An Efficient *In Vitro* Regeneration and Conservation Protocol of *Rauvolfia serpentina* (L.) Benth. Ex Kurz - An Endangered Medicinal Plant

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Abstract

In the present study, a well-structured in vitro protocol for direct organogenesis was developed using Murashige and Skoog medium, with nodal and shoot tip explants, to achieve rapid clonal multiplication without an intervening callus phase. Explants were cultured on MS medium supplemented with varying concentrations and combinations of auxins (NAA, IAA, and IBA) and cytokinins (BAP and Kinetin). The response was evaluated in terms of shoot initiation percentage, shoot length, and number of shoots per explant, multiplication percentage with shoot length and number of shoots per explant, and root initiation response. The most effective treatment for direct shoot initiation was MS medium supplemented with BAP and Kinetin (1.0 mg/l-5.0 mg/l). The best initiation percentage was achieved in apical shoots compared to nodal explants at a low Kinetin concentration (1.5 mg/l), for multiplication on MS medium fortified with BAP (1.5 mg/l-5.00 mg/l) in combination with Kinetin (0.5 mg/l) and IBA (0.5 mg/l), which induced a high frequency of multiple shoots per explant. Rooting was achieved on MS liquid medium with NAA (0.5mg/l-2.0mg/l) in combination with IAA and IBA 0.5 mg/l. In vitro-raised shootlets were harvested, treated with IAA and IBA (1.0 mg/l and 1.5 mg/l) for a specific duration, and transplanted to pots containing a media mixture (Vermiculite and cocopeat @ 1:1), and studied for *ex vitro* rooting efficiency.

Keywords: *In vitro* Propagation, *Rauvolfia serpentina*, direct organogenesis, conservation protocol, endangered medicinal plant

Introduction

Rauvolfia serpentina is a perennial shrub of the Apocynaceae family, widely known as Indian snakeroot or sarpagandha. Sarpagandha is a perennial, evergreen undershrub that reaches up to one meter in height. The stem is woody, with leaves containing milky latex, arranged three per Node, measuring 10 to 15 cm in length and 3 to 5 cm in width. The lateral veins are 8-12 pairs, and the petiole is approximately five millimeters long. Inflorescence is corymbose cymes and axillary with five to seven flowers with red Calyx and white-colored corolla. The fruits are shiny, purplish-black, two-lobed, while the seeds are small, typically measuring around 2mm - 4mm in length and 1-2 mm in width, appearing oblong to slightly ovoid with a rough, wrinkled surface texture that aids in seed coat protection.

The internal structure of seeds features a straight embryo embedded in a horny endosperm, enabling dormancy periods up to several months due to an impermeable stony seed coat. Rhizomes are pale brown to cream-colored, stubby, branched, typically measuring 2cm - 10 cm long and 5mm - 22mm thick, often curved, with a bitter taste and a characteristic acrid odor. Its root is a rich source of Indole alkaloids (Klushnichenko *et al*., 1995), such as reserpine, ajmaline, and serpentina, which support their use in antihypertensive and antiarrhythmic medicine, as well as in the treatment of fever, wounds, insomnia, epilepsy, and vertigo (Eashan Mukherjee *et al.,* 2020). There is a strong demand for dried root of *Rauvolfia* for the drug market, at 20,000 tons/year (Paturkar and Khobragade, 2016).

Habitat and Ecological niche

Sarpagandha originates in tropical and subtropical Asia and occurs both in the wild and under cultivation across numerous humid regions at low to mid elevations. Sarpagandha *(Rauvolfia serpentina)* is native to tropical and subtropical Asia and is now found both in the wild and under cultivation in many humid, low-to mid-elevation regions. In India, it is fairly widespread in suitable forest habitats but has declined in the wild in many areas and is locally threatened or endangered. *Sarpagandha* prefers moist to wet low land and sub-mountain tropical forests, forest understories, bamboo thickets, secondary growth, riverbanks, and other shady, well-drained sites. It is mostly found at low elevations but can occur up to around 2000-2100m in humid, shaded conditions**.**

Availability and cultivation

Wild populations have been heavily exploited for root harvest, resulting in their designation as threatened or endangered in multiple Indian states, where forest collection is completely restricted. To fulfill commercial demand, sarpagandha is now widely cultivated on a broad scale, with planting materials, including seedlings and rooted cuttings, commercially available from multiple suppliers, particularly in India's medicinal plant trade hubs. The (IUCN) International Union for Conservation of Nature, Red Listed Categories are intended to be an easily and widely understood system of classification for the species at high risk from regional to global extinction, and

to provide an explicit and objective framework for the classification of species according to their extinction risk. One such red-listed medicinal plant assessed as endangered in India is sarpagandha, botanically known as *Rauvolfia serpentina* (L.). Benth. ex Kurz belonging to the family Apocynaceae. In India, it is distributed from Himachal to Arunachal Pradesh. Its presence is also recorded in the Western and Eastern Ghats, as well as in Eastern and Central India.

Tissue culture approach

Wild populations of *R. serpentina* are primarily threatened by overharvesting and low natural seed viability (Ahmed *et al.,* 2008). *R. Serpentina* is cultivated more through micropropagation rather than natural methods for conservation and mass multiplication, as the natural method of Propagation is associated with challenges like the availability of seeds due to abortive embryos, leading to poor germination and slow multiplication. This technique also conserves elite varieties and supports the conservation of endangered medicinal plants by reducing pressure on wild populations.

Tissue culture approach in *R. serpentina* refers to the in vitro culture of explants on nutrient-enriched media to obtain large numbers of genetically stable, disease-free plants, maintaining genetic fidelity and pharmaceutical quality for extraction without intense strain on the wild population. These approaches support both ex situ conservation and the rapid production of disease-free plantlets.

Micropropagation positively affects alkaloid yield and quality, enabling the production of plantlets with alkaloid content similar to or higher than that of field-grown plants. It facilitates mass propagation of medicinal plants under controlled conditions, preserving or even improving the production of therapeutically valuable alkaloids. The controlled in vitro environment minimizes variation that occurs in field conditions, resulting in a more stable and pure alkaloid composition. The current research focuses on the mass propagation of *Rauwolfia serpentina* using apical shoots and nodes as explants in *in vitro* condition, maintaining plant tissue in a laboratory setting to keep it free of microbes and ensure its development. Micropropagation enables the preservation of biodiversity in native flora through potential scientific approaches, such as *ex situ* conservation in a medicinal garden and micropropagation via tissue culture.

Materials and Methods

A detailed description of the materials utilized and the methods followed for the present study is provided hereunder.

Experimental study plant

Rauvolfia serpentina (L).

Voucher code number SVUH: 0473 was issued for *Rauvolfia serpentina* by the Department of Botany, S.V. University, Tirupati, Chittoor district, Andhra Pradesh, India.

Collection of plant material

Saplings of *R. serpentina* were collected from Yogi Vemana University, Kadapa, Andhra Pradesh. planted and maintained in the Herbal Garden of the Department of Biosciences and Sericulture, Sri Padmavati Mahila Visvavidyalayam (Women's University), Tirupati. The apical shoot tips and nodal explants were collected from healthy plants and used in the present experimental studies.

In Vitro Experimental Study

Seed germination, Direct organogenesis of shoot tip and nodal explants, and callus cultures from leaves have been accomplished in the present work. An assembly line of work includes Washing and storage of glassware, media preparation, sterilization, inoculation, and maintenance of cultures in the growth chamber.

Experimental study area

In vitro studies on *Rauvolfia serpentina* were conducted in the Tissue Culture Laboratory of the Department of Biosciences and Sericulture, SP MahilaVisvavidyalayam, Tirupati, Andhra Pradesh, India.

Washing, storage, and sterilization of glassware

Culture vessels were immersed in sulphuric acid (0.16% conc.) for 10-14 hrs and washed with detergent labolene. Then cleaned under a jet flow of running tap water and immediately rinsed with distilled water 2-3 times. These were allowed to dry, then autoclaved at 15 psi for 15 min. The cooled glassware is then stored in dust-free store-wells. Prior to preparation, the required glassware was oven-dried at 80°C for 2 hours. The contaminated vessels were first decontaminated by autoclaving them at 15 lbs/in^2 for 20 minutes.

Media for apical shoot tip, node, and rhizogenesis In the present study, MS medium was used for initiation, multiplication, and rooting using apical shoots and nodal explants. To prepare 1000 ml of medium, 8 gm of agar (Himedia) was added to 500 ml of deionized, sterile water (Type 1, Millipore). When agar was dissolved, 30 g of sucrose was added, and, as required, other additives, vitamins, and amino acids were dispersed into the medium. To this mixture, the required volumes of stock solutions were added, and the mixture was adjusted to 1000 ml with autoclaved distilled water. The pH of the media was adjusted to 5.6-5.8 with 1 N NaOH or HCl prior to autoclaving. The media were steam-sterilized by autoclaving at 15 psi. Then, media of different concentrations and combinations were dispersed into culture bottles/test tubes.

***In vitro* Rooting**
In vitro grown shootlets of *R.serpentina* were freshly transferred to liquid rooting media fortified with NAA (0.4-2.0 mg/l) in combination with IBA and IAA (0.5 mg/l). The well-formed root system plantlets were gently removed from the media and washed with lukewarm water. The rooted plantlets were successfully transplanted to potting culture, a mixture of vermiculite and cocopeat (1:2 ratio).

***In vitro* studies of shoot regeneration of Rauvolfia serpentina**
The study was undertaken to investigate standardized protocols for effective *in vitro* regeneration of R. *serpentina* from shoot tips and nodal explants.

Shoot tip and Nodal explant
Healthy apical shoots and nodal bud explants, collected from the herbal medicinal garden, were maintained at the Department of Biosciences and Sericulture. 6-8 mm shoot tips and 4-5 cm nodal bud explants were excised from the one-year-old field-grown mother plants of *R.serpentina*.

Collection of explants
One day before excising the explants, healthy shoots were selected and sprayed with a 1% Bavistin (Carbendazim 50% WP) solution (w/v). Shoot tips and nodal bud explants were excised and immediately placed in 0.5 % Bavistin solution.

Surface sterilization for Shoot tip and Node
Explants were collected in 0.5 % Bavistin (Carbendazim 50 % WP) and gently stirred for 2 min. Then rinsed with autoclaved distilled water for a few minutes followed by Tween - 20 + savlon for 3 min, and rinsed with water for 3-4 times up to 2-3 min followed by 0.75 % $Hgcl_2$ and 70 % Ethanol each explant up to 30 sec - 1 min and immediately followed by water for about 2-3 min separately for each explant.

Initiation for shoot tip and nodal explants
To study the effect of auxin and cytokinins combination on initiation of apical shoots, explants were inoculated on MS media supplemented with cytokinins alone, BAP (1.0-5.0 mg/l), and Kinetin (1.0-5.0 mg/l).

Elongation and multiplication for the shoot tip and Node
For multiplication, newly emerged microshoots were inoculated onto MS media supplemented with a combination of Cytokinins (BAP and Kin) and Auxin (IBA). The combinations include: BAP (1.5-5.0 mg/l) with Kinetin (1.0 mg/l) and BAP (1.5-5.0 mg/l) with IBA (0.5 mg/l).

***In vitro* rooting**
In vitro grown shootlets of *R.serpentina* were transferred to MS rooting liquid media fortified with NAA (0.4-2.0 mg/l) in combination with IBA and IAA (0.5 mg/l). The well-formed root system plantlets were gently removed from the media and washed with lukewarm water. The rooted plantlets were successfully transplanted into a potting mix of vermiculite and cocopeat (1:2 ratio).

Rooting in *ex vitro* condition
In vitro raised shootlets were harvested and then treated with IBA and IAA alone (1.0 mg/l and 1.5 mg/l) for about 15-20 min. Microshoots without treatment served as the control. Treated shootlets were transplanted into pots containing autoclaved rooting mixture (Vermiculite and cocopeat @ 1:1). These were irrigated with half-strength MS basal solution at 7-day intervals.

Plant Growth Hormones
For initiation, apical shoot tips and nodal explants were transferred onto MS media fortified with different concentrations of cytokinin, i.e., BAP (1.0 mg - 5.0 mg/l) and Kinetin (1.0 mg - 5.0 mg/l).
Growth hormones for Multiplication: Cytokinins, BAP (2.0-3.0 mg/l), and Kinetin (1.0-1.5 mg/l) were used for shoot multiplication and elongation. For *in vitro* rooting, growth hormones NAA (0.4-2.0 mg/l) were used in combination with IBA and IAA (0.5 mg/l).

Inoculation
All the required materials for inoculation were transferred to the inoculation chamber. Prior to inoculation, UV light was switched on for 30 min and, after switching off, the chamber was left for another 30 min. While working, hands were sanitized with 70 % alcohol. The surface-sterilized explants were then placed in sterile petri dishes and inoculated into the required media.

Culture conditions
The cultures were incubated in a growth room under controlled conditions: 25±20 °C, 60-70% relative humidity, and a 16 h photoperiod/day at a photon flux density of 15-20 µE m2/s-1 from white fluorescent tubes.

Subculture

Subcultures were carried out at regular intervals on fresh nutrient medium. Based on the response, cultures were subcultured as and when required for further development.

Data Analysis

The experiments were repeated three times, and data analysis was performed. The experiments were conducted with at least 15 replicates per treatment. One replicate means one explant. Each experiment was repeated four times. The results are expressed as mean ± standard deviation of four experiments. Observations were recorded regularly. The present data was analyzed using a Two-Way ANOVA (Analysis of variance) in SPSS (version 20.0). Similarly, the remaining parameters were analyzed using ANOVA, and statistical significance was calculated.

Results

Initiation from apical shoots

Surface-sterilized apical shoots were inoculated in MS medium containing BAP (1.0 mg/l to 5.0 mg/l) and alone Kinetin (1.0mg/l-5.0 mg/l). The shoot initiation percentage ranged from 56.67% to 96.67%, with a maximum of 96.67% at BAP 3.0 mg/l. The lowest initiation percentage (56.67%) was recorded at 1.0 mg/l, while the longest shoots (4.13cm) were reported at BAP 3.0 mg/l. In contrast, the shortest shoot length (4.03cm) was recorded at 1.0 mg/L. The highest (5.33) number of shoots per explant was recorded at BAP 3.0 mg/l, and the lowest (3.00) was observed at BAP 1.0 mg/l.

Apical shoots were inoculated onto MS medium fortified with Kinetin (1.0mg/l-5.0mg/l). The initiation percentage ranged from 70.00% to 96.67%. In Kinetin -supplemented medium, the highest (96.67%) initiation response was recorded at 1.5 mg/l kinetin, and the lowest was recorded at 5.0 mg/l. The highest (7.13cm) shoot length was recorded at 1.5 mg/l, and the lowest was recorded at 5.0 mg/l. The highest (6.33) number of shoots per explant was recorded at 1.5 mg/l, and the lowest (2.33) at 5.0 mg/l. The results are presented in the Table no.1.

Table 1: Effect of BAP and Kin on initiation of *Rauvolfia serpentina* from apical shoots on full-strength MS medium

S.No	Growth Hormone Concentration (mg/L)		% of shoot Initiation	Shoot Length (cm)	Number of Shoots
	BAP	Kin			
1.	1.0	-	56.67±3.33	4.03±0.88	3.00±0.00
2.	1.5	-	63.33±3.33	5.00±0.12	4.33±0.33
3.	2.0	-	66.67±3.33	5.80±0.23	4.33±0.33
4.	2.5	-	93.33±3.33	7.00±0.23	5.67±0.33
5.	3.0	-	96.67±3.33	4.13±0.13	5.33±0.33
6.	4.0	-	90.00±5.77	6.47±0.17	5.67±0.33
7.	5.0	-	86.67±3.33	6.23±0.15	5.33±0.33
8.	-	1.0	80.00±0.00	3.70±0.06	4.00±0.57
9.	-	1.5	96.67±3.33	7.13±0.24	6.33±0.33
10.	-	2.0	90.00±0.00	4.07±0.12	4.67±0.33
11.	-	2.5	86.67±3.33	3.87±0.07	3.67±0.33
12.	-	3.0	83.33±3.30	3.53±0.07	3.33±0.33
13.	-	4.0	76.67±3.33	2.43±0.12	2.67±0.33
14.	-	5.0	70.00±.0.00	2.13±0.09	2.33±0.33
Variables * Treatments			**F- 22.205 P-0.000 Sig****		

Each value represents the average of 3 replications (n=3); explants treated with BAP and Kinetin. p<0.05 Significant at 0.05 level p<0.01 significant at 0.01 level p>0.05 Not significant

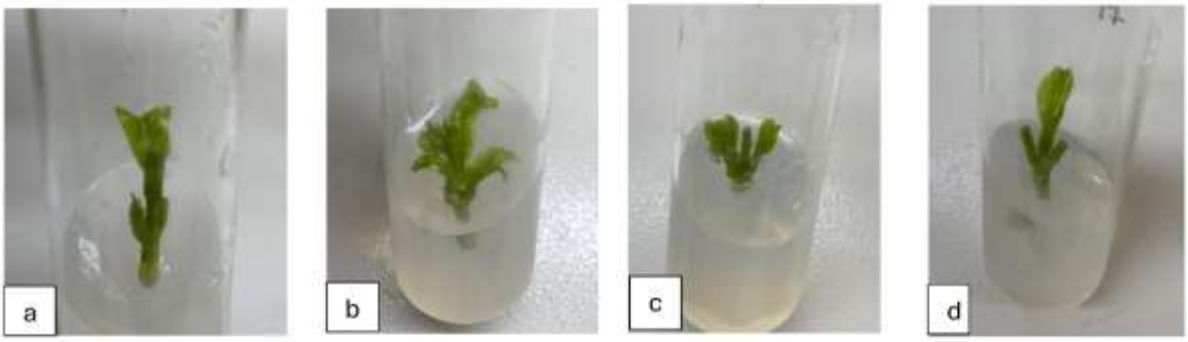

Figure 1 Initiation from apical shoots after one week of inoculation. a, b. BAP-supplemented medium. c, d. Kinetin-supplemented medium.

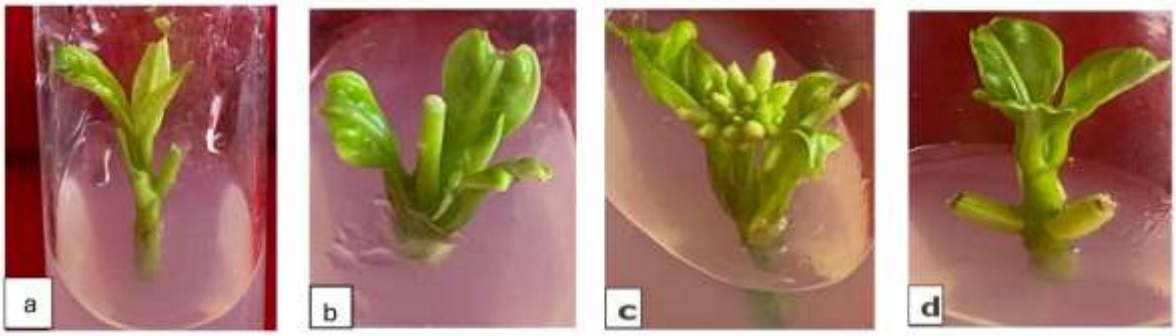

Figure 2 Initiation from apical shoots after two weeks of inoculation. a, b. BAP supplemented medium. c, d. Kinetin-supplemented medium

Initiation from nodal explants

Nodal segments inoculated in MS fortified with BAP alone (1.0 to 5.0mg/l) and Kinetin (1.0 to 5.0mg/l). The shoot initiation percentage ranged from 53.3% to 93.3%. Achieving a maximum of 93.3% at 4.0 mg/l and a lowest (53.33%) at 1.0mg/l. Nodal explants with the highest shoot length (5.23cm) were observed at 2.5 mg/l BAP, and the lowest (3.03cm) recorded at 1.0mg/l. The highest number of shoots per explant (5.67) was recorded at 4.0 mg/l, and the lowest (3.67) at 1.0 mg/l and 1.5 mg/l.

In Kinetin-supplemented medium, the percentage of initiation ranged from 63.3% to 96.67%. Maximum initiation percentage (96.67%) was observed at 2.5mg/l and the lowest 63.33% at 5.0mg/l. The maximum shoot length of 5.77 cm was reported at 2.5mg/l and the least (30.7cm) was recorded at 5.0mg/l. The number of shoots was also found to be high (5.67) at 2.5 mg/l, and the lowest number (3.33) of shoots was observed at 1.0mg/l and 5.0mg/l. The results are presented in the Table no.2.

Table 2: Effect of BAP and Kin on initiation of *Rauvolfia serpentina* from nodal explants on full-strength MS medium

S.No	Growth Hormone Concentration (mg/L)		% of shoot Initiation	Shoot Length (cm)	Number of Shoots
	BAP	Kin			
1	1.0	-	53.33±3.33	3.03±0.09	3.67±0.33
2	1.5	-	56.66±3.33	3.87±0.07	3.67±0.33
3	2.0	-	73.33±3.33	4.67±0.07	4.67±0.33
4	2.5	-	90.00±5.77	5.23±0.09	5.33±0.33
5	3.0	-	90.00±0.00	5.10±0.10	5.00±0.00
6	4.0	-	93.33±3.33	5.07±0.03	5.67±0.33
7	5.0	-	76.66±3.33	4.87±0.09	4.33±0.33
8	-	1.0	66.67±3.33	3.43±0.15	3.33±0.33
9	-	1.5	70.00±0.00	3.77±0.17	4.33±0.33
10	-	2.0	83.33±3.33	5.47±0.09	4.67±0.33
11	-	2.5	96.67±3.33	5.77±0.09	5.67±0.33
12	-	3.0	86.66±3.33	5.37±0.09	5.33±0.33
13	-	4.0	73.33±3.33	4.47±0.12	5.00±0.57
14	-	5.0	63.33±3.33	3.07±0.09	3.33±0.33
Variables * Treatments			**F value 8.119 P value -0.000 Sig-****		

Each value represents the average of 3 replications (n=3); explants treated with BAP and Kinetin. p<0.05 Significant at 0.05 level p<0.01 significant at 0.01 level p>0.05 Not significant

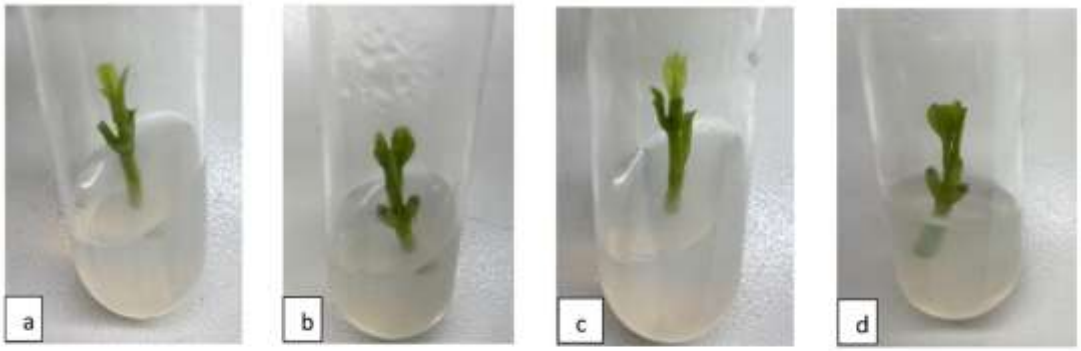

Figure 5 Initiation from nodal explants after one week of inoculation. a, b. BAP-supplemented medium. c, d. Kinetin-supplemented medium.

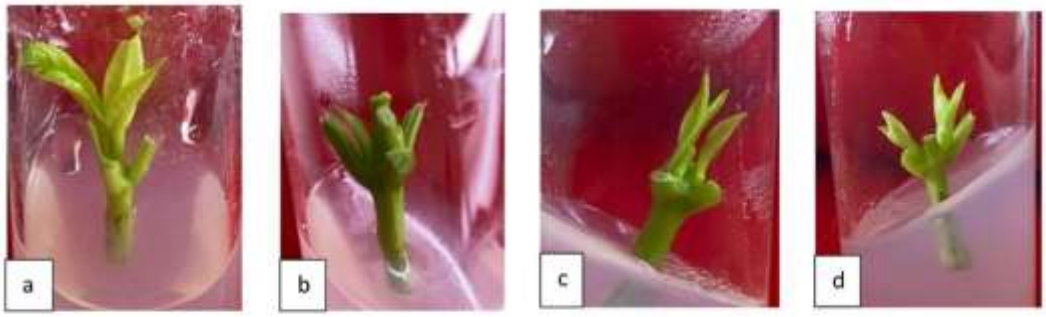

Figure 6 Initiation from nodal explants after two weeks of inoculation, a, b. BAP supplemented medium. c, d. Kinetin-supplemented medium

Multiplication from shoot tip explants

Initiated shootlets were subcultured to the multiplication medium containing a combination of growth hormones: BAP 1.5mg/l -5.0 mg/l and kinetin 1.0 mg/l.

In BAP with Kinetin combination, the highest (97.78%) multiplication rate was recorded at 2.5+1.0 mg/l, and the lowest (73.00%) initiation rate was observed at 5.0+1.0 mg/l. The maximum shoot length (5.80cm) was observed at 2.5+1.0 mg/l, and the lowest (3.37cm) at 5.0+1.0mg/l. whereas the number of shoots per explant was also found to be maximum (5.33cm) at 2.5+1.0 mg/l, and the lowest (3.00) at 1.5+1.0 mg/l.

BAP in combination with IBA yielded the highest response (97.78%) at 4.0+1.0 mg/l and the lowest (77.55%) at 1.5+1.0 mg/l. The highest (9.00cm) shoot length was recorded at 4.0+1.0 mg/l, and, in contrast, the lowest (4.13cm) was observed at 1.5+1.0 mg/l. The highest (7.33) number of shoots per plantlet was recorded at 5.0+1.0 mg/l, whereas the lowest (3.67) was recorded at 1.5+1.0 mg/l.

In BAP+IBA (1.5mg/l-5.0mg/l+0.5 mg/l) supplemented medium, maximum shoot length (9.00cm) was observed at 4.0+0.5mg/l, whereas the plantlets with an increased number of shoots per explant were observed at BAP+IBA 5.0mg/l+0.5 mg/l. The results are presented in the Table no.3.

Among the concentrations and combinations of growth hormones, BAP in combination with Kin & IBA was later effective in rapid multiplication, with increased shoot length and a higher number of shoots per explant. The highest concentration of cytokinin (BAP) with low auxin favors maximum shoot length with a larger number of shoots.

Table 3. Effect of growth hormones BAP + Kin & BAP + IBA on Multiplication of *Rauvolfia serpentina* from apical shoot explants.

S.No	Growth Hormone Concentration (mg/L)		% of multiple shoot formation	Shoot Length (cm)	Number of Shoots
	BAP + Kin	BAP + IBA			
1.	1.5 + 1.0	-	75.33±2.33	3.60±0.57	3.00±0.58
2.	2.0 + 1.0	-	91.11±4.44	5.23±0.15	4.33±0.33
3.	2.5 + 1.0	-	97.78±2.22	5.80±0.12	5.33±0.33
4.	3.0 + 1.0	-	91.11±2.22	5.13±0.09	4.67±0.33
5.	4.0 + 1.0	-	84.44±2.22	3.80±0.12	3.67±0.33
6.	5.0 + 1.0	-	73.00±4.04	3.37±0.09	3.33±0.33
7.	-	1.5 + 0.5	77.55±4.55	4.13±0.09	3.67±0.33
8.	-	2.0 + 0.5	82.22±2.22	5.33±0.13	4.00±0.00
9.	-	2.5 + 0.5	84.44±2.22	5.86±0.12	4.33±0.33

10.	-	3.0 + 0.5	91.11±2.22	7.70±0.15	5.67±0.33
11.	-	4.0 + 0.5	97.78±2.22	9.00±0.12	6.67±0.33
12.	-	5.0 + 0.5	95.55±2.22	8.80±0.15	7.33±0.33
Variables * Treatments			**Fvalue-10.684 p-value-0.000 sig-****	**Fvalue-224.972 p-value-0.000 sig-****	**f-value-15.446 p-value-0.000 sig-****

Each value represents the average of 3 replications (n=3); explants treated with BAP + Kin and BAP + IBA combination of plant growth regulators ± indicate the standard error values. p<0.05 Significant at 0.05 level p<0.01 significant at 0.01 level p>0.05 Not significant

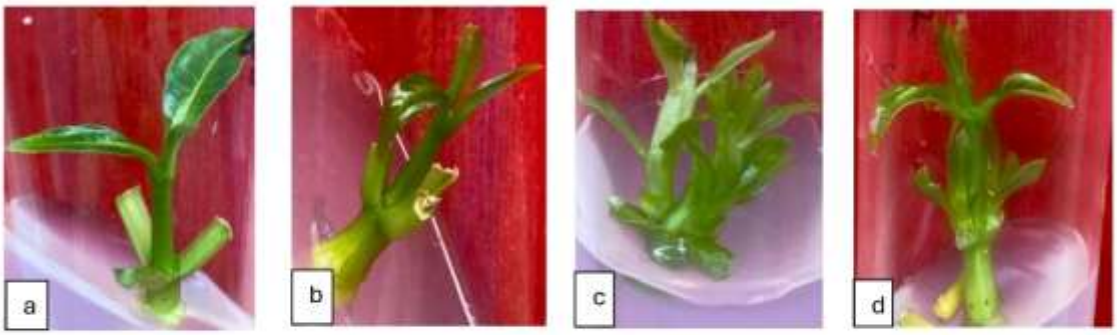

Figure 3 Shoot multiplication from apical shoots after one week of inoculation. a, b. BAP supplemented medium. c, d. Kinetin supplemented medium.

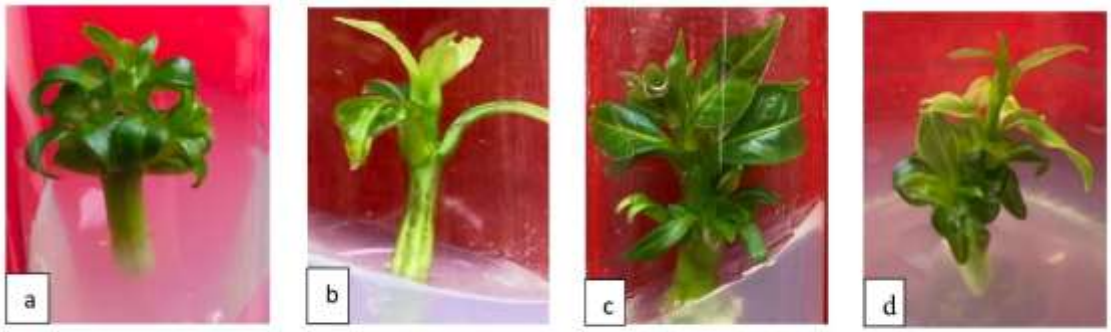

Figure 4 Shoot multiplication from apical shoots after two weeks of inoculation. a, b. BAP supplemented medium. c, d. Kinetin supplemented medium.

Multiplication from nodal explants

Initiated nodal explants were cut into bits and inoculated onto MS medium fortified with BAP 1.5mg/l -5.0mg/l in combination with kinetin 1.0mg/l and BAP 1.5 mg/l - 5.0 mg/l with IBA 0.5mg/l.

In BAP+Kin fortified medium, the highest (84.44%) multiplication rate was recorded at 3.0+1.0 mg/l, and the lowest (64.00%) at 5.0+1.0 mg/l. The highest shoot length (5.00cm) was recorded at 3.0+1.0mg/l and the lowest (3.27cm) at 1.5+1.0 mg/l. Shootlets with a larger number (4.67) of shoots were reported at 3.0+1.0 mg/l, and fewer (2.67) number of shoots/shootlets were recorded at 5.0+1.0 mg/l.

In BAP+IBA-supplemented medium, the maximum (97.78%) multiplication percentage was recorded at 5.0+1.0 mg/l, whereas the lowest (62.00%) was recorded at 1.5+1.0 mg/l. Shoot length was observed to be highest (6.77 cm) at 4.0+0.5 mg/l BAP+IBA, and lowest (4.47cm) shoot length was recorded at 1.5+1.0 mg/l. The number of shoots per explant at 4.0+0.5 mg/l was high (5.67), and the lowest number (2.33) was recorded at 1.5+1.0 mg/l. The results are presented in the Table no.4

Table 4. Effect of growth hormones BAP + Kin & BAP+IBA on Multiplication of *Rauvolfia serpentina* from node explants.

S.No	Growth Hormone Concentration (mg/L)		Initiation percentage	Shoot Length (cm)	Number of Shoots
	BAP + Kin	BAP + IBA			
1	1.5 + 1.0	-	64.33±4.33	3.27±0.07	3.33±0.33
2	2.0 + 1.0	-	79.89±3.94	4.13±0.09	4.33±0.33
3	2.5 + 1.0	-	80.00±0.00	4.63±0.12	4.33±0.33
4	3.0 + 1.0	-	84.44±2.22	5.00±0.12	4.67±0.33
5	4.0 + 1.0	-	68.33±2.33	3.80±0.15	3.00±0.00
6	5.0 + 1.0	-	64.00±2.00	3.60±0.06	2.67±0.33
7.	-	1.5 + 0.5	62.00±2.00	4.47±0.12	2.33±0.33
8.	-	2.0 + 0.5	68.33±2.33	5.00±0.12	3.00±0.00
9.	-	2.5 + 0.5	79.89±3.94	5.47±0.12	3.67±0.33

10.	-	3.0 + 0.5	88.88±2.22	6.10±0.10	4.67±0.33
11.	-	4.0 + 0.5	95.55±2.22	6.77±0.15	5.67±0.33
12.	-	5.0 + 0.5	97.78±2.22	6.73±0.20	5.67±0.33
Variables * Treatments			**f-value-21.680 pvalue-0.000 sig-****	**f-value-37.85 p-value0.000 sig-****	**f-value-19.600 p-value0.000 sig-****

Each value represents the average of 3 replications (n=3); explants treated with BAP+kin &BAP+ IBA combination of plant growth regulators± indicates the standard error values, p<0.05, Significant at 0.05 level, p<0.01, significant at 0.01 level, p>0.05, not significant

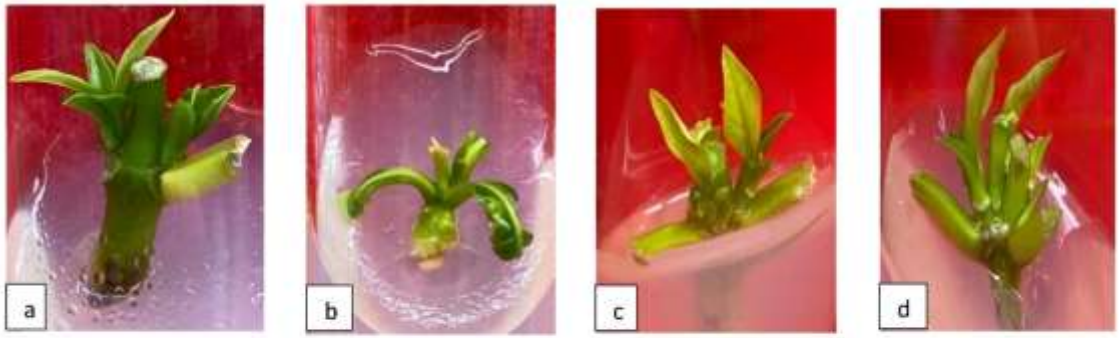

Figure 7 Shoot multiplication from nodal explants after one week of inoculation. a, b BAP supplemented medium. c, d. Kinetin supplemented medium.

Figure 8 Shoot multiplication from nodal explants after two weeks of inoculation. a, b. BAP supplemented medium. c, d. Kinetin supplemented medium.

In vitro root initiation from shoot tip explants

Well-developed multiple shoots were inoculated into the liquid rooting medium fortified with root hormones: NAA (0.4 mg/l-2.0 mg/l) in combination with IBA 0.5 mg/l, or NAA (0.4 mg/l-2.0 mg/l) in combination with IAA 0.5 mg/l.

In the NAA+IBA-fortified medium, the root initiation percentage ranged from 60.00% to 73.33%. The highest (73.33%) rooting response was observed at 1.0mg/l+0.5 mg/l, while the lowest (60.00%) rooting response was observed at 2.0mg+0.5mg/l. The root length was highest (3.27cm) at 0.8 mg/l-0.5 mg/l and lowest (1.13cm) at 2.0 mg/l+0.5 mg/l. The number of roots/shootlets reported the highest (6.67) at 1.0 mg/l + 0.5 mg/l, and the lowest (3.33) at 2.0 mg/l + 0.5 mg/l.

In NAA+IAA-supplemented medium, the rooting initiation percentage ranged from 57.78% to 71.11%. The highest (71.11%) rooting response was recorded at 1.0 mg/l + 0.5 mg/l, and the lowest (57.78%) at 2.0 mg/l + 0.5 mg/l. The highest (5.50cm) root length was recorded at 1.0 mg/l + 0.5 mg/l, and the lowest (2.57cm) at 0.4 mg/l + 0.5 mg/l. The shootlet with the highest (5.33) number of roots was recorded at 0.8mg/l+0.5mg/l and the lowest (3.33) number of roots/shootlets was recorded at 2.0mg/l+0.5mg/l. The results are presented in the Table no.5

The rooting response of shoots was significantly higher when the rooting medium was supplemented with NAA in combination with IAA, as reflected by root initiation, a greater number of roots/shoot, and improved root length, indicating that IAA-containing media are more effective for root induction and root development than NAA+IBA. The NAA + IBA combination produced fewer, shorter roots, indicating a lower rooting response. NAA+IBA's role in assisting adventitious root formation may be attributed to the differential stability and transport properties of auxins in the plant system.

IAA is a natural auxin with elevated bioactivity, and when combined with NAA, it seems to synergistically enhance cell division and differentiation in the root initiation zone of shoots. There is only a slight difference in the rooting response when IAA and IBA are combined with NAA. The results are consistent with the reports of Bokade *et al.,* (2024), who supported the IBA role in the medium in scaling up rhizogenesis.

Table 5. Effect of growth hormones NAA + IBA & NAA+IAA on Root initiation of *Rauvolfia serpentina* from apical shoot explants

S.No	Growth Hormone Concentration (mg/L)		Initiation percentage	Root Length (cm)	Number of Roots
	NAA + IBA	NAA + IAA			
1	0.4 + 0.5		62.22±2.22	1.97±0.88	4.33±0.33
2	0.8 + 0.5		71.11±2.22	3.27±0.15	5.33±0.33
3	1.0 + 0.5		73.33±0.00	2.73±0.24	6.67±0.33
4	1.5 + 0.5		66.66±0.00	1.70±0.25	3.67±0.33
5	2.0 + 0.5		60.00±0.00	1.13±0.09	3.33±0.33
6.		0.4 + 0.5	60.00±0.00	2.57±0.12	3.67±0.33
7.		0.8 + 0.5	68.88±2.22	2.87±0.09	5.33±0.33
8.		1.0 + 0.5	71.11±2.22	5.50±0.26	4.67±0.33
9.		1.5 + 0.5	64.44±2.22	4.90±0.21	4.33±0.33
10.		2.0 + 0.5	57.78±2.22	3.80±0.15	3.33±0.33
Variables * Treatments			**f-vale-0.000 p value-1.000 sig-NS**		

Each value represents the average of 3 replications (n=3); explants treated with NAA+IBA and NAA+IAA combination of plant growth regulators± indicates the standard error values
p<0.05 Significant at 0.05 level p<0.01 significant at 0.01 level p>0.05 Not significant

In vitro root initiation from nodal explants

In vitro grown shootlets from nodal explants of *R.serpentina* were transferred to MS rooting liquid media fortified with NAA (0.4 mg/l -2.0 mg/l) in combination with IBA(0.5mg/l) and IAA (0.5 mg/l).

In NAA+IBA-fortified medium, the root initiation percentage ranged from 57.78% to 71.11%. The maximum (71.11%) rooting response was observed at NAA 1.5 mg/l + IBA 0.5 mg/l. The least response (57.78%) was reported at NAA 2.0 + IBA 0.5 mg/l. The maximum (3.63cm) root length was observed at 1.0mg/l+0.5 mg/l, and the lowest (2.23cm) root length was recorded at 0.4mg/l+0.5mg/l. The number of roots/shootlets was observed to be highest (6.33) at the 1.0mg/l+0.5mg/l concentration and lowest (4.00) at 0.4mg/l+0.5mg/l. *R. serpentina* showed a strong rooting response in a medium supplemented with NAA and IBA. A low concentration of NAA combined with a low concentration of IBA enhanced the rooting response.

In NAA(0.4 mg/l – 2.0 mg/l) + IAA (0.5 mg/l) the rooting response varied between 48.88%-68.88%. The maximum (68.8%) root initiation at 1.0 mg/l + 0.5 mg/l and the least response (48.8%) at 2.0 mg/l + 0.5 mg/l wereobserved. Shootlets with maximum (4.70cm) root length were observed at 1.0mg/l+0.5mg/l and the lowest (3.27cm) root length was recorded at 2.0 + 0.5mg/l. The number of roots was observed to be high (5.33) at 0.8mg/l+0.5mg/l, and the lowest (3.33) number of roots/shootlets was recorded at 2.0mg/l+0.5mg/l. The results are presented in the Table no.6

The results clearly showed the strongest rooting response in the NAA+IBA-supplemented medium compared to the NAA+IAA-supplemented medium, which is in line with the studies of Vaishnavi Zade *et al*., (2024). The root-forming ability fell below 50% in the NAA+IAA-supplemented medium.

Table.6 Effect of growth hormones NAA + IBA & NAA+IAA on Root initiation of *Rauvolfia serpentina* from node explants.

S.No	Growth Hormone Concentration (mg/L)		Initiation percentage	Root Length (cm)	Number of Roots
	NAA + IBA	NAA+IAA			
1	0.4 + 0.5	-	60.00±0.00	2.23±0.20	4.00±0.58
2	0.8 + 0.5	-	64.33±4.33	2.67±0.33	5.67±0.33
3	1.0 + 0.5	-	68.88±2.22	3.36±0.20	6.33±0.33
4	1.5 + 0.5	-	71.11±2.22	3.47±0.20	5.67±0.33
5	2.0 + 0.5	-	57.78±2.22	2.87±0.09	4.33±0.33
6.	-	0.4 + 0.5	55.55±2.22	3.37±0.22	5.33±0.33
7.	-	0.8 + 0.5	62.22±2.22	4.33±0.20	6.33±0.33
8.	-	1.0 + 0.5	68.88±2.22	4.70±0.26	7.33±0.33
9.	-	1.5 + 0.5	64.44±2.22	3.77±0.12	5.67±0.33
10.	-	2.0 + 0.5	48.88±2.22	3.27±0.18	4.33±0.33
Variables * Treatments			**f-vale-1.070 p value-0.397 sig-NS**		

Each value represents the average of 3 replications (n=3); explants treated with NAA+BAP & NAA+IAA of plant growth regulators± indicates the standard error values. p<0.05 Significant at 0.05 level p<0.01 significant at 0.01 level p>0.05 Not significant

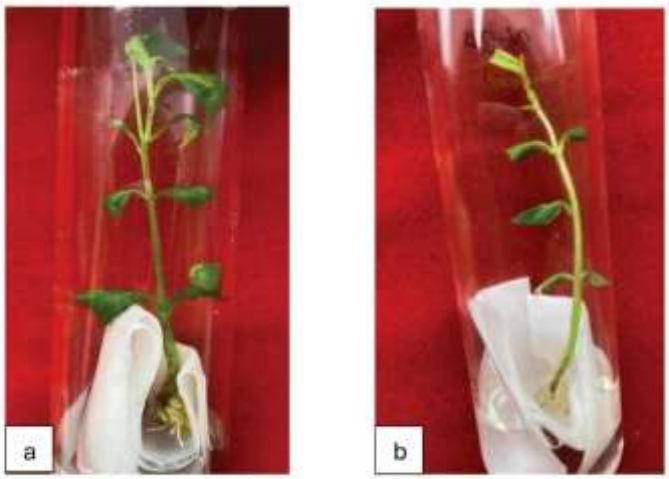

Figure 9 *In vitro* rooting of apical shoot and nodal explant. a. Apical shoot b. Nodal explant.

Figure 11 Acclimatization of *in vitro* grown plantlets. a. Apical shoot. b. Node

Ex vitro rooting from nodal explants.

Explants were treated with auxins IBA and IAA alone at 1.0 and 1.5 mg/l for about 15 min. Then, these treated shootlets were placed in ex vitro rooting media containing a 1:1 mixture of cocopeat and vermiculite, and the results are presented in Tables no.7.

Table. 7 Effect of growth hormones alone, IBA and IAA on *ex vitro* Root initiation of *Rauvolfia serpentina* from apical shoots and Node explants

S.No	Growth Hormone Concentration (mg/L)		Root Initiation percentage from apical shoots	Root Initiation percentage from node explants	Root Length (cm)	Number of Roots
	IBA	IAA				
Apical shoots						
1	1.0		53.33±6.66	-	4.17±0.12	7.33±0.33
2	1.5		73.33±6.66	-	5.40±0.12	8.67±0.33
3		1.0	46.67±6.66	-	4.27±0.18	0.58±0.33
4		1.5	66.67±6.66	-	3.83±0.12	0.58±0.33
Node explants						
1.	1.0		-	46.67±6.66	4.00±0.12	6.33±0.33
2.	1.5		-	66.67±6.66	4.17±0.12	5.67±0.33
3.		1.0	-	53.33±6.66	3.83±0.09	5.67±0.33
4.		1.5	-	60.00±0.00	3.60±0.06	4.67±0.33
Variables * Treatments			**Fvalue-0.000 P value-1.000 Sig-NS**	**Fvalue-1.333 P value-0.282 Sig-NS**		

Each value represents the average of 3 replications (n=3); explants treated with BAP and IBA combination of plant growth regulators± indicates the standard error values. p<0.05 Significant at 0.05 level p<0.01 significant at 0.01 level p>0.05 Not significant

In the IBA and IAA (1.0 and 1.5 mg/l), the rooting response of apical shoots varied from 53.33 to 73.33%. The maximum (73.33) initiation was recorded with IBA alone at 1.5 mg/l, followed by IAA alone at 1.0 mg/l (66.67), and the lowest was recorded with IAA alone at 1.0 mg/l (46.67). Shootlets with maximum (5.40 cm) root length and number of rootlets (8.67) were noticed in IBA at 1.5 mg/l, and minimum length (3.83cm) in IAA at 1.5 mg/l, and the least number (6.67 cm) in IAA at the concentration 1.0 mg/l.

In the IBA and IAA (1.0 and 1.5 mg/l), the rooting response from nodal explants varies from 46.67 to 66.67%. The maximum (66.67%) initiation was observed with IBA alone at 1.5 mg/l, followed by IAA alone at 1.0 mg/l (53.33), and the lowest was recorded with IAA alone at 1.0 mg/l (46.67). Shootlets with maximum (4.17 cm) root length were noticed at 1.5 mg/l IBA, and minimum length (3.60 cm) in IAA at 1.5 mg/l. The highest NAA (0.4-2.0 mg/l) in combination with IBA and IAA (0.5 mg/l).

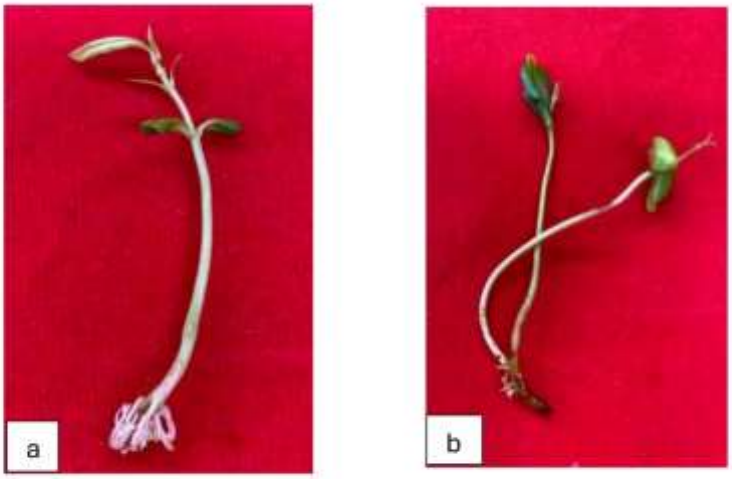

Figure 10 *Ex vitro* rooting of apical shoot and nodal explant. a. Apical shoot b. Nodal explant.

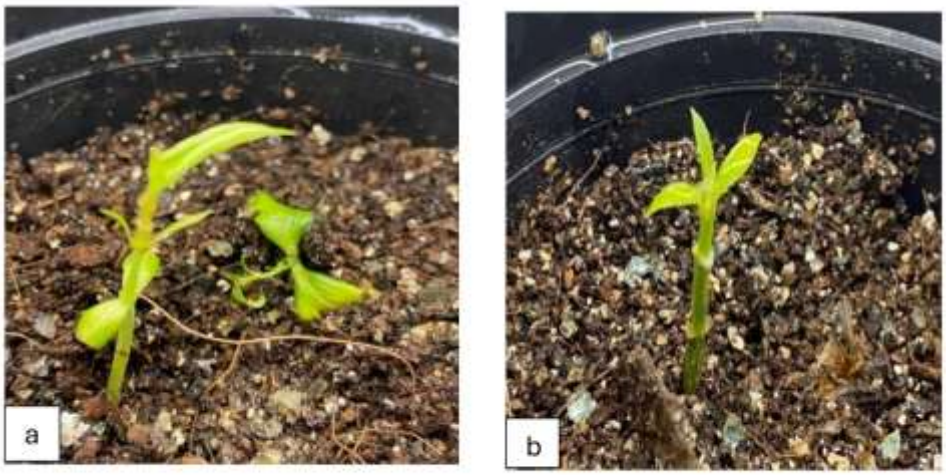

Figure 12 Acclimatization of *ex vitro* grown shootlets. a. Apical shoot. b. Node

Discussion

Among the different concentrations and combinations evaluated for shoot initiation percentage, those in BAP-supplemented medium were relatively low. In contrast, Kinetin was more effective than BAP in inducing shoots from shoot tip explants, especially at lower concentrations. The results clearly indicated that Kinetin-driven rapid shoot initiation, whereas BAP enhanced shoot vigor. A moderate kinetin concentration produced the highest initiation percentage, shoot multiplication, and shoot length, while a higher BAP concentration supported initiation but reduced shoot quality and induced callus formation.

The present findings of the study diverge from those of Rossa Yunita *et al.,* who suggested that BAP was more effective in inducing shoot initiation. Current work is carried out with shoot tips and nodal explants for direct regeneration, in which shoot tip explants are more responsive than nodal explants. Among the two cytokinins tested for initiation response, Kinetin exhibited optimal efficacy for direct regeneration from both shoot tip and nodal explants. Kinetin was also found to be effective in increasing the number per explant and the longest shoot length. Kinetin also influenced the development of lateral buds by promoting rapid cell division and breaking dormancy.

Very low concentration of cytokinins (BAP) failed to induce bud break, while high concentration triggered callus formation instead of shoot development. These observations contrast with the findings of Artiwahurwagh *et al*., (2022), Khan *et al.*, (2018), and Mahapatra *et al.,* (2024), who reported that the combination of growth hormones induced the fastest response.

BAP and kinetin combination was effective for shoot multiplication in numerous plant species. BAP at a low concentration (2.5 mg/l) combined with 1.0 mg/l kin promoted a rapid multiplication response, which progressively declined with increasing BAP concentration. The present reports are in accordance with the results reported by Arti *et al.,* (2022).

But in the present study, the combined effect of BAP and IBA elicited the highest multiplication response in the sarpangandha species. Among both concentrations and combinations, elevated shoot multiplication percentage was observed for BAP+Kin and BAP+IBA; however, BAP+Kin showed stronger multiplication at low concentration compared to BAP+IBA. Whereas BAP+IBA is effective in inducing rapid multiplication with a greater number of shoots than BAP+Kin. In both explants, the apical shoot tips were found to be more responsive than the nodal explants.

Regeneration of multiple shoots was rapid at the initial stage but subsequently slowed under the BAP+Kin combination. In contrast, BAP+IBA combinations effectively induced multiple shoots from nodal explants. The present findings are on par with the responses of Arti Wahurwagh *et al.,* (2022) and Vandana Jain *et al.,* (2002).

The rooting response of shoots was significantly higher when the rooting medium was supplemented with NAA in combination with IAA, as reflected by root initiation, a greater number of roots/shoot, and improved root length, indicating that IAA-containing media are more effective for root induction and root development than NAA+IBA. The NAA + IBA combination produced fewer, shorter roots, indicating a lower rooting response. NAA + IBA's role in assisting adventitious root formation may be attributed to the differential stability and transport properties of auxins in the plant system.

IAA is a natural auxin with elevated bioactivity, and when combined with NAA, it seems to synergistically enhance cell division and differentiation in the root initiation zone of shoots. There is only a slight difference in the rooting response when IAA and IBA are combined with NAA. The results are consistent with the reports of Bokade *et al.,* (2024), who supported the IBA role in the medium in scaling up rhizogenesis.

In nodal explants, the in vitro rooting media fortified with NAA + IBA, the root initiation percentage ranged from 57.78% to 71.11%. The results clearly showed the strongest rooting response in the NAA+IBA-supplemented medium compared to the NAA+IAA-supplemented medium, which is on par with the studies of Vaishnavi Zade et *al.,* (2024). The root-forming ability fell below 50% in the NAA+IAA-containing medium. This suboptimal growth indicates auxin-specific media for a strong rooting response, supported by the studies of Jyoti Kumari and Jai Kumar (2019).

Ex vitro rooting from nodal explants.

Gradual acclimatization from *in vitro* to *ex vitro* conditions is an essential step, as immature cuticles and stomata cause them to wilt under low humidity and low light intensity. The *ex vitro* plants survive better and undergo rapid acclimatization to the natural environment, as per Annapurna and Rathore (2010). The present reports are in accordance with the findings of Shekhawat and Manokari (2015).

When the *in vitro* grown plantlets were exposed to the outside environment, they experienced sudden desiccation and death. In order to overcome the physiological limitations, gradual acclimatization for two to three weeks was followed by a proper potting mixture, which facilitates the better growth of *in vitro* grown plantlets. Plants produced *ex vitro* were comparatively stronger, with more shoots per shootlet, higher rooting efficiency, and better survival rate than *in vitro*-grown plants.

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