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# OPTIMIZATION OF CRITICAL FACTORS INFLUENCING *IN-VITRO* MULTIPLICATION, HYDROPONIC ESTABLISHMENT AND DIFFERENTIAL GENE EXPRESSION ANALYSIS IN *WITHANIA COAGULANS* (L.) DUNAL.

**Mansi Singh Bisen[#], Bipin Maurya[#], Sneha Singh, Sabitri Kumari, Pajeb Saha, Nidhi Rai and Shashi Pandey Rai***

Laboratory of Morphogenesis, Centre of Advance Study in Botany, Department of Botany, Faculty of Science, Banaras Hindu University, Varanasi - 221 005, Uttar Pradesh, India.

[#]First & Second Author contributed equally

*Corresponding author e-mails : shashi.bhubotany@gmail.com; pshashi@bhu.ac.in

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**ABSTRACT**

This study was undertaken with the aim to optimize *in-vitro* propagation techniques and hydroponic establishment in *Withania coagulans* (L.) Dunal to address the challenges posed by its declining population and limited natural propagation, while also elucidating gene expression related to withanolide biosynthesis. *W. coagulans* is a critically endangered medicinal plant species valued for its pharmacological properties. Declining population and limited natural propagation have posed a serious challenge to its conservation. In this study, different phytohormonal concentrations and plant organs have been investigated for their effects on germination and growth of the plant. Shoot apical meristems and nodes were selected as explants and cultured in various media compositions enriched with growth regulators. BAP+NAA combination exhibited the highest shoot and node growth. Additionally, organic additives like orange juice enhanced shoot regeneration. Root regeneration was optimized with 2mg/L IBA supplementation. Somatic embryo maturation was maximized with 0.5mg/L ABA and 3% Sucrose. Furthermore, we confirmed clonal uniformity among tissue culture-raised plants. Gene expression analysis focused on four key genes involved in withanolide biosynthesis (HMGR, FPPS, SMT1 and SQS) across node, shoot apical meristem (SAM), somatic embryo (SE) and (SR) explants with HMGR predominantly expressed in nodes, SMT1 in roots and SQS in somatic embryos. Meanwhile, FPPS showed consistent expression across all explant types. Overall, this study elucidates the effects of plant growth regulators and other organic additives for physiological processes and gene expression in *W. coagulans*, providing in sights for its controlled propagation and utilization of bioactive compounds.

***Key words :*** Medicinal plant, *In-vitro* propagation, Withanolides, Genetic polymorphism, Phytohormones.

## Introduction

*Withania coagulans* (L.) Dunal, commonly known as “Indian Rennet” or “Paneer Booti,” is a critically endangered medicinal plant species belonging to the Solanaceae family (Khan *et al*., 2021). This plant has drawn considerable interest from both traditional herbal medicine practitioners and contemporary researchers due to its wide variety of pharmacological activities, such as analgesic, anti-inflammatory and anti-diabetic actions (Gupta *et al*., 2022). However, the conservation status of *W. coagulans* has raised concerns due to their declining population, habitat loss and limited natural propagation (Gilani *et al*., 2009). The sustainable utilization and effective conservation of *W. coagulans* necessitate the development of efficient propagation methods, particularly in vitro techniques, which offer the potential to rapidly generate a large number of genetically uniform plants (Nandini *et al*., 2019). Nonetheless, *in-vitro* propagation of this species has been hindered by its low germination rate, making it a challenging subject for research and propagation efforts (Rathore *et al*., 2012). The establishment of an effective protocol for *in-vitro*

propagation is crucial not only for the preservation of this valuable medicinal plant but also for meeting the growing demand for its therapeutic compounds.

Phytohormones, including auxins and cytokinins, play pivotal roles in regulating various physiological and developmental processes in plants (Giron *et al*., 2013). Their influence on in-vitro propagation has been widely documented, indicating that the manipulation of phytohormonal concentrations could potentially enhance the germination and growth of *W. coagulans in vitro* (Murugesan and Senthil, 2017). Furthermore, the selection of appropriate plant organs, such as seeds, cotyledons, or shoot tips, for initiating *in-vitro* cultures can significantly impact the success of propagation protocols (Murashige, 1974). The primary mode of propagation for *W. coagulans* is using seeds, although the potential for seed establishment is constrained by the unisexual attributes of its flowers. The species' susceptibility to excessive harvesting and challenges in reproductive success have significantly elevated its susceptibility to total eradication. Presently, there is anabsence of documented instances involving the preservation of this plant through tissue culture methods in external environments (Schippmann, 2002).

In light of the critical conservation status of *W. coagulans* and the challenges associated with its in-vitro propagation, this study aims to examine the effect of different phytohormonal concentrations and plant organ selections on the germination and growth of *W. coagulans in vitro* (Kaur *et al*., 2022). By systematically analyzing the responses of this species to varying hormone levels and organ sources, we aspire to optimize a reliable protocol for *in-vitro* propagation. Such an achievement would not only contribute to the conservation of *W. coagulans*, but also provide a foundation for further research into its pharmacological potential and cultivation for medicinal and commercial purposes. In this paper, we present our experimental approach, methodologies and anticipated outcomes, shedding light on the potential avenues for enhancing the *in vitro* propagation of this critically endangered species. The results of this study are expected to contribute significantly to the fields of plant conservation, herbal medicine and sustainable utilization of medicinal plant resources.

## Materials and Methods

### Selection of explant

The seeds of *W. coagulans* were sourced from the Laboratory of Morphogenesis, Department of Botany at Banaras Hindu University, Varanasi, India. These seeds underwent a thorough washing process using running water for duration of 40 minutes. Subsequently, they were subjected to disinfection by dipping in 70% ethanol for a minute, followed by rinsing with sterile distilled water. Following this, the seeds were soaked in a solution comprising 5% (v/v) sodium hypochlorite and tween 20 for 15 minutes, succeeded by six sequential rinses with sterile distilled water. Thereafter, the treated seeds were introduced into Murashige and Skoog (MS) medium and placed in an incubation environment with temperatures maintained at 25–27°C, accompanied by a 16-hour photoperiod. The illumination, provided by cool white fluorescent bulbs, offered a light intensity of 25 mol $m^{-2}$ $s^{-1}$. Upon successful germination of the seeds, the resulting plantlets were cultivated in a medium containing half-strength MS. Shoot apical meristems (SAM) and nodes from *in vitro*-cultivated seedlings (4–5 weeks' post-germination) were used as an explant for further study.

### Different media concentration and explant

Experiments were conducted to establish optimized culture conditions for *W. coagulans* through the assessment of various media compositions and the utilization of distinct explants, namely the apical bud and nodal bud. These explants were chosen to evaluate their respective totipotency and responsiveness. The basal medium, which exhibited successful regeneration of explants, was then enriched with diverse combinations and concentrations of growth regulators, such as cytokinins, auxins and gibberellins. These growth regulators were selected based on the specific objectives of each experiment, aimed at investigating their impacts on the *in vitro* response of distinct explants. Control experiments were conducted using basal media without the inclusion of growth regulators. The growth regulators were prepared as stock solutions, including MS + BAP, MS + IBA, MS + GA, MS + SA, MS + JA, BAP + IAA, BAP + NAA, Kinetin + IAA, Kinetin + NAA, Agar (control) + 0.1 mg/L NAA + 2.0 mg/L BAP, Coconut water + 0.1 mg/L NAA + 2.0 mg/L BAP, Coconut milk + 0.1 mg/L NAA + 2.0 mg/L BAP, Orange juice + 0.1 mg/L NAA + 2.0 mg/L BAP and NAA + BAP + TDZ (mg/L). Elongation and culture induction were carried out using these hormonal solutions and sucrose concentrations ranging from 1% to 4%. To ensure consistent conditions, the pH of all media combinations was maintained to 5.8 ± 0.1 with 0.1N NaOH or 0.1N HCl before autoclaving at 121°C and 15 psi for duration of 20 minutes.

### Multiplication of shoot and regeneration of rooting and callusing

The cultures were maintained within a controlled environment at a temperature of 25±1°C under a

photoperiod of 16 hours, with a photosynthetic photon flux density (PPFD) of 25 µ mol $m^{-2}s^{-1}$ given by cool white fluorescent tubes (40 W; Philips, India). Nodal segments procured from plants cultivated in the field were subjected to a series of preparatory steps. Firstly, they were thoroughly cleansed using a 5% (v/v) Teepol solution, followed by surface sterilization with 70% (v/v) ethanol for duration of 30 seconds. Subsequently, the segments were treated with an aqueous solution of freshly prepared 0.1% (w/v) $HgCl_2$ for 3 minutes. The explants were then meticulously rinsed with sterile distilled water and introduced onto MS medium, supplemented with Kinetin (Kn) at different concentrations either independently or in combination. Furthermore, various concentrations of Plant Growth Regulators (PG) and Coconut Cream (CC) from Sigma (USA) were assessed alongside the optimal cytokinin concentration. Shoot buds that were initiated in the primary cultures were separated into clusters of 3-4 buds each, and subsequently, these clusters were cultivated on fresh medium to further enhance the proliferation of shoot buds.

Upon reaching a length of 2–3 cm, the *in vitro*-derived microshoots were subjected to a two-step rooting procedure. In the initial step, individual microshoots received a pulse treatment involving PGRs, administered either alone or in conjunction with Indole-3-butyric acid (IBA) and Phenylacetic acid (PAA) at concentrations of 10, 50 and 100 mg $l^{-1}$. This treatment was carried out for duration of 7 days on a liquid MS medium, facilitated by a filter paper bridge. The subsequent step entailed the transfer of the pre-treated microshoots onto a semi-solid medium comprised of ½ or ¼ strength MS medium supplemented with 3% sucrose, and further enriched with Indole-3-acetic acid (IAA), Naphthaleneacetic acid (NAA), IBA or PAA at concentrations ranging from 0.25 to 1 mg $l^{-1}$, either individually or in combination. The cultures were monitored over a period of 4 weeks to assess their development. For histological analysis, preparations were conducted as per the established protocol (refer to the appropriate reference). Once the plantlets had matured, they were delicately extracted from the containers, gently rinsed with water and subsequently transplanted into pots filled with a mixture comprising equal parts of garden soil and organic manure (1:1 ratio).

**Transfer to hydroponic and soil conditions**

For acclimatisation of the plants, the seedlings obtained through *in-vitro* culture were transferred onto the hydroponics system set up with half strength hoagland (0.25M $KH_2PO_4$, 0.25M $KNO_3$, 0.25M $Ca(NO_3)_2.4H_2O$, 0.25M $MgSO_4$, 1.43g/l $H_3BO_3$, 0.91g/l $MnCl_2.4H_2O$, 0.11g/l $ZnSO_4.7H_2O$, 0.04 g/l $CuSO_4.5H_2O$, 0.01g/l $H_2MoO_4.H_2O$, 8.9mM EDTA, 17mM $FeSO_4.7H_2O$ and 34mM KOH) (Rai *et al*., 2024). Simultaneously, seedlings were also planted in soil which was collected from the Botanical Garden at Banaras Hindu University, Varanasi and supplemented with sand and organic manure (2:1:1 w/w) in plastic cups which were half filled.

**Isolation of DNA and genetic fidelity test**

Genomic DNA samples were extracted from the foliage of randomly chosen regenerated *W. coagulans* plant. The plant material was subjected to pulverization in liquid nitrogen (maintained at -196°C) and subsequently preserved at -20°C for subsequent utilization in DNA extraction through the CTAB (Cetyltrimethylammonium bromide) method as described by Clarke (2009). To ascertain the clonal fidelity of the regenerated shoots, a panel of four Random Amplified Polymorphic DNA (RAPD) primers was employed. The Polymerase Chain Reaction (PCR) amplification was conducted following a specific thermal profile: initial denaturation at 94°C for 5 minutes, succeeded by 35 amplification cycles comprising denaturation at 94°C for 30 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 1 minute. The amplification procedure was concluded with a final extension step for 5 minutes at 72°C.

**Isolation of RNA and Gene expression analysis**

Fresh leaf tissues were meticulously collected to facilitate the subsequent isolation of RNA. The TRIZOL reagent (GIBCOBRL) was employed for RNA extraction, adhering closely to the established manufacturer's protocol. The concentration of the isolated RNA was determined through the employment of a Nano Drop spectrophotometer, manufactured by Thermo Scientific. The design of primers aimed at facilitating gene analysis was accomplished utilizing the Primer 3 software. To discern the gene expression patterns, a semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) approach was undertaken. This was conducted employing a Bio-Rad thermo cycler, and the intensities of the amplified RT-PCR products' bands were subjected to quantification on a 2% agarose gel. The Gel-Doc EZ imager, provided by Bio-Rad was used for this purpose, and the ensuing data analysis was facilitated using the Quantity One software (Bio-Rad). The data normalization process incorporated the endogenous gene ACTIN. The assessment of transcript levels was accomplished utilizing the comparative Ct (threshold cycles) method, as outlined by Pandey *et al*. (2016), which involves the formula $2^{-\Delta\Delta Ct}$.

**Statistical analysis**

All experimental procedures were replicated thrice and data gathered from these replicates underwent to analysis of variance (ANOVA), a statistically robust

method employed to evaluate the significance of observed variations using Duncan's multiple range test (DMRT) was used as a post-hoc analysis. The statistical software package SPSS Inc., Version 20.0, was utilized for executing these analyses, with a significance threshold set at $P \leq 0.05$, ensuring that only results meeting this criterion were deemed statistically meaningful. The graphical representation of the experimental findings was meticulously executed utilizing Graph Pad Prism (version 5.0).

## Results

### Effects of different media concentrations on germination

The evaluation of germination percentages revealed distinct trends across various treatments and time points. On the 14th day, the highest germination percentage was observed in the MS+ GA treatment, reaching 38%. Notably, the MS + BAP treatment also exhibited a considerable germination rate at this time point, recording a value of 30%. As the germination process continued, the 21st and 28th days show cased further fluctuations in germination percentages. MS+ GA treatment continued to demonstrate its positive influence, displaying the highest germination percentages ranging between 60-65% on the 21st and 28th days (Fig. 1). Following suit, the MS + BAP treatment exhibited a noticeable improvement in germination, with percentages ranging from 35-40% during the same periods.

### Assessment of media concentration on explants

In the case of shoot apical meristem (SAM) explants, the examination of shoots and nodes displayed the most prominent outcomes with the BAP + NAA combination (2.22 and 2.69) (Table 1). This represented a remarkable 6-fold enhancement when contrasted with the control, and a noteworthy 0.5-1.5-fold improvement in comparison to alternative combinations. Similarly, the utilization of Kn + NAA led to the highest shoot length values, measuring 13.27 cm and 12.85 cm, respectively. This exemplified a noteworthy 3-4-fold augmentation in comparison to BAP, and an appreciable 0.2-0.5-fold amplification when juxtaposed with alternative combinations.

Interestingly, analogous trends emerged when node explants were investigated. The application of BAP + NAA yielded a significant surge in the number of shoots (6.09) and nodes (7.81), show casing a notable 3-fold elevation relative to the control and an approximately 0.5-1.0-fold increase compared to alternative combinations. Furthermore, the highest shoot length was observed once more with the Kn + NAA treatment, displaying values of 13.32 cm and 12.69 cm, respectively. This corresponded to a substantial 3-4-fold rise when compared with BAP, and an appreciable 0.2-0.5-fold enhancement when measured against alternative combinations.

### Impact of plant growth hormones on shoot initiation

The study investigated the influence of various treatments involving organic additives and hormones on shoot regeneration in *W. coagulans*. Among the treatments, the most remarkable shoot regeneration was achieved with the application of orange juice supplemented with 0.1 mg/L NAA (α-naphthaleneacetic acid) (Fig. 2) and 2.0 mg/L BAP (6-Benzylaminopurine), yielding a regeneration rate of 8.0 (Fig. 3). This was closely followed by the Agar-based control treatment enriched with 0.1 mg/L NAA and 2.0 mg/L BAP, as well as the utilization of Coconut water supplemented with the same hormonal concentrations, both displaying regeneration indices of 8.0 and 6.0, respectively (Table 2). Furthermore, the *in vitro* growth of the shoots was evaluated in terms of various morphological parameters, including number of nodes, plant height, leaves and branches. The treatment that show cased the maximum plant height and number of nodes was the one utilizing MS medium supplemented with 2 mg/L BAP, with values of 6.36 cm and 5.66, respectively (Table 3). Conversely, the treatment employing MS medium enriched with 4 mg/L BAP demonstrated the highest number of leaves (8.66) and

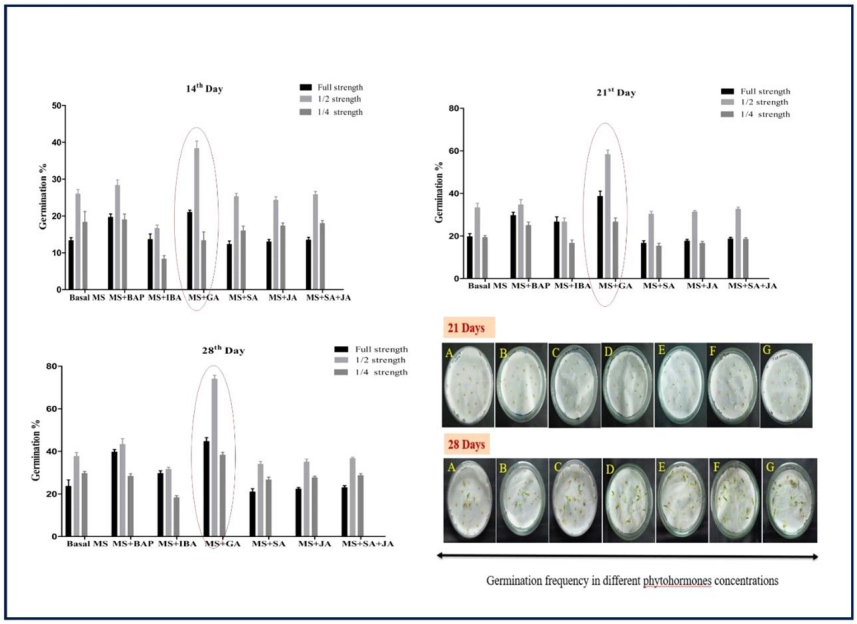


**Fig. 1 :** Effect of input variables on seed germination in *W. coagulans*.

**Table 1 :** Effects of different concentrations and combination of plant growth regulators on SAM and nodal explants after 4 weeks in *W. coagulans.*

| PGR concentration | SAM Explants | | | Nodal Explants | | |
|---|---|---|---|---|---|---|
| | No. of shoot | Shoot length | No. of nodes | No. of shoot | Shoot length | No. of nodes |
| **Control** | 1.35±0.03h | 5.03±0.02ab | 6.92±0.09gh | 2.05±0.00e | 7.68±0.06d | 8.07±0.05de |
| **BAP** | | | | | | |
| 2.22 | 7.59±0.06b | 3.47±0.05fgh | 6.74±0.08gh | 7.48±0.10ab | 3.04±0.03fg | 6.48±0.03fg |
| 4.44 | 5.76±0.06c | 4.33±0.04efg | 6.05±0.16h | 5.89±0.08bc | 3.87±0.00f | 6.82±0.09fg |
| 8.88 | 5.94±0.06c | 4.73±0.07hi | 3.81±0.09ij | 4.58±0.13d | 2.35±0.03fgh | 3.95±0.03ij |
| **Kn** | | | | | | |
| 2.32 | 3.37±0.11de | 10.64±0.02b | 8.27±0.10def | 2.32±0.03e | 8.78±0.10c | 6.67±0.13ef |
| 4.65 | 2.78±0.04ef | 11.24±0.03a | 4.04±0.06i | 2.28±0.06e | 6.88±0.04d | 4.21±0.03hi |
| 9.29 | 2.36±0.04efgh | 10.72±0.18b | 7.72±0.15defg | 1.98±0.01e | 10.21±0.11b | 7.29±0.01ef |
| **TDZ** | | | | | | |
| 2.27 | 2.64±0.12efg | 3.33±0.06gh | 4.42±0.04i | 2.99±0.05e | 3.36±0.12f | 4.21±0.13hi |
| 4.54 | 2.84±0.01ef | 2.68±0.03hi | 3.50±0.06ijk | 2.42±0.03e | 2.94±0.04fg | 2.91±0.04ij |
| 9.08 | 2.05±0.04fgh | 2.65±0.05hi | 2.45±0.09jk | 2.05±0.01e | 1.94±0.04gh | 2.29±0.04j |
| **BAP+IAA** | | | | | | |
| 2.22+2.85 | 6.02±0.01c | 4.84±0.06ef | 7.42±0.09fgh | 5.88±0.03c | 5.55±0.03e | 6.66±0.08ef |
| 2.22+5.71 | 3.43±0.06d | 6.72±0.10cd | 7.74±0.14efgh | 2.27±0.04e | 8.91±0.13cd | 6.86±0.05ef |
| **BAP+NAA** | | | | | | |
| 2.22+2.69 | **9.62±0.13a** | **13.27±0.02a** | **16.33±0.15a** | **7.81±0.09a** | **13.00±0.05a** | **14.63±0.19a** |
| 2.22+5.37 | 8.78±0.09a | 12.85 ±0.03a | 14.60±0.04b | 6.09±0.03c | 5.50±0.11e | 10.25±0.21c |
| **Kn+ IAA** | | | | | | |
| 2.32+2.85 | 6.02±0.07bc | 7.22±0.20cd | 11.94±0.06c | 7.22±0.10ab | 5.57±0.12e | 12.01±0.18b |
| 2.32+5.71 | 3.93±0.12d | 8.06±0.30c | 9.35±0.07d | 5.57±0.03d | 7.58±0.24d | 9.67±0.27cd |
| **Kn+ NAA** | | | | | | |
| 2.32+2.69 | 1.56±0.03fgh | 4.74±0.06ef | 9.10±0.16de | 2.05±0.05e | 5.61±0.06e | 10.03±0.05c |
| 2.32+5.71 | 1.22±0.06gh | 5.25±0.09e | 8.00±0.21defg | 1.73±0.02e | 12.84±0.19ab | 10.65±0.11bc |

**Table 2 :** Effect of different organic additives on shoot regeneration.

| Organic additive | Concentration | Numbers of regenerated shoots |
|---|---|---|
| Agar (control) +0.1 mg/L NAA + 2.0 mg/L BAP | 0.8 g | 8 |
| Coconut water+0.1 mg/L NAA + 2.0 mg/L BAP | 15mL | 6 |
| Coconut milk+0.1 mg/L NAA + 2.0 mg/L BAP | 15mL | 2 |
| Orange juice+0.1 mg/L NAA + 2.0 mg/L BAP | 8ml | 10 |

branches (4.66).

**Impact of varying concentrations of plant growth hormone (IBA) on root regeneration**

The investigation into the influence of varying concentrations of plant growth hormones on root regeneration revealed significant disparities in root morphology and health (Fig. 4). Notably, the application of MS medium enriched with 2 mg/L indole-3-butyric acid (IBA) yielded the most substantial outcomes. The root length achieved under this treatment was notably high, measuring 5.4 units. Concurrently, the root number reached 5.73, and a root regeneration percentage of 65% was observed. Comparatively, the utilization of MS medium supplemented with 1 mg/L, 3 mg/L and 4 mg/L IBA exhibited comparatively lower values for root length, root number, and root regeneration percentage in the range of 2.4-3.7, 3.23-4.11 and 30%-45%, respectively (Table 4). Control plants, not subjected to hormone treatment, displayed the least favorable results for the aforementioned parameters.

**Effect of different ABA and sucrose concentrations on maturation of somatic embryos**

The investigation into the impact of varying concentrations of abscisic acid (ABA) and sucrose on

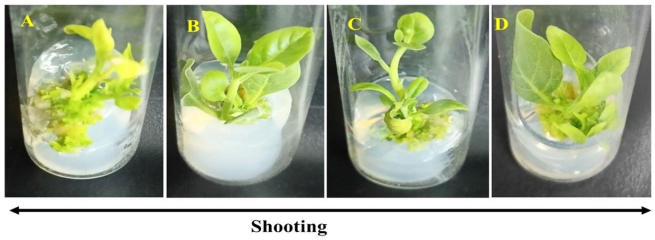


**Fig. 2 :** Shooting in different organic additives (a) Agar (b) Coconut water (c) Coconut milk (d) Orange juice.

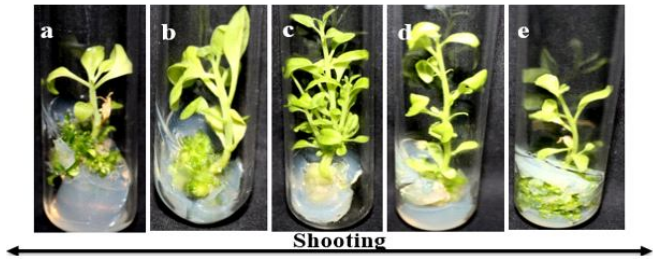


**Fig. 3 :** Shooting in different concentrations of BAP (a) Basal MS-Media (b) MS+ 1 mg/L BAP (c) MS+2mg/L BAP (d) MS+3mg/L BAP (e) MS+4 mg/L BAP.

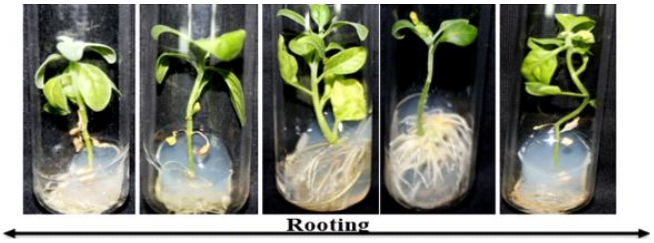


**Fig. 4 :** Rooting in different concentration of IBA (a) Basal MS-Media (b) MS+ 1 mg/L IBA (c) MS+2mg/L IBA (d) MS+3mg/L IBA (e) MS+4 mg/L IBA.

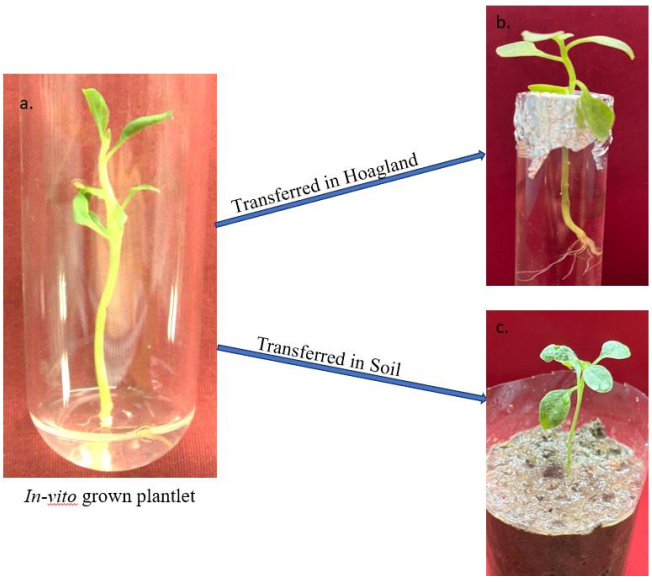


**Fig. 5 :** Establishment of plants for acclimatisation (a) *In-vitro* grown plantlet (b) Plantlet transferred in half strength hoagland solution (c) Plantlet transferred in soil.

frequency of 48.5% was attained with the utilization of 0.5 mg/l ABA, representing the maximum maturation response. Additionally, the application of 0.1 mg/l ABA resulted in a significant maturation frequency of approximately 31.4%. In the case of sucrose, a concentration of 3% yielded the highest maturation frequency of somatic embryos, registering at 60.2% (Table 6).

**Table 3.** Different concentrations of BAP used in this study and their effect on shoot regeneration.

| Treatment | Shoot length (cm) | No. of nodes | No. of leaves | No. of branches |
|---|---|---|---|---|
| Basal MS-Media | 3.43±0.29a | 2.33±0.3a | 2.66±0.25a | 1.33±0.158a |
| MS+ 1 mg/L BAP | 4.33±0.13b | 3.33±0.258b | 4.66±0.258b | 2.33±0.328b |
| MS+2mg/L BAP | 6.36±0.18d | 5.66±0.358d | 8.66±0.36d | 4.66±0.136cd |
| MS+3mg/L BAP | 5.03±0.21c | 3.33±0.33bc | 5.66±0.308bc | 3.66±0.189c |
| MS+4 mg/L BAP | 3.23±0.113a | 2.66±0.304a | 4.333±0.358b | 3.33±0.254bc |

**Table 4 :** Effect of different concentrations of IBA on root regeneration.

| Treatment | Root length (cm) | Root number | Rooting % |
|---|---|---|---|
| Basal MS-Media | 2.83±0.279a | 2±0.12a | 4±3.07a |
| MS+ 1 mg/L IBA | 3.46±0.279ab | 4.5±0.23b | 19±2.76b |
| MS+2mg/L IBA | 5.4±0.149e | 20.73±0.14e | 65±1.45e |
| MS+3mg/L IBA | 3.66±0.184bc | 19.13±0.35d | 58±2.78d |
| MS+4 mg/L IBA | 3.73±0.91cd | 12.77±0.32c | 30±1.53c |

the maturation of somatic embryos (SE) in *W. coagulans* yielded noteworthy outcomes (Table 5). Notably, the highest frequency of SE maturation was achieved under specific ABA and sucrose concentrations. A maturation

**Acclimatisation of plants under hydroponic and soil conditions**

For further growth and development, in-vitro cultivated plantlets were shifted on to hydroponic set up as well as soil condition. Our studies demonstrated that the plantlets in hydroponic set up showed better response as compared to those grown in soil conditions. The plantlets in hydroponic set up exhibited a 17% increment in morphological parameters, i.e., total root biomass and total shoot biomass comparative to soil conditions (Fig. 5).

**Analyze the genetic fidelity**

In the assessment of clonal fidelity for the

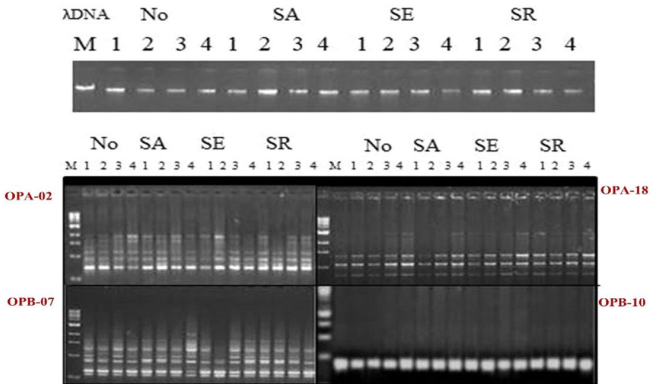


**Fig. 6 :** DNA fingerprints profile withOPA-02, OPA-18, OPB-07 and OPB-10 primer.

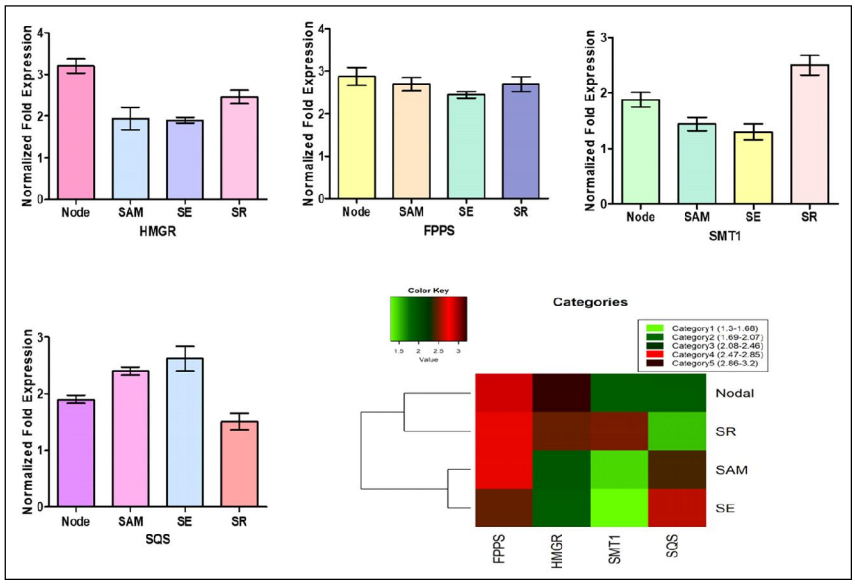


**Fig. 7 :** Analysis of gene expression pattern by RT-PCR and qRT-PCR analysis of genes involved in the withanolide biosynthetic pathway. Each expression was performed in three biological replicates. [(HMG-CoA reductase *HMGR*), (farnesyl pyrophosphate synthase *FPPS*), (Squalene/phytoene synthase *SQS*), (sterol methyl transferase 1 *SMT1*)].

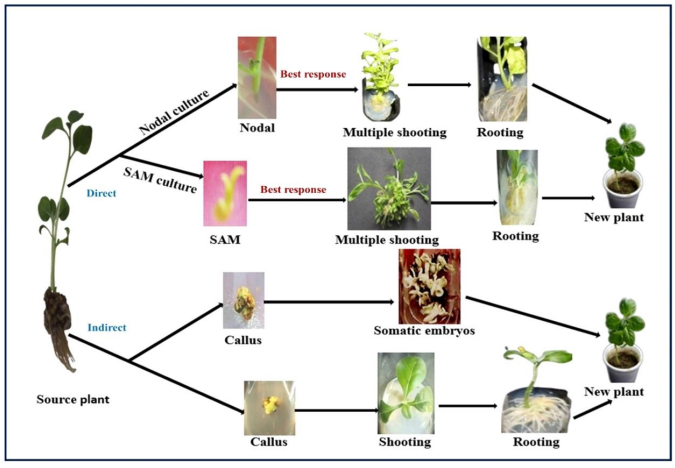


**Fig. 8 :** Summary of efficient regeneration and multiplication of *W. coagulans* by nodal, SAM, somatic embryos and multiple shoots in this study.

regenerated shoots, Random Amplified Polymorphic DNA (RAPD) analysis was employed (Fig. 6). Out of the four randomly selected primers, two exhibited the generation of distinct and consistently reproducible DNA amplification products. In total, 210 amplification products were discerned across the tested samples. Specifically, the primers OPA-02 and OPA-18 displayed highly reproducible banding patterns. The fingerprinting profiles obtained from the regenerants were characterized by a monomorphic nature, indicating a lack of genetic variation among tissue culture-raised plants (Table 7).

### Gene expression analysis

The investigation delved into the expression levels of four key genes associated with the withanolide biosynthetic pathway, namely HMGR, FPPS, SMT1 and SQS. These analyses were conducted across four distinct explant types: node, shoot apical meristem (SAM), somatic embryos (SE) and root (SR). The expression profiles were measured to ascertain potential variations in gene expression across the different tissue sources. Comparatively, the HMGR gene displayed a notable difference in expression among the explants, with the highest expression level recorded in the node explants. The HMGR expression in node explants was approximately 1-fold higher compared to the other three explant types. In the case of the SMT1 gene, a distinct pattern emerged, show casing its highest expression in the root (SR) explants. The expression of SMT1 in root explants was recorded as approximately 1.2-fold higher compared to that in node explants, which exhibited the next highest expression. Conversely, the SQS gene displayed a differential expression pattern, with the somatic embryo (SE) explants exhibiting the highest levels of expression, followed by the shoot apical meristem (SAM) explants. Interestingly, the expression of the FPPS gene appeared to be relatively consistent across all four explant types, with no notable variations in expression levels observed (Fig. 7).

## Discussion

The variation in germination percentages observed in this study underscores the significant impact of growth regulators on the germination process of *W. coagulans*. The distinct responses of germination to different treatments and time points suggest the complex interplay

**Table 5 :** Effect of different concentrations of ABA on maturation of somatic embryo.

| Concentration of ABA ($mgl^{-1}$) | Maturation frequency (%) | Pre-maturation germination of SEs (%) | Germination of mature SEs (%) |
|---|---|---|---|
| Control | 18.0± 5.0b | 10.4 ± 3.4c | 0.0±0.001a |
| 0.01 | 8.7 ± 1.21a | 36.5 ± 4.2d | 30.5 ± 2.0d |
| 0.1 | 31.4 ± 2.2cd | 1.0±0.1a | 1.0±0.1ab |
| 0.5 | 48.5 ± 1.1e | 0.01±0.001ab | 58.6 ± 2.1e |
| 1.0 | 30.1 ± 2.0cd | 0.01±0.1a | 0.01±0.2bc |

**Table 6.** Effect of different concentrations of sucrose on the maturation of somatic embryo.

| Concentration of sucrose (%) + constant Full strength MS media | Maturation frequency (%) | Pre-maturation germination of SEs (%) | Germination of mature SEs (%) |
|---|---|---|---|
| 1 | 10. 3 ± 1.9a | 9.4 ± 3.7c | 46.6 ± 3.0b |
| 2 | 22. 3 ± 4.3b | 19.4 ± 9.4d | 78.6 ± 7.0d |
| 3 | 60.2 ± 3.1d | 0.0 ± 0.01a | 55.5 ± 2.8ab |
| 4 | 31.4 ± 3.6c | 1.0 ±0.1ab | 1.0±0.01a |

**Table 7 :** List of primers used for genetic fidelity analysis of different *in vitro*-grown explants of *W. coagulans.*

| S. no. | Primer name | Sequence | *In vitro* grown plants | The number of bands amplified | Number of polymorphic bands | % Polymorphism |
|---|---|---|---|---|---|---|
| 1 | OPA-02 | TGCCGAGCTG | Nodal | 6 | 0 | 0 |
| | | | SAM | 6 | 1 | 16.67 |
| | | | SE | 5 | 2 | 40 |
| | | | SR | 5 | 2 | 40 |
| 2 | OPA-18 | AGGTGACCGT | Nodal | 3 | 0 | 0 |
| | | | SAM | 5 | 2 | 40 |
| | | | SE | 4 | 1 | 25 |
| | | | SR | 5 | 2 | 40 |
| 3 | OPB-07 | GGTGACGCAG | Nodal | 6 | 1 | 16.67 |
| | | | SAM | 6 | 1 | 16.67 |
| | | | SE | 6 | 3 | 50 |
| | | | SR | 5 | 2 | 40 |
| 4 | OPB-10 | CTGCTGGGAC | Nodal | 2 | 0 | 0 |
| | | | SAM | 2 | 0 | 0 |
| | | | SE | 2 | 0 | 0 |
| | | | SR | 2 | 0 | 0 |

between exogenously supplied plant growth regulators and the inherent physiological processes governing germination (Ahmed Nimir *et al*., 2014; Xiao *et al*., 2019). The substantial germination percentages recorded for the MS+ GA treatment, particularly on the 21st and 28th days, could be attributed to the role of gibberellins in promoting seed germination. Gibberellins are known to play a pivotal role in breaking seed dormancy and promoting cell elongation, which are crucial processes during germination (Shu *et al*., 2016). The observed higher germination percentages for MS + BAP treatment on the 14th day and its continued moderate improvement on the subsequent days might be indicative of the role of cytokinin in enhancing cell division and differentiation, contributing to overall germination success. These results highlight the potential of optimizing germination conditions through the application of specific growth regulator combinations, providing valuable insights for the

propagation and cultivation of *W. coagulans* (Tripathi *et al*., 2018).

The observed variations in shoot and node proliferation, as well as shoot length, among the different hormonal treatments and explant types, underscore the influential role of exogenously applied growth regulators on the morphogenic processes of *W. coagulans* (Singh *et al*., 2019). The significant enhancements achieved through the BAP + NAA combination for both SAM and node explants suggest a synergistic effect of these two growth regulators in promoting shoot and node formation (Premkumar *et al*., 2016 and Wulandari *et al*., 2021) (Fig. 8). The pronounced shoot elongation recorded with the Kn + NAA treatment is indicative of the specific impact of this combination on cell elongation and differentiation. This result corroborates prior knowledge of cytokinin (Kn) and auxin (NAA) interactions in stimulating cell division and elongation, a phenomenon essential for shoot development. The consistency in trends observed for both explant types reinforce the notion that certain growth regulator combinations hold promise for the *in vitro* propagation of *W. coagulans* (Rathore *et al*., 2016).

The diverse treatments explored in this study exemplify the significant role that organic additives and hormone combinations can play in influencing shoot regeneration and subsequent growth in *W. coagulans* (Dehvari-nagan *et al*., 2021). The enhanced shoot regeneration observed with orange juice supplementation, particularly in conjunction with NAA and BAP, underscores the potential of natural additives in promoting morphogenic responses (Manawatu *et al*., 2014). This may be attributed to the abundance of bioactive compounds within orange juice that stimulate cell division and differentiation, key processes in shoot regeneration. The comparably favourable outcomes achieved with the Agar-based control and Coconut water treatments highlight the efficacy of these media compositions in providing the necessary nutrients and hormonal cues for shoot regeneration (Manawadu *et al*., 2014; Hamdeni *et al*., 2022). These results correlate with the established roles of NAA and BAP in promoting shoot formation and growth. The variations in plant height, node number, leaf count, and branch number among different treatments further accentuate the intricate interplay between exogenously applied growth regulators and endogenous plant physiology (Morales-Payan *et al*., 2000). The treatment containing 2 mg/L BAP displayed remarkable shoot elongation and increased node formation, while the treatment enriched with 4 mg/L BAP exhibited an enhanced branching pattern and leaf production.

The evident influence of varying concentrations of the plant growth hormone IBA on root regeneration underscores the pivotal role of exogenous hormonal cues in the developmental processes of *W. coagulans* (Vailizadeh *et al*., 2011). Indole-3-butyric acid, a well-known auxin, plays a fundamental role in growth and initiation of roots, stimulating cell division and elongation in the root meristems. The substantial root length, root number, and root regeneration percentage observed in the MS+2 mg/L IBA treatment may be attributed to the optimal concentration of IBA promoting the initiation and elongation of root primordia (Ghimire *et al*., 2018). The observed diminishing trend in root characteristics as IBA concentrations deviate from this optimum is indicative of a hormonally regulated response. Higher IBA concentrations may lead to an imbalance in hormonal signaling, thereby negatively affecting root regeneration (Aloni *et al*., 2010).

The observed effects of abscisic acid and sucrose concentrations on somatic embryo maturation shed light on the regulatory role of these compounds in the developmental processes of *W. coagulans*. Abscisic acid, a plant hormone associated with stress responses and growth regulation, has been implicated in various developmental stages, including embryo development and maturation. The enhancement of somatic embryo maturation frequency with the application of ABA at 0.5 mg/l and 0.1 mg/l indicates a hormonally-mediated promotion of the maturation process (Mazri *et al*., 2020). The significant increase in somatic embryo maturation frequency observed at a sucrose concentration of 3% emphasizes the pivotal role of sucrose in supporting maturation processes. The higher sucrose concentration likely contributes to the provision of essential energy and nutrients required for embryo development, thereby enhancing maturation success (Komatsuda *et al*., 1992).

The verification of clonal fidelity in regenerated shoots is of utmost importance in plant tissue culture, ensuring the genetic stability and authenticity of the propagated plant material. RAPD analysis, a widely used molecular technique, offers insights into the genetic uniformity of plantlets derived from tissue culture (Razaq *et al*., 2013; Rathore *et al*., 2016). In this study, the successful amplification of distinct and reproducible products with specific primers provides evidence of the reliability and robustness of the RAPD technique for clonal fidelity assessment. The detection of monomorphic fingerprinting profiles in the regenerants is indicative of the high genetic similarity and consistency between the tissue culture-raised plants and the mother plant (Kaur *et al*., 2021; Fatima *et al*., 2012). This signifies the preservation of the genetic makeup during the tissue culture propagation

process. The absence of genetic variation is crucial in maintaining the true-to-type nature of propagated plants, which is essential for the preservation of desirable traits and the production of authentic plant material (Kasagana and Karumuri, 2011).

The examination of gene expression patterns across different explant types provides valuable insights into the regulation of the withanolide biosynthetic pathway in *W. coagulans*. The distinct expression profiles observed for the HMGR, SMT1, SQS and FPPS genes emphasize the intricate genetic and molecular control mechanisms underlying the production of withanolides, essential bioactive compounds with medicinal significance (Singh *et al*., 2018 and Singh *et al*., 2023). The elevated expression of the HMGR gene in the node explants suggests a potential role for these tissues in the regulation of withanolide biosynthesis. Node explants may serve as important sites for the initiation and regulation of metabolic pathways leading to the production of withanolides (Mishra *et al*., 2016). The heightened expression of the SMT1 gene in the root (SR) explants implies a specialized role for this tissue in the biosynthesis of specific withanolide compounds.

Further, the observed higher expression in roots may be linked to the production of withanolides with distinct physiological functions associated with root development and function (Sabir *et al*., 2013). The elevated expression of SQS in the somatic embryo (SE) explants aligns with the dynamic cellular processes during embryogenesis that could necessitate increased production of withanolides, potentially to support stress tolerance and development. In contrast, the relatively uniform expression of the FPPS gene across all explant types suggests that this gene may not exhibit pronounced tissue-specific regulation and could play a more generalized role in withanolide biosynthesis (Dhar *et al*., 2015).

## Conclusion

Thus, this study conclusively provides a comprehensive understanding of the intricate interactions between exogenously applied growth regulators and the inherent physiological processes governing the organogenesis and development of *W. coagulans*. Notably, the substantial germination percentages observed with MS+GA treatment highlight gibberellin involvement in seed germination by breaking dormancy and promoting cell elongation. Furthermore, the influence of growth regulators on node and shoot proliferation, elongation, and somatic embryo maturation is evident. The BAP + NAA combination demonstrates a synergistic effect, while Kn + NAA promotes shoot elongation through cytokinin and auxin interactions. Organic additives and hormone combinations underscore their potential in enhancing shoot regeneration and growth, particularly orange juice supplementation with NAA and BAP. The hormonally-mediated enhancement of somatic embryo maturation by abscisic acid and sucrose underscores their regulatory roles.

Moreover, the successful clonal fidelity verification through RAPD analysis confirms the preservation of genetic uniformity in tissue culture-raised plants, ensuring authentic propagation. Lastly, gene expression patterns elucidate the complex genetic control mechanisms underlying withanolide biosynthesis, with distinct roles of specific genes in different explant types. In summary, this study unveils intricate relationships between growth regulators, physiological processes, and gene expression in *W. coagulans*, offering valuable insights into its controlled propagation, cultivation and harnessing of its bioactive compounds.

### Author's contribution

MSB, BM and SPR devised the concept and outline for the manuscript. BM wrote the article, while MSB created the figures and tables. SS, PS, NR, and SK revised the manuscript. SPR conducted a thorough review and editing of the manuscript.

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### Competing interests

The authors confirm that there are no conflicts of interest associated with this publication.

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