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Full length Research

Intense Plantlet Regeneration through shoot-tip explants of *Rauvolfia micrantha* Hook. F. An Important Medicinal Endangered Plant

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Rauvolfia micrantha **species Hook. f. occupies a significant position within the biomedical sector due to its efficacy in reducing blood pressure. This is due to reserpine's presence in the roots' oleoresin component. Because of its low seed viability, sluggish seed germination rate, and vast genetic diversity, *R. micrantha* is difficult to cultivate commercially using conventional methods. By employing a semisolid nutrient culture medium, the current optimized protocol establishes aseptic cultures ideal for producing plantlets in an experiment. Shoot tip explants of *R. micrantha* were cultivated on MS medium supplemented with cytokinins TDZ /BAP/KN/ in varying concentrations (1.0-6.0 mg/L) in isolation. In contrast to all other concentrations of TDZ/KN/BAP alone, the TDZ of (4.0 mg/L) exhibited the greatest proliferation of shoot buds. As the concentration increased beyond 6.0 mg/L, the cytokinins' capacity to induce gradual growth of shoot buds diminished. On MS medium supplemented with 3.0 mg/L NAA, the laboratory-grown stalks produced additional roots. Consequently, the laboratory-grown plant, which was cultivated via shoot tip cultures, was transplanted into containers filled with garden soil and exposed to optimal light and shade conditions outside. When cultivated in a glasshouse or field conditions, 90–95% of the laboratory-grown plants survived, and 85% of the plants grown according to this protocol exhibited identical appearances. Following eight weeks in their newly transplanted containers, these plants produced flowers. The established protocol can be utilized to produce more true-to-type plants promptly.**

Keywords: *Rauvolfia micrantha* Hook. f., *In vitro* culture, Shoot tip explant, Plant growth regulators, Plant Regeneration, Rooting, and Hardening.

Abbreviation: **BAP** (Benzyl Amino Purine), **KIN** (Kinetin), **TDZ** (Thioduzuron), **NAA** (Napthalene acetic acid), (**PGR**) Plant Growth Regulators

INTRODUCTION:

Rauvolfia micrantha Hook. f. (Apocynaceae) is a perennial arboreal shrub that may be found in the Tonnarelli and Travancore hills of the Western Ghats in southern India, specifically at an elevation of 600 meters. The roots of the plant contain a variety of alkaloids, including (Antihypertensive drugs work through various mechanisms to reduce blood pressure. Some common classes of antihypertensive medications include: Diuretics, Beta-Blockers) antihypertensive, hypertensive, and tranquilizer compounds such as ajmalicine,

reserpine, sarpagine, reserpine, and serpentine (Ajmalicine: Source: Ajmalicine, also known as raubasine, is an indole alkaloid found in plants such as *Rauvolfia serpentina* (Indian snakeroot) and Catharanthus roseus (Madagascar periwinkle).

Reserpine: Source: Reserpine is an alkaloid derived from the roots of *Rauvolfia serpentina*. Action: Reserpine has been historically used as an antihypertensive medication due to its ability to deplete neurotransmitters, such as norepinephrine and serotonin, from nerve terminals. By depleting these neurotransmitters, reserpine reduces sympathetic nervous system activity, leading to vasodilation and a subsequent decrease in blood pressure. Sarpagine: Source: Sarpagine is a type of alkaloid found in the same plant family as reserpine, particularly in species like *Rauvolfia serpentina*. (Anonymous, 1969). Furthermore, it serves as a substitute for the root of *Rauvolfia serpentine* in commercial plots that are being increasingly supplied to American purchasers (Youngken, 1954). *R. micrantha* is utilized as a substitute for *Rauvolfia serpentina* in the traditional Indian medicinal system known as Ayurveda, namely for the treatment of various neurological disorders, notably in the region of Kerala (Sahu, 1979). According to Sahu (1979), the plant has been characterized as rare and distinctive among the southern forests of the Western Ghats. The decrease of *R. micrantha* in its natural habitat can be attributed to several factors, including the presence of endemic regions, limited geographical distribution, small populations in remote locations, and human-induced pressures on forest ecosystems. Conventional multiplication is afflicted by issues such as low seed viability, restricted germination, and the scarcity and delayed development of roots in seedlings and vegetative cuttings. Considering the urgent need for conservation, the process of in vitro multiplication may be achieved using shoot tip explant cultures, as demonstrated by Sudha and Seeni in 1996. Plant tissue culture is a well-recognized biotechnological technique utilized for the rapid proliferation of medicinal plants with the aim of commercialization, conservation (Nadeem *et al.,* 2000), and cryopreservation (Kitto, 1997). (Decruse *et al.,* 1999) conducted the study. Somatic embryogenesis and organogenesis have been identified as the primary processes for the clonal growth of exceptional medicinal plant species (Gary and Brent, 1986).

Somatic embryogenesis has been found to enhance plant production in some situations, resulting in larger quantities and improved quality of secondary metabolites (Gastaldo *et al.,* 1994). In vitro regeneration of plants belonging to the *Rauvolfia* species has predominantly been achieved through organogenesis, as documented by Sarker et al. (1996), Sudha and Seeni (1996), and Patil and Jayanthi (1997). However, somatic embryogenesis has been reported as a less common method of regeneration in *Rauvolfia vomitoria* (Tremouillaux-Guiller and Chenieux, 1991) and Rauvolfia caffra (Tremouillaux-Guiller and *Chenieux,* 1991). According to Upadhyay *et al.* (1992), Traditionally, the process of embryogenesis has predominantly relied on the utilization of plant tissues such as cotyledons, hypocotyls, leaves, or internodes. However, roots have been infrequently employed for this purpose (Vuorela *et al.,* 1993). The use of root segment culture, organogenesis, and somatic embryogenesis techniques has proven to be highly advantageous in the context of genetic transformation investigations involving *Agrobacterium rhizogenic*. This experimental system is also suitable for investigating cell differentiation and the regulatory mechanisms behind totipotency in plants.

The present study clarifies a micropropagation technique for *R. micrantha*, whereby shoot tip/meristem explants are used as the primary source for the generation of multiple shoot buds through direct culture.

MATERIALS AND METHODS

Explants of the shoot tips of *R. micrantha* were collected from young, healthy branches of a plant that was one year old and was growing at the research location in the Department of Botany at Osmania University Hyderabad in Telangana state. Some of the shoot tip explants had axillary buds while others did not. During a five-minute treatment with a 5% teapot solution, the clippings were thoroughly rinsed with running water from the faucet. They were cleaned well with running tap water, sterilized on the exterior with 0.1% w/v Mercuric chloride ($HgCl_2$) for 4-5 minutes, and then rinsed with sterile distilled water at least three times before use. Pieces of sterile nodes were dried on sterile filter paper before being inoculated.

Culture media and culture conditions:

The explants of the shoot tip and meristem were then inoculated into an MS medium (Murashige and Skoog (1962) that contained 30 gm/L of sucrose and was fortified with varying doses of cytokinin BAP/KN/TDZ (Table 1). Before adding 0.8% agar to any of the media, the pH of all the media was adjusted to 5.8 and the containers were autoclaved at 121^0 C under 15 pounds per square inch for 15–20 minutes. After the medium had been solidified with 0.8% agar (Difcobacto), it was ready to be used. We used MS+ BAP/KN/TDZ medium at concentrations ranging from (1.0 to 6.0 mg/L) for the cultivation of differentiating explants. The temperature in the culture tubes was maintained at 25^0 C, which is 20^0 C higher than the ambient temperature. The

photoperiod was set at 16 hours, and the light source was white fluorescent light (40-50). After a total of six weeks of culture, the axillary shoots were transplanted into a rooting medium. This was done after the shoots had multiplied.

RESULTS AND DISCUSSION

Auxiliary buds are considered the most suitable explants for efficient micropropagation due to their possession of a meristem, which may be rapidly induced to develop into shoots while maintaining the clonal fidelity of the parent plant. Supplementary bud culture has been documented as a method of micropropagation for various plant species, such as *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.* 2003). Consequently, shoot tip segment explants possessing an auxiliary bud were employed to stimulate the proliferation of many shoots and facilitate their development. The findings derived from the cultures of axillary and shoot tip buds are displayed in (Table 1). The findings pertain to the proliferation of many branches and roots. The results of growing *R. micrantha* with various combinations of hormones exhibited significant variability. During the initial week following the inoculation, the axillary buds exhibited signs of increased activity. Subsequently, in the second and third weeks, distinct shoots emerged, characterized by the presence of leaves and internodes. Following the injection, the axillary buds exhibited signs of activation within the initial week. The survival rate of *R. micrantha* explants derived from shoot tip segments of the wild plant exhibited significant variability across different seasons. Based on the latest research findings, the collection of explants from field-grown plants was conducted at various intervals throughout the year in order to ascertain the most favorable season for the initiation of the culture. Explant samples obtained throughout the months of August and October exhibited a reduced duration for sprouting and accelerated proliferation of shoot buds in comparison to samples acquired during other months.

Effect of TDZ:

The results of growing *R. micrantha* shoot tip/ meristem explants on MS media supplemented with TDZ at dosages ranging from (1.0 to 6.0 mg/L) are presented in (Figure 1A) and shown in (Table 1) respectively. Not only did the medium containing TDZ at a dosage of 4.0 mg/L produce the maximum number of shoots (6.0±0.32 shoots/explant), but it also showed the highest proportion of responsive cultures (65%). The rate of shoot bud multiplication was found to decrease as the concentration of TDZ increased to (5.0 mg/L), and the rate of shoot multiplication was found to decrease when the concentration of TDZ increased above (6.0 mg/L). Both results were found to be consistent with the hypothesis that an increase in TDZ concentration would hurt the rate of shoot multiplication (Figure 1a).

Effect of KIN:

It was reported what happened when the shoot tip of *R. micrantha* was grown on MS medium that was augmented with KN at concentrations ranging from (1.0 to 6.0 mg/L). The result at (4.0 mg/L) KIN indicated the greatest proportion of responsive cultures (7.0±0.35) when compared to the results of the tests that were done with various doses. KIN at (1.0, 2.0, and 3.0 mg/L) produced (3.3± 00.25), (5.6± 0.32), and (5.2± 0.35) shoots/explant, respectively; however, more shoots were regenerated from shoot tip explants at (4.0 mg/L) KIN (7.0± 0.35) shoots/explant), followed by (4.0 mg/L) KIN. (Table 1) displays the responses that were collected from a total of 42, 46, and 52 distinct cultures.

Effect of BAP:

It was possible to see the results of growing *R. micrantha* shoot tip explants on MS medium that had been supplemented with BAP at dosages ranging from 1.0 to 6.0 mg/L. After growing the shoot tip explants on MS media, these observations were observed after the growth. When compared to the other concentrations that were investigated, the BAP dosage of 4.0 mg/L yielded the highest proportion (7.0± 0.42) of responsive cultures. Even though a larger number of shoots were generated from shoot tip explants at BAP concentrations of 5.0 mg/L (6.0 shoots/explant) than at BAP concentrations of 6.0 mg/L (6.8±032 shoots/explant), respectively, it should be noted that this difference was not statistically significant. When BAP was present at dosages of (1.0, 2.0, and 3.0 mg/L), the number of shoots that were produced from each implant ranged anywhere from 3.0 to 7.0, with a standard deviation of 5.0±0.32. The following is a breakdown of the percentages of civilizations that participated in the survey: 48, 52, and **67%** respectively: (See Table 1, Figure 1B).

In Vitro Rooting:

The fully formed and healthy shoots were then transplanted into MS medium that had been enhanced with various quantities of NAA (ranging from 0.5 to 4.0 mg/L). This was done to observe the effects of the NAA on the shoots. However, only 58% of plants produced

Table 1. Evaluation of plant growth regulator after eight 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) *
TDZ			
1.0	46	3.0 ± 0.32	3.0 ± 0.42
2.0	50	4.2 ± 0.35	3.2 ± 0.75
3.0	60	5.4 ± 0.32	5.4 ± 0.32
4.0	65	6.0 ± 0.32	6.2 ± 0.36
5.0	54	5.8 ± 0.36	5.2 ± 0.36
6.0	40	4.5 ± 0.23	4.5 ± 0.23
KIN			
1.0	42	3.3 ± 0.25	4.0 ± 0.85
2.0	46	5.6 ± 0.32	4.8 ± 0.32
3.0	52	5.2 ± 0.32	5.2 ± 0.32
4.0	64	7.0 ± 0.35	6.0 ± 0.35
5.0	68	5.3 ± 0.32	7.3 ± 0.32
6.0	57	4.2 ± 0.23	5.2 ± 0.23
BAP			
1.0	48	3.0 ± 0.32	5.0 ± 0.32
2.0	52	4.6 ± 0.32	5.8 ± 0.42
3.0	67	5.0 ± 0.32	6.6 ± 0.62
4.0	72	7.0 ± 0.42	7.5 ± 0.52
5.0	60	6.0 ± 0.32	8.5 ± 0.42
6.0	54	6.8 ± 0.32	6.8 ± 0.72

Table 2. Analysis of the impact of MS+ NAA (1.0-4.0 mg/L) on *R. micrantha* micro shoots capability to establish roots 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	36	3.0 ± 0.32	3.0 ± 0.42
1.0	40	5.2 ± 0.35	3.2 ± 0.75
2.0	58	6.4 ± 0.32	5.4 ± 0.32
3.0	50	5.0 ± 0.32	6.2 ± 0.36
4.0	44	5.8 ± 0.36	5.2 ± 0.36

roots with (6.4± 0.32 roots/explants) (Table 2) (Figure 1c), which indicates that this concentration of NAA was optimal for root production (Figure 1c). Abundant histogenesis was observed on 2.0 mg/L NAA, which responded with (6.4± 0.32 roots/explants) compared to NAA concentrations of (0.5-2.0 mg/L). Adjustment to climate After the rooted plantlets had been withdrawn from the culture medium, the agar that had adhered to the plantlets' roots was removed by washing the roots under water that was dripping from a tap. After that, the seedlings were transplanted into poly pots that had been previously filled with wet vermiculite, and they were housed in a growth environment that had a temperature of 28^0 and a relative humidity of 70–80 percent. After a period, equal to three weeks, they were moved into poly bags that held a mixture of soil, sand, and manure in the proportion of (1: 1: 1). After that, these bags remained inside a shade house for an additional three weeks as the experiment continued. The Hogland solution was used to hydrate the potting mix that the seedlings were growing in once every three days for a total of three weeks (Figure 1d). We were able to effectively regenerate shoots from shoot tip cultures by growing them on an MS medium that had various amounts of cytokinin added to it. These cytokinins included BAP, KIN, and TDZ. This resulted in the successful production of plants. As a growth regulator, TDZ induced a considerably larger number of shoots at a concentration of 4.0 mg/L compared to BAP/KIN at the same concentration.

On the other hand, it was found that neither lower nor

Figure 1. Direct *in vitro* shoot buds' proliferation of *R. micrantha*. (a) Formation of multiple shoots on MS+TDZ (4.0) mg/L from shoot tip, (b) Proliferation of multiple shoots on MS+BAP (4.0mg/L) from shoot tip, (c) rooting of individual shoots on MS+IBA (3.0mg/L) (d) hardening of plantlet.

greater concentrations of BA were optimal to produce the highest number of regenerated shoots. It was previously discovered that BA was essential for shoot regeneration in a few plants (Stefaan *et al.,* 1994; Faisal *et al.,* 2006, 2007). This is like what was discussed before in this paragraph. It was also demonstrated that Kin and 2iP are suitable for shoot regeneration; however, the reaction was substantially less than the one that was brought about by BA. Similar effects were discovered from the culture when it was grown on a medium that included Kn and 2iP. These findings were also substantiated by research that used *Mucuna pruriens* (Faisal *et al.,* 2006). In addition to this, it was found that the number of shoots reduced when the BA concentration was increased to a level that was higher than what was the appropriate level. This discovery is in line with what Haw and Keng (2003) discovered in an earlier study project they conducted in 2003. Explants that had been grown in a medium with increased levels of BA had shrunken nodes, vitreous leaves, and the formation of callus at the cut edge. This was notably noticeable at the proximal ends of the shoot tip explants. In the case of *Peganum harmala* (Saini and Jaiwal, 2000) and *Holostemma ada kodien* (Martin, 2002), similar findings were documented. The effect of accumulated auxin at the basal cut ends probably led to an increase in cell proliferation, which ultimately resulted in the formation of callus. This was especially true when cytokinins were present in the environment. It was hypothesized by Preece et al (1991) that species with a strong apical dominance were more likely to produce callus at the basal cut ends of shoot tip explants when grown on a cytokinin-enriched medium. The regeneration of an in vitro plant from a shoot-type explant is depicted in Figure 1A. The cultivation of *R. micrantha* and the

establishment of plants involve two steps: a) Induce the development of multiple shoots using MS medium supplemented with 6.0 mg/L BAP. b) Manage the growth of several shoots that have been cultured on MS medium with 4.0 mg/L TDZ_the plantlet becoming more robust in contrast, was more successful in boosting shoot bud proliferation. This was the case when comparing the two treatments. It's possible that BAP was the sole factor that effectively caused higher plantlet regeneration than any of the other hormonal treatments, given that it was used in conjunction with all the hormonal combinations and concentrations that were tested. According to Gulati and Jaiwal (1930) and (Chandra, and Pal1995), BAP was observed to increase the frequency of regeneration. In the case of *Rotula aquatic*, Sebastian *et al.* (2002) found that BAP was successful in both the induction of bud break and the promotion of shoot proliferation. In the case of *Rotula aquatic*, the use of BAP was discovered to be effective in the induction of bud break and shoot proliferation. Shoot tip explants were also used in the experiment (Shekhawat and Galston1983) to improve the rates of shoot multiplication in several plants. According to the findings of Tivarekar and Eapen (2001), explants of immature cotyledonary nodes were responsible for a high rate of plant regeneration in a variety of different species. Rhizogenesis was facilitated by the incorporation of NAA into the MS medium, albeit at a lower concentration than had been planned at the beginning. The greatest number of roots (6.0±0.32) and the greatest frequency of root growth (65%) were both accomplished on MS medium with 3.0 mg/L of NAA. After being properly hardened off for four weeks on a planting substrate of choice within the growth chamber, the regenerated plantlets that had grown shoots and roots were ultimately planted in natural soil (Figure 1D). shown any obvious alterations as a consequence of the shift in circumstances. This observation is consistent with a few other findings made in a manner analogous to it (Singh *et al.,* 2006; Faisal *et al.,* 2006 and 2007).

CONCLUSION:

In conclusion, the research conducted on the micropropagation of *R. micrantha* has yielded significant and promising results, paving the way for enhanced cultivation and conservation of this vital medicinal plant. The optimization of growth regulators and culture media has provided a highly efficient method for propagating *R. micrantha*, which holds great promise for the pharmaceutical and herbal medicine industries.

The utilization of shoot tip explants cultured on a medium typically intended for woody plants, augmented with (4.0 mg/L) of Thidiazuron (TDZ), has proven to be the most effective condition for the growth of *R. micrantha*. This finding is particularly noteworthy, as it not only increases the propagation success rate but also ensures the health and vitality of the propagated plants. As a result, it becomes feasible to cultivate and conserve *R. micrantha* on a larger scale.

The significance of these discoveries extends to the broader domain of medicinal plant research. *R. micrantha* is known for its rich reservoir of bioactive compounds, some of which have demonstrated potential in the development of therapeutic drugs and herbal remedies. With the newfound ability to efficiently propagate *R. micrantha*, the accessibility of these valuable compounds for research and development is greatly enhanced.

The successful micropropagation of *R. micrantha* opens the doors to sustainable farming practices and conservation efforts, potentially reducing the pressure on wild populations of this species. As medicinal plants continue to gain importance in the pharmaceutical and alternative medicine sectors, the increased availability of *R. micrantha* can contribute to the production of safer, more consistent, and reliable herbal medicines. Moreover, this research underscores the importance of plant tissue culture techniques in advancing the propagation and conservation of valuable plant species, offering hope for the sustainable utilization of botanical resources and the continued discovery of novel therapeutic compounds. This breakthrough has the potential to significantly impact the future of medicinal plant research and the development of new drugs and herbal remedies.

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