specimen cultivated in the research field of the Department of Botany at Osmania University in Hyderabad.

To ensure the establishment of aseptic cultures, the stem node segments underwent a systematic sterilization process. Initially, the cuttings were washed thoroughly with running tap water, followed by a 5-minute treatment with 5% teapot solution. Subsequently, external sterilization was performed using 0.1% w/v Mercuric chloride ($HgCl_2$) for 4-5 minutes, followed by rigorous rinsing with sterile distilled water at least three times. This multi-step sterilization protocol is a crucial element of the methodology, ensuring the removal of contaminants and the creation of a sterile environment for subsequent culture initiation.

One notable aspect of the methodology is the careful drying of sterile node pieces on sterile filter paper before inoculation. This step ensures the elimination of excess moisture, contributing to the prevention of potential contamination during the inoculation process. This attention to detail in handling and preparing the explants adds a layer of precision to the protocol, enhancing the chances of successful micropropagation.

Culture media and culture conditions:
The selected explants were introduced into a Murashige and Skoog (MS) medium enriched with 30 gm/L of sucrose and fortified with varying concentrations of cytokinins, namely Thidiazuron (TDZ), Benzylaminopurine (BAP), and Kinetin (KN), in combination with Indole-3-acetic acid (IAA) as detailed in Table-1. To solidify the medium, 0.8% agar (Difcobacto) was incorporated. Before the addition of agar, the pH of the medium was adjusted to 5.8, and subsequently, the containers were autoclaved at 121°C under 15 pounds per square inch for 15–20 minutes. This stringent autoclaving process ensures the elimination of any potential contaminants, establishing a sterile environment crucial for successful micropropagation.

For the differentiation of explants, media formulations were employed: MS+BAP/KN/TDZ. The culture tubes containing these media were maintained at a temperature of 25°C and 20°C above room temperature, complemented by a photoperiod of 16 hours under white, fluorescent light (40-50). This carefully controlled environment provides the optimal conditions for the initiation and development of axillary shoots.

Following six weeks of cultivation, the axillary shoots that demonstrated successful multiplication were transferred to a specialized medium designed to induce root development. This phase marks a crucial transition in the micropropagation process, aiming to establish a robust root system in the cultured plantlets. The medium for root induction is tailored to facilitate the efficient and uniform development of roots, ensuring the viability and adaptability of the micro-propagated plantlets**.**

3. Results and Discussion

In successful micropropagation endeavors, the choice of explants plays a pivotal role. Auxiliary buds, due to their pre-existing meristem, are preferred as they readily develop into shoots while maintaining clonal fidelity. This approach has been successfully employed in various plant species, demonstrating its applicability and effectiveness. Notable examples include *Prosopis chilensis* (Caro *et al.,* 2002*), Ilex dumosa* (Luna *et al.,* 2003), *Spilanthes acmella* (Haw and Keng, 2003), *Mucuna pruriens* (Faisal *et al.,* 2006), and *Tylophora indica* (Faisal *et al.,* 2007).

In this study, stem nodal segments with auxiliary buds were selected as explants for multiple shoot induction and proliferation, as outlined in (Table 1). This choice aligns with the recognized success of using auxiliary bud cultures in various plant species for efficient micropropagation. The findings from axillary and nodal bud cultures, as presented in (Table-1), underscore the successful formation of numerous shoots and roots in *R. micrantha*. The experiments involving different hormonal combinations exhibited varied outcomes, reflecting the complex nature of plant responses to such stimuli.

Upon inoculation, the axillary buds exhibited activity within the first week, with noticeable new shoots, leaves, and internodes becoming distinguishable by the second and third weeks. The survival rate of *R. micrantha* explants, obtained from nodal segments of wild plants, exhibited significant variability depending on the season. This prompted a systematic exploration of the most suitable time of year for optimal culture development. The observations revealed that explants collected during August and October exhibited a shorter time for sprouting and a rapid expansion of shoot buds. This suggests a season-dependent influence on the success and efficiency of micropropagation in *R. micrantha*.

Effect of TDZ:
The investigation into the impact of Thidiazuron (TDZ) concentration on the micropropagation of *R. micrantha* revealed distinct patterns in culture response and shoot development. At (0.5 mg/L) TDZ, 48% of cultures responded, showcasing an average of (5.0 ± 0.32) shoot buds per explant with a corresponding shoot length of (5.0 ± 0.32). Progressing to (1.0 mg/L) TDZ, the response percentage increased to 52%, accompanied by an elevated average of (5.6 ± 0.32) shoot buds per explant and (5.8 ± 0.42) average shoot length. The trend continued, reaching an optimal response at 4.0 mg/L TDZ, where 72% of cultures responded, yielding an average of (7.0 ± 0.42) shoot buds per explant and (7.5 ± 0.52) average shoot length. Concentrations ranging from 2.0 mg/L to 6.0 mg/L also resulted in high responses, maintaining a percentage of responding cultures between 60% and 67%, with corresponding high average shoot bud numbers and lengths. However, at extreme concentrations of 8.0 mg/L, the response percentage and shoot characteristics exhibited a relative decline.

Table I: Analysis of plant growth regulators on *in vitro* multiple shoots induction from nodal explants of *R. micrantha* on MS medium with various concentrations of BAP, KIN, and TDZ after 8 weeks of culture

Growth hormone concentration (mg / L)	% Of cultures responding	The average number of shot buds/explant (S.E) *	The average number of shot lengths (S.E) *
BAP			
0.5	46	4.0 ± 0.32	3.0 ± 0.42
1.0	50	5.2 ± 0.35	3.2 ± 0.75
2.0	60	5.4 ± 0.32	5.4 ± 0.32
4.0	65	6.0 ± 0.32	6.2 ± 0.36
6.0	54	5.8 ± 0.36	5.2 ± 0.36
8.0	40	5.5 ± 0.23	4.5 ± 0.23
KIN			
0.5	42	4.3 ± 0.25	4.0 ± 0.85
1.0	46	5.6 ± 0.32	4.8 ± 0.32
2.0	52	6.2 ± 0.32	5.2 ± 0.32
4.0	64	7.0 ± 0.35	6.0 ± 0.35
6.0	68	5.3 ± 0.32	7.3 ± 0.32
8.0	57	4.2 ± 0.23	5.2 ± 0.23
TDZ			
0.5	48	5.0 ± 0.32	5.0 ± 0.32
1.0	52	5.6 ± 0.32	5.8 ± 0.42
2.0	67	6.0 ± 0.32	6.6 ± 0.62
4.0	72	7.0 ± 0.42	7.5 ± 0.52
6.0	60	8.0 ± 0.32	8.5 ± 0.42
8.0	54	6.8 ± 0.32	6.8 ± 0.72

Effect of BAP:

The investigation into the micropropagation of *R. micrantha* revealed a substantial impact of growth hormone concentration, specifically Benzylaminopurine (BAP), on the overall culture response, the average number of shoot buds per explant, and the length of shoots. Across the range of BAP concentrations tested, distinct patterns emerged. At 0.5 mg/L, 46% of cultures responded, with an average of (4.0 ± 0.32) shoot buds per explant and an average shoot length of (3.0 ± 0.42). As the BAP concentration increased to (4.0 mg/L), the response percentage peaked at 65%, accompanied by an increase in both the average number of shoot buds (6.0 ± 0.32) and shoot length (6.2 ± 0.36). Beyond this concentration, at 8.0 mg/L, the response percentage decreased to 40%, with a corresponding decline in the average number of shoot buds (5.5 ± 0.23) and shoot length (4.5 ± 0.23). The optimal BAP concentration for achieving the highest percentage of responding cultures and High shoot development was identified as approximately 4.0 mg/L. Moreover, adjustments within the range of 2.0 mg/L to 6.0 mg/L maintained High responses, emphasizing the critical role of precise hormone concentration in the micropropagation protocol. These findings underscore the importance of fine-tuning growth hormone levels for the successful *in vitro* culture of *R. micrantha*.

Effect of KIN:

The impact of Kinetin (KIN) concentration on the micropropagation of *R. micrantha* is evident from the presented data. At 0.5 mg/L KIN, 42% of cultures responded, demonstrating an average of (4.3 ± 0.25) shoot buds per explant and an average shoot length of (4.0 ± 0.85). Progressing to 1.0 mg/L KIN, the response percentage increased to 46%, with an elevated average of (5.6 ± 0.32) shoot buds per explant and (4.8 ± 0.32) average shoot length. The trend continued, reaching 52% of cultures responding at 2.0 mg/L KIN, showcasing an average of (6.2 ± 0.32) shoot buds per explant and (5.2 ± 0.32) average shoot length. A further increase in the response percentage was observed at 4.0 mg/L KIN, where 64% of cultures responded, with an average of (7.0 ± 0.35) shoot buds per explant and (6.0 ± 0.35) average shoot length. While the response percentage remained high at 6.0 mg/L KIN (68%), the average number of shoot buds decreased to (5.3 ± 0.32), but the average shoot length increased to (7.3 ± 0.32). At 8.0 mg/L KIN, the response percentage decreased to 57%, displaying an average of (4.2 ± 0.23) shoot buds per explant and (5.2 ± 0.23) average shoot length. (Fig-1)

***In Vitro* Rooting**:

The experiment involved transplanting fully developed and robust shoots of *R. micrantha* into MS media enriched with varying concentrations of NAA (0.5 to 4.0 mg/L). Remarkable histogenesis was observed at 3.0 mg/L NAA, resulting in 96% of plants producing an optimal 6.0 ± 0.32 roots per explant (Table 2). This concentration of NAA proved to be optimal for root production. After the rooted plantlets were removed from the culture media, they underwent acclimatization. The agar from the roots was washed off, and the plantings were transferred to poly pots filled with pre-soaked vermiculite. These pots were placed in a growth room with a temperature of 28^0C and a relative humidity of 70–80 percent for three weeks. Subsequently, they were moved to poly bags containing a mixture of soil, sand, and manure, and maintained in a shade house for an additional three weeks. During this period, the seedlings were watered with Hogland's solution every three days (Fig-1).

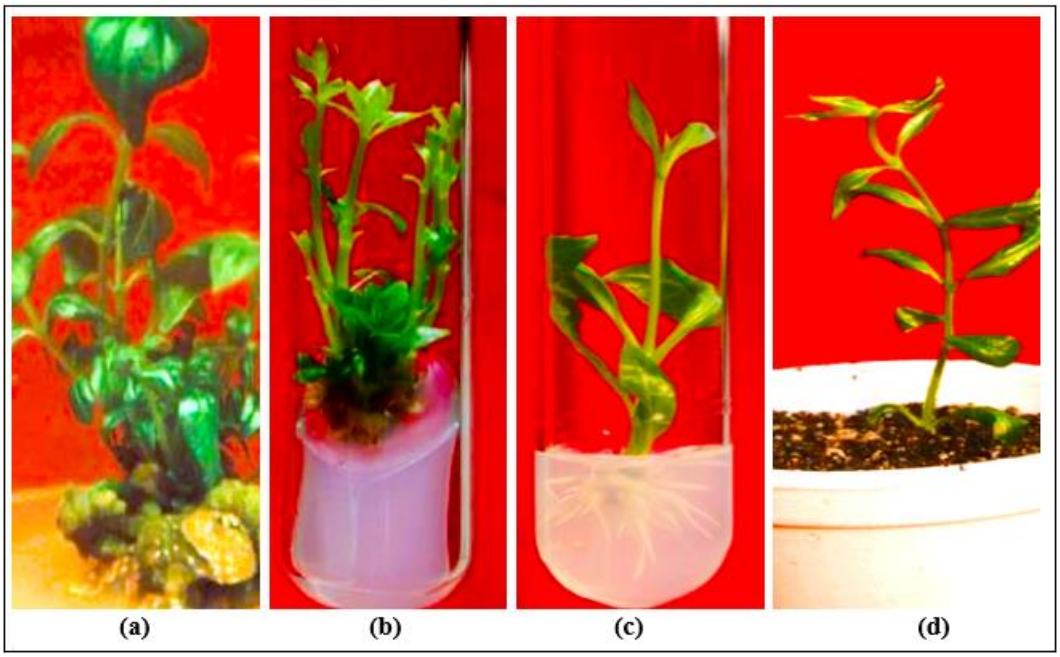

Figure 1: *In vitro* Plant let regeneration from stem node explant Culture and plant establishment of *R. micranthaa*) Direct multiple shoots developed on MS + 6.0 mg/L TDZ. b) Direct multiple shoots developed on MS+6.0 mg/L BAP) Rooting of individual micro shoots on MS+NAA (3.0mg/L) Fig. f) hardening of plantlet

Table 2: Evaluation of the impact of MS+ NAA (1.0-4.0mg/L) on the formation of roots from micro shoots of *R. micrantha* after four weeks

PGR concentration (mg / L)	% Of cultures responding	Mean number of roots/shoot (S.E) *	Mean root length (cm) shot lengths (S.E) *
NAA			
0.5	46	4.0 ± 0.32	3.0 ± 0.42
1.0	50	5.2 ± 0.35	3.2 ± 0.75
2.0	60	5.4 ± 0.32	5.4 ± 0.32
3.0	65	6.0 ± 0.32	6.2 ± 0.36
4.0	54	5.8 ± 0.36	5.2 ± 0.36

4. Discussion

Successful regeneration of shoots from nodal bud cultures was achieved on MS medium fortified with various cytokinins (TDZ, BAP, and Kn), with TDZ at 6.0 mg/L showing significantly greater shoot numbers compared to BAP/KIN at the same concentration. Higher concentrations of BA were not found suitable for optimal shoot regeneration. Similar findings were reported for shoot regeneration in various plants. The action of accumulating auxin at the basal cut ends, leading to the development of callus, was observed when BA concentration exceeded the ideal level. In conjunction with 2.0 mg/L TDZ, (0.5 mg/L) IAA + BAP (2.0 mg/L) was less effective in promoting shoot bud proliferation. Among all hormonal combinations and concentrations, BAP was the most effective in inducing greater plantlet regeneration. Nodal explants, especially immature cotyledonary nodes, played a crucial role in achieving high rates of plant regeneration.

The inclusion of NAA in MS medium facilitated rhizogenesis, with (3.0 mg/L) NAA yielding the highest number of roots (6.0 ± 0.32) and a 65% frequency of root development. Regenerated plantlets with fully formed shoots and roots were successfully acclimatized and transitioned to natural soil without any discernible changes. These findings align with previous research in the field (Singh *et al.,* 2006; Faisal *et al.,* 2006 and 2007).

5. Conclusion

In conclusion, the novel approach of micropropagation for *R. micrantha* utilizing stem node segments has proven to be successful. The careful selection of healthy plant material, systematic sterilization procedures, and meticulous attention to detail in explant handling contribute to the efficiency of the protocol. The choice of explants, specifically stem nodal segments with auxiliary buds, has demonstrated successful multiple-shoot induction and proliferation. The investigation into the impact of growth hormone concentrations, including TDZ), BAP and KIN, revealed distinct patterns in culture response and shoot development. Optimal concentrations for achieving high response percentages and high shoot characteristics were identified for each hormone. Notably, BAP exhibited effectiveness in inducing greater plantlet regeneration across various concentrations. Furthermore, successful *in vitro* rooting was achieved by transplanting shoots into a medium enriched with NAA, with the optimal concentration identified as 3.0 mg/L. The subsequent acclimatization process ensured the successful transition of plantlets to natural soil.

The study highlights the importance of the seasons on the efficacy of micropropagation in *R. micrantha*, finding August and October as ideal months for rapid development and metabolic activity. These findings stress the importance of paying close attention to exact hormone concentrations, careful explant selection, and environmental factors for optimal *R. micrantha* micropropagation. Overall, the study provides useful insights into plant tissue culture, setting the basis for future research and micropropagation procedures for *R. micrantha*.

References

[1] Anonymous. (1969). Pharmacognosy of Indigenous Drugs. (Vol. 1). Central Council for Research in Indian Medicine and Homoeopathy.

[2] Caro, L., Galleguillos, M., Barrientos, L., & Palma, J. (2002). Micropropagation of *Prosopis chilensis* (Mol.) Stuntz from young and mature plants. In Vitro Cellular & Developmental Biology - Plant, 38(5), 466-470.

[3] Chandra M and Pal A, (1995). Differential responses of two cotyledons of Vigna radiate L. In vitro Plant Cell Rep, 15 - 63-67

[4] Chaturvedi, H. C., Chandra, N., & Mohan, J. S. S. (1981). *In vitro* preservation of germplasm of some medicinal plants. Plant Cell Reports, 1(2), 43-45.

[5] Decise, S: Seri. S Pushpangadan, P(1999). Effect of cryopreservation in seed rotation of selected rare medicinal plants of India. *Seed Sci Techal* 27:50)-50s, 1999,

[6] Decruse, S. W., Krishnan, P. N., & Peter, K. V. (1999). Micropropagation of the medicinal plant *Rauvolfia serpentina* Benth. ex Kurz from nodal segments of mature plants. Plant Cell Reports, 18(1-2), 91-96.

[7] Faisal M, Siddique I, Anis M (2006). In vitro rapid regeneration of plantlets from nodal explants of *Mucuna pruriens* a valuable medicinal plant. Ann. Appl. Biol., 148: 1-6

[8] Faisal, M., Alatar, A. A., Ahmad, N., Anis, M., Hegazy, A. K., & El-Sherif, F. (2007). *In vitro* regeneration of *Tylophora indica* (Burm. f.) Merrill from leaf explants. *In Vitro* Cellular & Developmental Biology - Plant, 43(4), 362-367.

[9] Gary, A Tre, H. M. (1996) Establishing a micropropagation system for American Kinng (*Pans quinquefolium*). Hart Science 21 212-236; 1986.

[10] Gary, C. M., & Brent, N. K. (1986). Somatic embryogenesis in *Rauvolfia serpentina*. Plant Cell Reports, 5(1), 18-21.

[11] Gastaldo, C., Papadakis, C., & Rigaud, J. (1994). Somatic embryogenesis in *Rauvolfia vomitoria.* Plant Cell Reports, 13(11), 643-647.

[12] Gulati A and Jaiwal PK, (1930). Culture condition affecting plant generation from cotyledons of (*Vigna radiate* L.) Plant cell Tissue Organ Cult, 23 - 1-7.

[13] Haw AB, Keng CL (2003). Micropropagation of *Spilanthes acmella* L., a bio-insecticide plant, through the proliferation of multiple shoots. J. Applied Hort., 5: 65–68.

[14] Ilahi I, Rahim F, Jabeen M (2007). Enhanced clonal propagation and alkaloid biosynthesis in the culture of Rauwolfia. Pak. J. Plant Sci., 13: 45-56.

[15] Kitto, S. L. (1997). Cryopreservation of in vitro grown shoot tips of *Rauvolfia serpentina*. Plant Cell Reports, 16(11), 783-787.

[16] Lee, K.; Zapata Aries, J Brantere, H.: Aura, H (1997). Histology of somatic embryo initiation and organogenesis from thine explant of Afins app: Plant Cell Tres. Organ Cult 511-8, 1997,

[17] Luna, C. M., Martínez, M. M., Monterubbianesi, G., & Gonzalez, A. M. (2003). In vitro plant regeneration from leaf explants of Ilex dumosa. In Vitro Cellular & Developmental Biology - Plant, 39(1), 78-81.

[18] Martin KP (2002). Rapid propagation of *Holostemma ada-kodien* Schult., a rare medicinal plant, through axillary bud multiplication and indirect organogenesis. Plant Cell Rep., 21: 112-117

[19] Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

[20] Nadeem, M., Palni, L. M. S., Purohit, A. N., & Pandey, H. (2000). In vitro conservation of threatened medicinal plant *Rauvolfia serpentina* Benth. ex. Kurz: A case study. In Vitro Cellular & Developmental Biology - Plant, 36(6), 524-528.

[21] Patil, V. R., & Jayanthi, M. (1997). In vitro regeneration from hypocotyl and leaf explants of *Rauvolfia tetraphylla* L. Plant Cell Reports, 16(9), 652-656.

[22] Preece JE, Huttreman CA, Ashby WC, Roth PL (1991). Micro and cutting propagation of silver maple. I. Results with adult and juvenile propagules. J. Am. Soc. Hortic. Sci., 116: 142-148

[23] Sahu, T. R. (1979). Medicinal Plants of Manipur. Bulletin of Medico-Ethno-Botanical Research, 1(1-4), 1-33.

[24] Saini R, Jaiwal PK (2000). In vitro multiplication of *Peganum harmalaan* important medicinal plant. Indian J. Exp. Biol., 38: 499-50

[25] Sarker, K. P. Talam. A lalan, R. Hoque, A. Janler, 0, L fa (1996) in vitro propagation of *Rauvolfia serpertina* through tissue culture. Planta Med 2338-390, 1996

[26] Sarker, R. H., Chakravarty, A. K., & Choudhury, B. (1996). Micropropagation of *Rauvolfia tetraphylla* L. Biologia Plantarum, 38(3), 427-431.

[27] Sebastian DP, Benjamin S, Hariharan M, 2002. Micropropagation of *Rotula aquatic* L our – An important woody medicinal plant. Phytomorphol.52: 137-144.

[28] Sharma, A. K., Kulkarni, K. S., & Shukla, Y. N. (1993). Somatic embryogenesis and plant regeneration from root explants of *Rauvolfia tetraphylla* L. Current Science, 65(5), 409-411.

[29] Sharma, K. Yang, E C Thorps, T. A (1991) Histology of shoot bud integrity from seedling net segments of Brassica napus L. Ann. Hot.71-461-400 1991

[30] Shekawat NS, Galston AW, 1983. Isolation, culture, and regeneration of moth bean, *Vigna aconitifolia* leaf protoplasts. Plant Science Letters, 32 - 43–51.

[31] Singh AK, Sharma M, Varshney R, Agarwal SS, Bansal KC (2006). Plant regeneration from alginate to encapsulated shoot tips of *Phyllanthus amarus* Schum and Thonn, a medicinally important plant species. In Vitro Cell. Dev. Biol. Plant., 42: 109-113

[32] Sudha, C. G Seeni, S.(1996) In vivo propagation of *Rauvolfia micrantha*, a rare medicinal plant. Plant Cell Tiss Organ Cult. $4/243-24 1996
[33] Tivarekar S and Eapen S, (2001). High-frequency plant regeneration from immature cotyledon of mung bean, Plant cell Tissue Organ Cult, 66 - 227-230.
[34] Tremouillaux-Guiller, J., & Chenieux, J. C. (1991). Plant regeneration from protoplast-derived calluses of *Rauvolfia vomitoria*. Plant Cell Reports, 9(2), 96-99.
[35] Upadhyay, S., Bhojwani, S. S., & Maheshwari, N. (1992). Regeneration of plants from callus cultures of *Rauvolfia tetraphylla*. Plant Cell Reports, 11(5-6), 221-225.
[36] Vuorela, H., Häkkinen, S., Oksman-Caldentey, K. M., & Rischer, H. (1993). Micropropagation of root cultures - an overview. Plant Cell, Tissue and Organ Culture, 33(3), 165-182.
[37] Youngkhen, HW Malabar *Rauvolfia,micrantha* Hd F. J. Am. Pharm Asse 43:141-143, 1954