PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol x(x): xx–xx
https://doi.org/10.14719/pst.2858

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RESEARCH ARTICLE

Efficient protocol for micropropagation of medicinal forest tree Shyonak (*Oroxylum indicum)* by silver nitrate promoted high frequency shoot proliferation

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OPEN ACCESS

ARTICLE HISTORY

Received: 10 August 2023
Accepted: 20 November 2023

Available online
Version 1.0 : 28 March 2024

Check for updates

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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Publisher's Note: Horizon e-Publishing Group remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS, UGC Care, etc See https://horizonepublishing.com/journals/index.php/PST/indexing_abstracting

CITE THIS ARTICLE

Joshi N, Nailwal TK ,Dutta S. Efficient protocol for micropropagation of medicinal forest tree Shyonak (*Oroxylum indicum*) by silver nitrate promoted high frequency shoot proliferation. Plant Science Today (Early Access). https://doi.org/10.14719/pst.2858

Abstract

Oroxylum indicum, a significant medicinal plant in the Indian Himalayan Region (IHR), faces ecological and economic challenges due to high demand for its traditional herbal remedies, leading to overexploitation. To combat this, the authors have developed an *in vitro* propagation protocol aimed at achieving robust shoot proliferation. Using nodal segments from *in vitro* germinated seedlings, the best results were obtained with a medium containing only benzyladenine (BA) at 22.2 µM. Despite basal callus formation and reduced shoot numbers, the addition of silver nitrate improved shoot proliferation and overall plant health. For *in vitro* root induction and proliferation, a medium enriched with Indole-3-acetic acid (IAA) at 2.85 µM proved the most effective. Successful transplantation of acclimatized plantlets to field conditions was achieved. This protocol not only aids *O. indicum* conservation but also holds socio-economic potential for Himalayan communities, as its bark is valuable in commercial Ayurvedic preparations, including Dashamoolarishta.

Keywords

shyonak; nodal segments; silver nitrate; shoot multiplication; root induction

Introduction

Oroxylum indicum (Family Bignoniaceae) holds significant economic, ecological, and medicinal value. It encompasses over 112 genera and more than 725 species of trees, shrubs, and vines found worldwide. *O. indicum* is a deciduous tree of small to medium size, widely distributed in tropical and subtropical regions. It is a native habitat of the Indian subcontinent, extending from the Himalayan foothills into Bhutan, South China, and the Malaysian ecozone (1). In India, it is prevalent in the Eastern and Western Ghats, as well as North East India, situated between latitudes 21°34´N to 29°50´N and longitudes 87°32´E to 97°52´E (2, 3).

It is commonly referred to as "Shyonaka" or "Indian trumpet flower" in English (4). This plant finds extensive use in traditional Ayurvedic medicine. Traditional knowledge from the Maram Naga village in Sonapat district, Manipur, highlights that the decoction of its bark has the potential to be a potent anticancer remedy, particularly effective against nasopharyngeal cancer (5). Local tribes in Nagaland, including Seema, Angami, and Lotha, incorporate young leaves into their diets to manage hypertension. Consuming raw seeds for hypertension control is a novel practice requiring further verification (3). The decoction of the root bark is

employed by tribal communities to treat jaundice. Additionally, studies have reported the hepatoprotective properties of the crude aqueous extract of *O. indicum* root bark against liver damage in experimental animals (6 - 10). The root is a vital component of traditional Ayurvedic formulations such as Dasamula, Brahma Rasayana, Dhanawatara, Awalwha, and Narayana Taila, among others (11, 12). In China, the seeds of *O. indicum* are utilized for liver and stomach issues, ulcers, and boils. In Burma, Vietnam, and the Philippines, the bark is employed to manage dysentery and rheumatism (13, 14).

Most of the Himalayan plants, which have either medicinal or aesthetic value, are facing the danger of extinction, and many of them are limited to very few places in their natural habitat. Uttarakhand is an Indian state known for its natural beauty and for its floral diversity. Due to the increased interference of man with nature, there is a gradual loss of state floral wealth. The situation is so alarming that only 432 plants of *O. indicum* are present in Uttarakhand, and many of them are on the verge of extinction. Under such circumstances, the plant tissue culture technique might be a savior in raising the number of these plants through the *in vitro* method.

The plant reproduces naturally through seeds, which typically germinate at the onset of the rainy season. During their early stages, seedlings require a moderate amount of shade. However, it's worth noting that seed production is limited, and the viability of these seeds is quite low. These challenges associated with natural propagation, coupled with unregulated harvesting for medicinal purposes, have led to its classification as an "endangered" species in certain states of India (15). The scarcity of *O. indicum* can be attributed to factors such as high demand in the pharmaceutical industry, slash-and-burn agriculture practices, and habitat destruction. Given the significance of plants in various medicinal formulations and their limited presence in their natural environment, there is an urgent necessity to establish a reproducible propagation protocol for their cultivation.

Materials and Methods

Plant Material

The mature seeds were collected from plants cultivated at Fatehpur, Kaladhungi Road, Haldwani (340 m asl; 29° 14'49.7" N lat.; 79°27'02.8" E long) in late December 2012. The herbarium file of the plant sample was deposited for identification at the Botanical Survey of India, Nothern Regional Centre, Dehradun, India (Accession No. 115360).

Explant Preparation

Seeds were aseptically germinated *in vitro* to obtain sterile seedlings, which served as the source plants for this study. The seed coats were mechanically removed, and the seeds were initially washed in running tap water for 30 minutes. Subsequently, they were immersed in a solution containing Tween-20 surfactant (6-7 drops) for 15 minutes with gentle agitation. After thorough rinsing, the seeds were treated with a fungicide solution (0.5% w/v bavistin) for 20 minutes with continuous shaking. The seeds were then transferred to a sterilized LAF, where the explants were cleaned five times with sterile Millipore water under aseptic conditions. Surface disinfection was carried out using mercuric chloride ($HgCl_2$, 0.1% w/v) for 5 minutes, followed by five subsequent rinses (2 minutes each) with sterile Millipore water.

Before inoculation, any remaining water on the surface of the explants was removed using autoclaved filter papers. These seeds were cultured under aseptic conditions on Murashige and Skoog (MS) medium having nil growth hormone (MS_{00}) and MS medium containing GA_3 and casein hydrolysate, augmented with 3% w/v sucrose and 0.8% *w/v* agar.

In vitro seed germination

Sterile seeds were inoculated in MS basal medium (PGR-free) and supplemented with different concentrations of GA_3 and casein hydrolysate in order to increase the germination rate. All media contained agar (0.8%, w/v) and sucrose (3%, w/v). Seeds in MS basal medium were used as a control (Table 1). Seed germination frequency (% germination) was calculated by the following formula:

Data for percentage germination and the number of days required for germination were recorded after one week of

$$\% \text{ germination} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds on seed germination medium}} \times 100$$

seed inoculation. These *in vitro* germinated seedlings were further used as explants.

Shoot induction

Table 1. Effect of GA_3 and casein hydrolysate on *in vitro* seed germination in *O. indicum*

Concentration of GA_3 and Casein (mg/L)	Percentage germination	Number of days required for germination
Control	19.94 ± 3.34^{a}	24.20 ± 0.37^{f}
Casein		
01.0	33.3 ± 5.28^{ab}	21.80 ± 0.58 ^{e}
02.0	41.65 ± 3.73^{b}	19.50 ± 0.43^{d}
Casein + GA_3		
00.0 + 04.0	23.28 ± 4.09^{a}	20.2 ± 0.37^{d}
00.0+ 08.0	33.3 ± 5.28^{ab}	19.6 ± 0.51^{d}
01.0+ 04.0	63.3 ± 6.23^{c}	16.8 ± 0.58^{c}
02.0+04.0	69.96 ± 6.23^{c}	15 ± 0.32^{b}
02.0+ 08.0	93.32 ± 4.09^{d}	11.6 ± 0.75^{a}

Note. Values are represented as the mean of triplicate ± SE, significant value at $p<0.05$ was calculated according to Duncan's multiple range- Posthoc test by using the statistical package SPSS (Statistical Package for Social Science). Value followed by the same letter in a column is not significantly different. Control- PGR-free medium. Data were recorded after 4-5 days of seed inoculation. These experiments were performed five times; six seeds were used per treatment.

Cotyledonary nodes possessing a short hypocotyl, which were derived from seedlings grown *in vitro*, were carefully excised and employed as explants to initiate and propagate shoots. These explants were introduced into an aseptic MS medium supplemented with various concentrations of plant growth regulators (PGRs) as indicated in Table 2. Every medium formulation contained sucrose (3% w/v) and agar (0.8% w/v). Following a four-week incubation period post-inoculation, data regarding the mean number of shoots per explant, the average length of shoots, and the average number of leaves per explant were meticulously recorded. The MS basal medium was employed as the control. Subsequent to this initial assessment, regular sub-culturing was conducted every four weeks on a medium of identical composition.

Effect of growth adjuvant

Table 2. Shoot induction from cotyledonary nodes taken from *in vitro* grown seedlings in MS medium supplemented with GA_3 and Casein hydrolysate.

PGRs(µM)	Avg. no. of shoots/ explant	Avg. shoot length (cm)	No. of leaves / explants
Control (without PGRs)			
BA			
	1.00± 0.01^{a}	0.79 ± 0.08^{a}	2.00 ± 0.01^{a}
8.88	1.22 ± 0.11^{ab}	1.61 ± 0.11^{b}	2.51 ± 0.15^{a}
17.76	1.66 ± 0.19^{b}	1.76 ± 0.18^{b}	3.11 ± 0.59^{a}
22.2	3.95 ± 0.33^{c}	6.23 ± 0.47^{c}	7.20 ± 0.99^{b}
BA+NAA			
0.00+2.68	1.14 ± 0.095^{ab}	1.23± 0.163^{ab}	2.30 ± 0.06^{a}
8.88+2.68	1.55 ± 0.223^{ab}	1.34± 0.091^{ab}	2.17 ± 0.43^{a}
17.6+2.68	1.22 ± 0.11^{ab}	1.41± 0.074^{ab}	2.11 ± 0.39^{a}
22.2+2.68	1.11 ± 0.11^{ab}	1.15 ± 0.03^{ab}	1.77 ± 0.29^{a}

Note. The presented values represent the mean of triplicate measurements ± standard error. Significance at the p=0.05 level was determined using Duncan's multiple range posthoc test through the statistical software SPSS (Statistical Package for Social Science). If values share the same letter within a column, they are not considered significantly different. The control group utilized a medium without plant growth regulators (PGRs). Each treatment group consisted of nine explants. The experiment was conducted in triplicate, with a total of 27 explants, and data were collected 30 days after inoculation.

MS medium containing 6-benzyladenine (BA 22.2µM) alone was initiated to be the best PGR for multiplying the shoots; however, basal callus development and a limited average number of shoots per explant were observed in it. Therefore, $AgNO_3$ (SN, v/v 1-10mg/L) was tested with BA for elimination of these problems. To study the further effect on the enhancement of shoot multiplication rate, sterilized SN was added to autoclaved media (Table 3).

Table 3. Effect of $AgNO_3$ on shoot Proliferation

PGRs (µM)	Avg. no. of shoots/ explant	Avg. shoot length (cm)	No. of leaves/ explants
Control (without PGRs)	0.66 ± 0.01^{a}	1.14±0.46^{a}	0.77 ± 0.30^{a}
BA			
4.43	1.33 ± 0.19^{a}	1.23 ± 0.07^{a}	1.77 ± 0.22^{ab}
8.88	1.25 ± 0.13^{a}	1.24 ± 0.03^{a}	1.33 ± 0.10^{ab}
13.32	1.88 ± 0.48^{a}	2.21 ±0.13^{ab}	2.66 ± 0.38^{bc}
BA+ $AgNO_3$			
4.43+2.94	3.88 ± 0.48^{b}	3.13 ± 0.12^{b}	3.99 ± 0.38^{c}
4.43+5.88	16.20 ± 0.99^{c}	6.85 ± 1.34^{c}	11.77 ± 1.35^{d}
4.43+11.76	3.66 ± 0.19^{b}	2.86 ± 0.44^{ab}	2.10 ± 0.29^{ab}
8.88+2.94	1.33 ± 0.19^{a}	2.04 ± 0.19^{ab}	1.11 ± 0.11^{ab}

Note. Values are the mean of triplicate ± SE significant value at $p<0.05$ calculated according to Duncan's multiple range- Posthoc test by using the statistical package SPSS (Statistical Package for Social Science). Value followed by the same letter in a column is not significantly different. Control: PGR-free medium. Each treatment consisted of nine explants in each. The experiment was performed in triplicates. In total, 27 explants were used, with 9 explants for each treatment, and the data was taken after 30 days of inoculation.

In vitro root induction and proliferation

Well-grown micro-shoots, about 3-5 cm in length, were exposed to continuous exposure of auxins, i.e., indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), in $MS_{1/2}$ or MS basal medium for root induction and proliferation. The medium was supplemented with varying concentrations of IBA and IAA alone. Among the used auxins, indole acetic acid (IAA, 2.85 µM) was the most effective and induced maximum roots after 20 days of inoculation in a half-strength MS medium (Table 4).

Table 4. Effect of IBA and IAA on root induction in *in vitro* regenerated shoots

PGRs	Concentration (µM)	Root
Control		–
IBA	1.23	_
	2.46	+
	4.92	+ +
IAA	1.42	+
	2.85	+ + +
	5.71	_

+ + +: assumed as 100% response; + + : assumed as 75% response; + : assumed as 50% response; _ : no root induction

Hardening and acclimatization

Mature, well-established plants with substantial root systems were carefully extracted from the culture bottles after a six-week cultivation period. The plant roots were gently cleansed under a continuous stream of tap water to eliminate any residual clarigel. Subsequently, these plantlets were transplanted into thermacol cups (12 x 8 cm^2) filled with a sterile mixture of soil, sand, and farmyard manure (in a ratio of 3:1:1, w/w). To maintain optimal humidity levels, they were covered with plastic bags punctured with small holes and placed within a controlled culture room environment (maintained at 25°C ± 2°C, with a 16-hour light/8-hour dark photoperiod). These transplants were watered on alternate days.

After a growth period of 20 days, the polybags covering the thermacol cups were removed, and the plants were left undisturbed for an additional 40 days. Subsequently, they were transplanted into mud pots (15 x 20 cm^2) containing garden soil and placed in a greenhouse environment for a period of 12 weeks to facilitate acclimatization. Once adequately hardened, the plants were transferred to a polyhouse for a three-week transitional phase before being exposed to direct sunlight.

Data Analysis

The experiments were conducted following a fully randomized design to assess the impact of various treatments. Each treatment group consisted of nine explants, and this setup was replicated three times. Statistical analysis was carried out using the SPSS software (Statistical Package for Social Science, version 17). Analysis of variance (ANOVA) was employed, and the statistical significance of the results was assessed using Duncan's multiple range post hoc test. Significance was considered at a level of $p<0.05$.

Results

Effect of GA_3 and Casein Hydrolysate on seed germination:

In order to get rapid germination of seeds, GA_3 (0-8mg/L) and casein hydrolysate (0-2mg/L) were added to the MS basal medium. Seeds inoculated on MS medium supplemented with GA_3 and casein hydrolysate showed a significant increase in germination percentage within 10-15 days after inoculation as compared to the seeds inoculated in MS basal medium without GA_3 and casein hydrolysate. The mean value of germination percentage revealed that the addition of GA_3 and casein hydrolysate to the basal medium was effective for all combinations used. The germination percentage increased with an increase in the concentration of GA_3 and casein hydrolysate. The addition of GA_3 (8 mg/L) and casein hydrolysate (2 mg/L) to the MS basal medium increased the germination percentage up to 93.32 percent in a minimum number of days (11.6 days) (Table 1). Different stages of seed germination are shown in Figure 1 (a - d). The results indicate that casein hydrolysate and GA_3 were both effective in increasing the rate of germination, along with a decrease in the number of days required for germination. GA_3 and casein hydrolysate in combination were found to give the best response in a short duration with a maximum percentage of germination. Seeds inoculated in MS basal medium also showed germination, but the response was delayed (24.2 days), with a germination percentage of only 19.94.

Shoot induction:

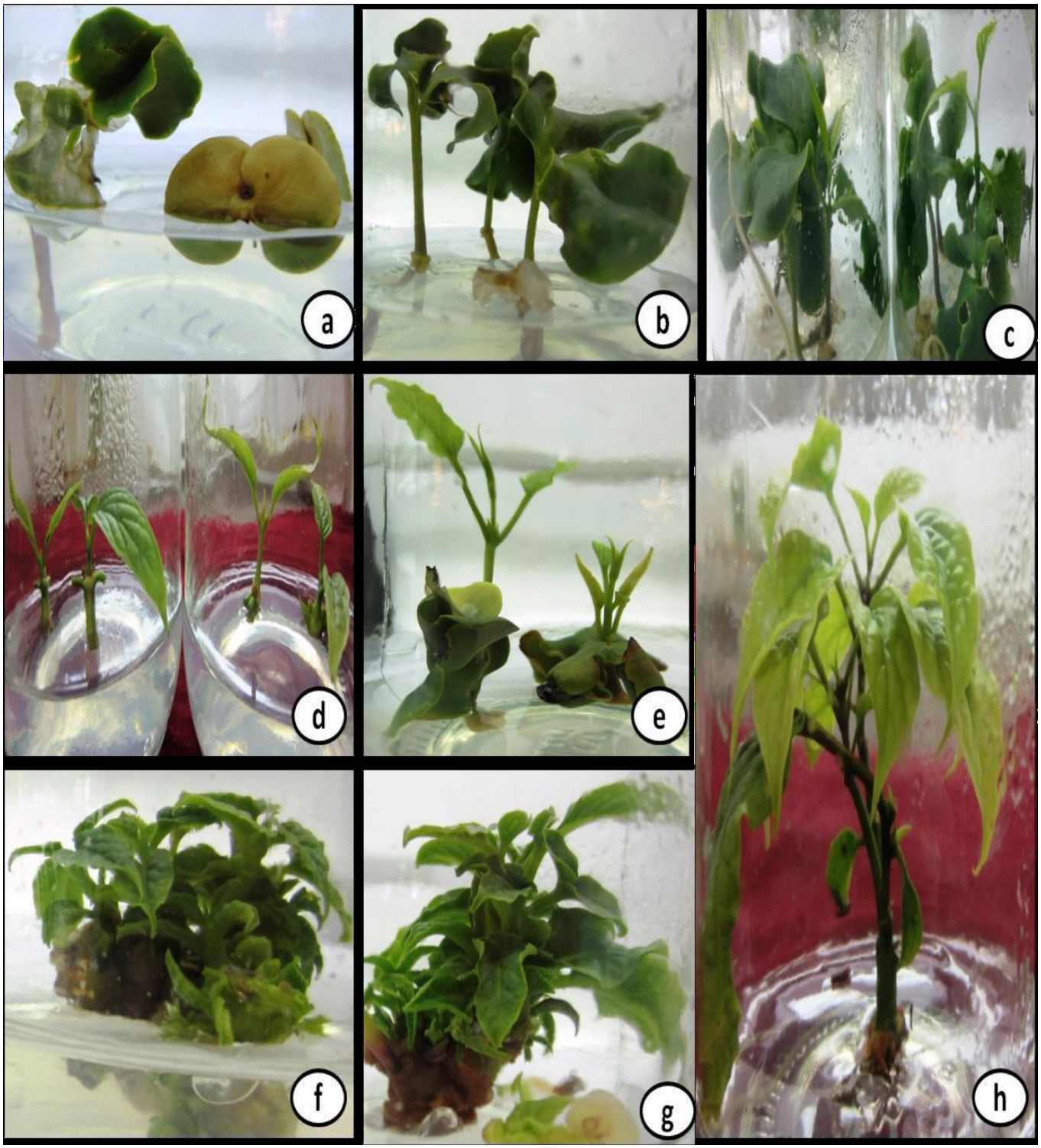
To see the synergistic effect of the cytokinin–auxin combination on shoot induction and multiplication, a cotyledonary node with a short hypocotyl of an *in vitro* germinated seedling was used as the explant. Nodal segments failed to induce morphogenesis in the MS basal medium; they remained green for up to 15 days, gradually became brown, and finally died after 30 days of inoculation. No bud breaks were observed from cotyledonary node explants in the medium without growth regulators. Incorporation of cytokinin (BA) and auxin (NAA) showed bud break and shoot growth (Table 2, Figure 1, e). Among the concentrations and combinations of BA, (22.2 µM) was found to be the most effective concentration for shoot proliferation when used alone, as this was able to produce a greater number of healthier shoots (3.95 ± 0.330) per explant in MS medium. The shoot length and number of leaves also increased to 6.23 ± 0.466 and 7.2 ± 0.986, respectively, when BA was used in the MS medium for shoot induction. BA (22.2 µM) was found to be optimum for shoot induction but did not facilitate massive shoot proliferation, and the number of leaves was found to be less and yellow in color. Therefore, a growth adjuvant was used.

Effect of growth adjuvant on shoot proliferation:

To optimize the number of shoots per explant, silver nitrate ($AgNO_3$) was used as an adjuvant. For multiple shoot induction, the in-vitro regenerated plantlets were transferred to the MS medium supplemented with BA alone and in combination with $AgNO_3$. The effect of continuous supplementation of PGRs on direct shoot proliferation was observed up to three subculture stages of 20 days each. In order to find the optimum concentration of $AgNO_3$ for best shoot proliferation, different concentrations of $AgNO_3$ and BA were used. The addition of $AgNO_3$ to the medium significantly enhanced shoot proliferation, as indicated in Table 3 and Figure 1 (f -g). From this experiment, it was revealed that shoot elongation and proliferation were enhanced sufficiently on BA (4.43 µM) with $AgNO_3$ (5.88 µM). However, the rate of shoot proliferation decreased at higher concentrations of $AgNO_3$ (11.76 µM).

MS medium supplemented with BA (4.43 µM) and (5.88 µM) significantly increased the number of shoots to 16.2 ± 0.986 shoots per explant with a height of 6.85 ± 1.33 cm and a greater number of green leaves (11.77 ± 1.35). So, the addition of $AgNO_3$ and BA to the MS basal medium had a positive effect on the growth and health of the whole plant. With the addition of $AgNO_3$ (5.88 µM) with BA (4.43 µM), the number of shoots just doubled (16.2 ± 0.986 shoots per explant), and these plantlets were further elongated in MS medium supplemented with BA (22.2 µM) (Figure 1, h).

Fig. 1. *In vitro* propagation of *O.indicum* through cotyledonary nodes



(a) Inoculation of seeds in MS medium supplemented with GA_3 (8 mg/l) and casein hydrolysate (2 mg/l). (b,c) *In vitro* germination of seeds after ten days (b) and 18 days (c). (d,e) Shoot induction from cotyledonary nodes of *in vitro* germinated plantlets of *O. indicum* after two weeks (d) and three weeks (e) of inoculation in MS medium supplemented with BA (22.2 µM). (f,g) **.** *In vitro* shoot proliferation after four weeks of inoculation in MS medium supplemented with BA (4.43 µM) and growth adjuvant $AgNO_3$ (5.88 µM). The number of shoots increased to 16.2 ± 0.986 shoots/ explants after the addition of an adjuvant to the medium as compared to 8.22 ± 0.11 shoots/ explants without the addition of $AgNO_3$. (h) Shoot elongation after six weeks of incubation in MS medium supplemented with BA (22.2µM).

In vitro root proliferation

Well-grown shoots, about 3 -5 cm in length, were transferred to $MS_{1/2}$ or MS basal medium for root induction and proliferation. The medium was supplemented with varying concentrations of IBA and IAA alone. Among the used auxins, IAA (2.85 µM) was found to be the most effective and induced maximum roots after 20 days of inoculation in a half-strength MS medium (Table 4, Figure 3, a-c).

Acclimatization:

Well-rooted plantlets were transferred to the thermacol cups containing sterile soil, sand, and farmyard manure and then successfully acclimatized in culture room conditions (25 ± 2° C, 80% relative humidity) for five weeks with 70% survival (Figure 3, d–f). Then they were transferred to pots containing garden soil, kept in a polyhouse for further growth, and finally placed in direct sunlight.

Fig. 2. *In vitro* root induction and proliferation

(a,b) Root induction in well-developed shoots of *O. indicum* after 15-30 days of incubation in half- strength MS medium containing 4.92 µM IBA **(a)** and 2.85 µM IAA **(b)**, respectively. **(c)** Preparation of plantlets for acclimatization and hardening. **(d)** Acclimatization of *in vitro* raised plantlets of *O. indicum* in the greenhouse by sowing them in a mixture of sterile soil, sand, and farmyard manure (3:1:1). **(e)** Hardening under *ex-situ* conditions of plants after 25 days of acclimatization in the greenhouse.

Discussion

In the natural environment, the regeneration of *O. indicum* relies on factors such as seed viability, germination, and seedling establishment. The germination process involves a sequence of events, beginning with water absorption through imbibition, influenced by seed composition. This initial step is followed by embryo expansion, leading to the rupture of covering layers and the emergence of the radicle as the final phase of germination. Ultimately, root emergence occurs from the swelling radicle, while shoot emergence originates from the plumule.

Under natural conditions, *O. indicum* seeds typically germinate early in the rainy season, specifically from May to August. The success of seed germination is greatly influenced by prevailing weather conditions, which can affect the occurrence and extent of seed infections. Phytotoxins, substances produced by fungi, can inhibit seed germination, and seedlings are susceptible to fungal decay. In the Kumaun region of the Central Himalaya, fourteen seed-borne fungi have been isolated from *O. indicum* seeds. Seedlings of *O. indicum* require moderate shade during their early stages of growth. However, the species faces challenges such as low seed set and reduced seed viability, resulting in germination issues. Additionally, the scattered distribution of the plant and its reliance on bat pollination has become problematic due to declining bat populations, potentially jeopardizing the species due to poor pollination efficiency. Only 30% of the seeds have been found to be viable as a result.

Furthermore, extensive root harvesting for medicinal purposes, low seed viability, a slow rate of germination, and limited success in propagation through stem cuttings have imposed significant constraints on the natural populations of this tree. Reports have also indicated low fruit sets due to insufficient pollination, contributing to seed abortion in this species.

Given these challenges, this study aims to enhance the seed germination of *O. indicum*, considering the various factors impacting its natural regeneration and survival. Previously, the role of natural organic extracts,

including casein hydrolysate, in tissue culture mediums was studied. Previously, in the case of *O. indicum*, casein hydrolysate helped in the efficient regeneration response and proliferation of shoots (22, 23). In other studies, GA_3 and Zeatin were reported to have a significant effect on the germination of *O. indicum* seeds, and on comparing these two additives, GA_3 alone at lower concentrations (60 ppm) had to have a profound effect on seed germination percentage (70%) by breaking seed dormancy (24). Reports are also available on *Asparagus sprengeri*, where high concentrations of IAA and GA_3 had a significant effect on seed germination in dark conditions (25, 26). Casein is an organic nitrogen supplement that acts as a good source of reduced nitrogen and has widely been used in embryo culture. It can be a source of calcium, phosphorus, several microelements, and, importantly, a mixture of up to eighteen amino acids. The nitrogen deficiency can be cheaply fulfilled by its addition. Presumably, it contains some stimulatory factor yet unidentified (22). It has been suggested that the positive effect of reduced nitrogen (organic nitrogen) in comparison to inorganic sources is associated with the enhanced mobility of the former at lower energy cost in comparison to the latter (27). GA_3 might enhance the growth potential of the embryo, as reported by (28, 29). It may also induce the degradation of food reserves in the endosperm by stimulating hydrolytic enzyme activity, as suggested by (30, 31).The stimulation of hydrolytic enzyme activity by GA_3 contributes to the improvement of seed germination rate through triphasic action - by breaking dormancy, promoting germination, and inhibiting the abscisic acid effect (32).

Therefore, in the present study, the addition of GA_3 (8mg/L) and casein hydrolysate (2 mg/L) to the MS basal medium increased the germination percentage up to 93.32 percent in a minimum number of days (11.6 days). On the basis of the findings in Table 2 (i.e., avg. no. of shoots, avg. shoot length, and avg. no. of leaves), MS + BA (22.2 µM) was found to be the best medium. (23), reported shoot bud initiation from axillary bud and shoot initiation from callus in *O. indicum* (33) while studying shoot induction from apical and axillary bud, found BA and 2, 4-D as the best multiple shoot inducing PGRs. BA (3.0 mg/L) and NAA (0.5 mg/L) were considered best for shoot induction and proliferation by (15). However, Dalal and Rai, (34) used BA (8.87µM), IAA (2.85µM), and GA_3 (1.44µM) to obtain the high frequency of shoot induction and proliferation. From the findings of Table 3, it was revealed that shoot elongation and proliferation of shoots were enhanced sufficiently by the addition of growth adjuvant in MS basal medium BA (4.43 µM) with $AgNO_3$ (5.88 µM). The rapid enhancement in the number of shoots by the addition of $AgNO_3$ is attributed to the fact that $AgNO_3$ is a potent ethylene inhibitor. It inhibits ethylene activity through Ag^{++} ions by reducing the receptor capacity to bind ethylene (35). It acts as an inhibitor of 1-aminocyclopropane -1-carboxylic acid, which is a precursor of ethylene in the biosynthetic pathway (36). The incorporation of silver nitrate in the culture medium greatly improved the regeneration of many dicot and monocot cultures (37). It is considered to reduce shoot organogenesis *in vitro,* thereby increasing shoot proliferation when used with cytokinins such as TDZ (38-40). Silver nitrate has been used for shoot elongation in *Capsicum* (41). However, increased numbers of shoots in cucumber were achieved by the incorporation of silver nitrate (42). MS medium supplemented with sliver nitrate also enhanced the frequency of shoot regeneration in *Brassica* species (43).

Auxin has an effect on *in vitro* root induction and proliferation. IBA is the most common auxin used for root induction, yet in the current study, IAA, (2.85 µM) gave the best response in half MS medium (Figure 2- a, b). Studies on PGRs inducing roots in the plants also revealed that the best variant medium is MS with the addition of IAA, where the older roots are gradually replaced with the new ones (44). IAA is considered to be the most important native auxin in higher plants and is also, therefore, the most studied. Exogenous IAA, in some cases, can promote root elongation (45–47). All the *in-vitro* regenerated plants were acclimatized (Figure 2c, 2d) by sowing them in a mixture of sterile soil, sand, and farmyard manure. The plants were hardened well in the greenhouse and finally in the *ex-situ* conditions (Figure 2e).

Conclusion

This study presents an efficient protocol for the high-frequency regeneration of *O. indicum*, a valuable medicinal forest tree. Optimal shoot multiplication was achieved using MS medium supplemented with 22.2 µM BA, while 2.85 µM IAA in $MS_{1/2}$ medium proved ideal for in vitro root induction. The introduction of the growth adjuvant silver nitrate not only increased the rate of shoot multiplication but also improved plant height and the number of healthy leaves per explant. This protocol has the potential to reduce the indiscriminate exploitation of natural *O. indicum* populations, meeting the demands of pharmaceutical industries for various plant parts. It also offers an efficient *in vitro* system for *O. indicum* improvement, mass multiplication, and conservation.

Acknowledgements

The authors wish to sincerely acknowledge the Department of Biotechnology, Kumaun University, Nainital, Uttarakhand, India, for providing the necessary facilities and support for this study.

Authors' contributions

N J planned the design of the study, carried out the experiments, performed statistical analysis, and drafted the manuscript. T N revised the manuscript. All the authors approved and read the final manuscript.

Compliance with ethical standards

Declaration : The authors declare no conflict of interest.

Ethical issues: None.

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