

# Temporal variations in biochemical attributes and stress responses in Ashwagandha (*Withania somnifera* L. Dunal) during in vitro propagation

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## Research Article

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7.

# Abstract

Ashwagandha (*Withania somnifera* L. Dunal) is a medicinally important plant with high demand in Ayurveda and other indigenous medicinal systems. Conventional propagation through seeds is inadequate to meet pharmaceutical demand due to poor germination, pathogen susceptibility, and genetic heterogeneity. Micropropagation provides a reliable alternative to overcome these limitations. In the present study, efficient protocols were developed for both direct and indirect regeneration. Shoot tip explants regenerated directly on MS medium supplemented with 5.0 mg/L BAP, while callus cultures induced with 0.5 mg/L 2,4-D supported indirect regeneration. Complete plantlets were established on MS medium containing 2.0 mg/L kinetin (KIN) and 0.1 mg/L IAA. Prolonged *in vitro* culture induced morphological, biochemical, and genetic variations among regenerants. Total protein and flavonoid contents were elevated during early subcultures, whereas H<sub>2</sub>O<sub>2</sub> levels progressively declined with successive passages. Activities of ROS-scavenging enzymes such as superoxide dismutase (SOD) and ascorbate peroxidase (APX) increased significantly. Regenerated plants exhibited distinct morphological leaf variations, further supported by genetic fidelity analysis, which confirmed divergence from greenhouse-grown counterparts. To evaluate stress responses, transcript levels of oxidative and abiotic stress-related genes (SOD, CAT, MYB, and HSP70) were quantified. Among these, HSP70 showed a pronounced upregulation (6.93-fold) in regenerants. Moreover, withanolide A accumulation under heat stress was significantly enhanced, highlighting tissue culture-induced metabolic shifts. Overall, this study establishes tissue culture-induced biochemical reprogramming, stress-gene upregulation, and secondary metabolite enhancement. These findings emphasize the need for monitoring physiological and genetic stability during *in vitro* culture of medicinal plants.

## Introduction

The use of medicinal plants in traditional medicines has been recognized worldwide for thousands of years (Singh et al., 2016). However, the demand for medicinal plants in herbal drug formulations has constantly increased in the post-COVID-19 era due to their relatively safer profiles, fewer side effects, and lower toxicity compared to synthetic drugs. Approximately two-thirds of the global population still relies on plant-based products for their primary healthcare (Singh et al., 2020). Among these, *Withania somnifera*, commonly known as Ashwagandha or Indian ginseng, belongs to the Solanaceae family and holds a pivotal role in Ayurvedic and indigenous medicinal systems (Singh and Mina 2025). It is a small, perennial, drought-tolerant C3 plant. It is widely cultivated in arid and semi-arid regions (Gaurav et al., 2023). Its cultivation extends across various regions of India including Madhya Pradesh, Uttar Pradesh, Punjab, Gujarat, Rajasthan, and more (Rout et al., 2011). The mature plant grows to a height of 1.5-2.0 meters. The flowers are greenish-yellow and borne in clusters at the leaf axils. The plant produces small, berry-like fruits that are red to yellow in color (Gaurav et al., 2023).

*W. somnifera* is specifically known for its diverse array of bioactive compounds with therapeutic properties including anti-inflammatory, anti-cancer, anti-stress, antianxiety, anticonvulsant (beneficial in both acute and chronic epilepsy), immunomodulatory, adaptogenic, endocrine, and cardiovascular

activities (Furmanowa et al., 2001). Various parts of the plant contain a wide range of chemical constituents such as alkaloids, amino acids, steroids, volatile oils, starch, reducing sugars, glycosides, withanol, chlorogenic acid, glucose, condensed tannins, and flavonoids (Uddin et al., 2012). Notably, the tender shoots are particularly rich in crude protein, calcium, and phosphorus. The primary bioactive components contributing to the medicinal properties of *W. somnifera* are withanolide A and withaferin A, which are steroidal lactones predominantly found in the roots and leaves (Tripathi et al., 2018).

Despite its immense pharmacological potential, the large-scale production of *W. somnifera* remains constrained due to its reliance on traditionally seed-based propagation. However, low seed viability, poor germination rates, and unproductive seeds have collectively hindered the efforts to meet the growing demand of the pharmaceutical industry (Kaur et al., 2021). The presence of inhibitory substances in seeds and fruit walls further reduces seed germination rates. Additionally, diseases such as blight and rot pose significant threats to plants under field conditions (Singh et al., 2017). Therefore, micro-propagation has emerged as a viable alternative for producing genetically identical and disease-free plantlets. *In vitro* propagation of *W. somnifera* offers year-round, season-independent production of plants (Shasmita et al., 2018). Several studies have reported robust and efficient micropropagation protocols using various explants of *W. somnifera* (Kaur et al., 2018, 2021; Nayak et al., 2013; Rani et al., 2014; P. Singh et al., 2017; Tata et al., 2019).

However, repeated subculture and prolonged maintenance of regenerants under tissue culture conditions often lead to substantial morphological, biochemical, genetic, or epigenetic variations caused by the tissue culture microenvironment (Bradaï et al., 2016; Ghosh et al., 2021). The tissue culture microenvironment, characterized by high concentrations of artificial growth hormones, inorganic nutrients, repeated mechanical injury during subculturing, excess humidity, accumulation of gases, and osmotic stress from excessive sucrose is considered the primary cause of tissue culture-induced variations (Bednarek and Orłowska, 2020; Rai et al., 2011; Sato et al., 2011). These conditions trigger the overproduction of reactive oxygen species (ROS) which can induce genetic and epigenetic changes through mechanisms such as DNA methylation, chromosomal rearrangements, and the introduction of deletions and substitutions in the genome, resulting in altered metabolic profile (Bednarek and Orłowska, 2020; Ghosh et al., 2021; Han et al., 2018; Krishna et al., 2016). These alterations can be identified through diverse morphological, cytological, biochemical, and molecular techniques (Bobadilla Landey et al., 2015; Kar et al., 2014; Pathak and Dhawan, 2012; Pérez et al., 2011). Among them, molecular marker techniques such as random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), and simple sequence repeats (SSRs) are most widely employed for studying the genetic stability of regenerated plants in *W. somnifera*. (Bamhania et al., 2013; Nayak et al., 2013; Shasmita et al., 2018). While useful variations responsible for agronomic traits have been extensively utilized in crop improvement, changes in the biochemical attributes of key bioactive compounds limit their applicability in the pharmaceutical industries (Aderibigbe and Anowai, 2020; Mishra et al., 2020; Iqbal et al., 2024). Therefore, identifying and maintaining genetically stable lines with uniform secondary metabolite profiles is imperative for their pharmaceutical utilization.

In this context, the present study was undertaken to investigate how prolonged subculturing and maintenance of regenerants under *in vitro* conditions affect the biochemical, morphological, and molecular characteristics of *W. somnifera*. We hypothesized that extended exposure to the tissue culture environment imposes severe physiological stress, leading to the overproduction of reactive oxygen species (ROS) which in turn causes variations in protein content, secondary metabolites (such as phenolics and flavonoids), and activity of antioxidant enzymes. To explore this, we evaluated ROS-scavenging enzyme activity, assessed genetic fidelity using molecular markers, analyzed the expression of stress-responsive and ROS-related genes via qRT-PCR, and quantified withanolide A content under heat stress conditions. Collectively, these analyses aim to deepen our understanding of tissue culture-induced stress responses and facilitate the development of optimized micropropagation protocols for the consistent production of genetically and biochemically stable lines suitable for pharmaceutical use.

## Materials and methods

### Plant materials and in vitro propagation

The fresh seeds of *W. somnifera* cv. Jawahar was collected from the medicinal plant garden of Ramakrishna Mission Ashrama, Narendrapur, Kolkata, India. Seeds were washed in running tap water for 2 minutes followed by washing with Tween 20 (4%; v/v) solution for 7 minutes. The seeds were disinfected with bavistin (0.5%, w/v, a systemic fungicide) for 7 minutes and surface sterilized with an aqueous solution of HgCl<sub>2</sub> (0.1%, v/v) for 3 minutes. After each treatment, seeds were thoroughly washed with sterile distilled water. Surface sterilized seeds were allowed to air dry under laminar air flow for 30 minutes.

### Culture medium and growth condition

Surface sterilized seeds were inoculated on semi-solid Murashige and Skoog medium (MS medium) (Murashige and Skoog, 1962) supplemented with 2% sucrose and 0.5% agar (solidifying agent). The pH of the medium was adjusted to 5.7 by using 1N of hydrochloric acid (HCl) and 1N of sodium hydroxide (NaOH) before autoclaving. The medium was heated with continuous shaking until the agar was uniformly dissolved and dispensed in a tissue culture bottle (approximately 50 ml in each bottle). Finally, the media-containing bottles were sterilized by autoclaving at 15 lb pressure and 121°C temperature for 15 minutes. Culture bottles inoculated with seeds were incubated at 25 ± 2°C for a 16-h light/8-h dark photoperiod regime and 55–60% relative humidity. *In vitro*-grown plants were used for further callus induction, shoot multiplication, and regeneration. Few seeds were also maintained in the greenhouse condition in earthen pots containing soil, sand, and vermiculite (1:1:8). These greenhouse-grown plants were used as a control for comparison with the *in vitro*-grown plants.

### Establishment of in vitro culture

The inoculated seeds were allowed to grow into shoots for 4–6 weeks. Healthy and actively dividing nodal segments were collected from *in vitro*-grown shoots. The nodal segments were inoculated on the

callus-inducing medium consisting of MS medium supplemented with 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) (Supplementary Table S1). The cultures were incubated initially in the dark at a suitable temperature for callus induction. Small pieces of callus were excised and cultured on MS media containing 2.0 mg/L 6-benzylaminopurine (BAP) with 0.1 mg/L indole-3-acetic acid (IAA) for shoot regeneration. Nodal segments were also used for direct shoot induction. For shoot induction, nodal explants were inoculated on MS media supplemented with 2.0% sucrose with 5.0 mg/L BAP. Complete plant development was induced using 2 mg/L Kinetin (KIN) with 0.1 mg/L IAA. The complete plant from callus and shoot is referred to as C and S, respectively and the value represents the number of subcultures (Fig. 1). Samples were sub-cultured every 45 days by transferring small pieces of callus or shoot to fresh medium to maintain the cultures. Subculturing was continued for up to nine subcultures in this study.

## **Estimation of total soluble proteins**

Total soluble protein content was determined according to the method of Lowry, using bovine serum albumin as a calibration standard. Reagent A (2% Sodium carbonate in 0.1N Sodium hydroxide) and Reagent B (1% Sodium potassium tartrate in 0.5% Copper sulfate) were prepared. 100 µl of samples were taken and volume made up to 1 ml. 5 ml of Reagent C (50 ml Reagent A + 1 ml of Reagent B, mixed properly before use) was added and mixed and incubated for 10 minutes. After incubation 0.5 ml of Reagent D (Folin-Ciocalteu reagent) was mixed and incubated for 30 min. at dark and the absorbance was measured at 660 nm. The concentration was calculated using the standard curve.

## **Estimation of hydrogen peroxide (HO)**

For hydrogen peroxide assay, 500 mg leaf and shoot tissues were homogenized with 5.0 ml of 0.1% chilled trichloroacetic acid (TCA) in the ice bath. The homogenate was centrifuged at 12,000 rpm for 15 minutes. 0.5 ml of supernatant was added with 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1.0 ml of 1 M potassium iodide. The absorbance of supernatant was measured at 390 nm using hydrogen peroxide as a standard (Sabir et al., 2012).

## **Determination of total phenolic and flavonoid content**

Total phenolic content and total flavonoid content were assayed using the protocol described by Khatun et al. (2008) and Chang et al. (2002) with slight modifications. Approximately 500 mg of tissues were homogenized with 5 ml of solvent. Methanol was used as a solvent for total phenolic and ethanol was used as a solvent for total flavonoid content estimation. Homogenate was centrifuged at 12,000 rpm for 15 minutes. The supernatant was used for subsequent analysis. For estimation of total phenolic content, 0.5 ml of supernatant was mixed with 2.5 ml of 10% Folin-Ciocalteu reagent followed by gentle mixing using a vortex. After that, 2.5 ml of 7.5% anhydrous sodium carbonate was added to this mixture and incubated for 30 minutes at room temperature in the dark. The absorbance was measured at 765 nm

using Gallic acid as a positive control. The amount of phenolic was expressed in Gallic acid equivalents. For estimation of total flavonoid content, 0.5 ml of supernatant was mixed with 0.1 ml of 10% aluminum chloride and 0.1 ml of 1M potassium acetate. Subsequently, the final volume (3 ml) was made using double distilled water. The mixture was kept aside for 30 minutes in the dark at room temperature. The absorbance of this mixture was measured at 415 nm. Quercetin was used as a positive control. The amount of flavonoid was expressed in terms of quercetin equivalents.

## **Determination of antioxidant enzyme activity**

### **Preparation of enzyme extract**

For assessment of different antioxidant enzyme activities, 1gm of fresh leaves and shoot tissues were collected from different cultures and were individually crushed in a pre-chilled mortar pestle with 10 ml of 0.1 M cold phosphate buffer (pH 7.0). The respective homogenates were then transferred into a fresh Eppendorf tube and centrifuged at 12,000 rpm for 15 min at 4°C. The resultant supernatants were used as enzyme extracts to be further utilized in assays and were kept in ice. Each parameter was analyzed using three replicates for each treatment.

### **Superoxide dismutase (SOD)**

Superoxide dismutase (SOD) activity of the samples was measured by calculating the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 20 µM riboflavin, 0.1 mM EDTA, 75 µM NBT and 50 µl crude enzyme. A blank was set without enzyme and NBT to calibrate the spectrophotometer while the reference control had NBT but no enzymes. All tubes were exposed to 400 w bulbs for 15 min and absorbance was read immediately at 560 nm.

### **Catalase (CAT)**

Catalase (CAT) activity was determined by using a reaction mixture containing 35 mM phosphate buffer (pH 7), 15 mM H<sub>2</sub>O<sub>2</sub>, and 100 µl enzyme extracts. Absorbance at 240 nm was read after adding the enzyme immediately every 10-sec interval for 90-sec enzyme activity was calculated using the molar extinction coefficient of H<sub>2</sub>O<sub>2</sub> (Chance and Maehly, 1955).

### **Ascorbate peroxidase (APX)**

The reaction mixture (3 ml) of ascorbate peroxidase (APX) contained 50 mM potassium phosphate buffer (pH 7), 0.5 mM ascorbic acid, 250 mM H<sub>2</sub>O<sub>2</sub>, and 0.225 ml of supernatant. Oxidation of ascorbic acid was followed by a decrease in absorbance at 290 nm. The enzyme activity was calculated using the molar extinction coefficient of H<sub>2</sub>O<sub>2</sub> (Nakano and Asada, 1981).

# Morphological analysis

*In vitro*-grown plantlets including A9, C9, and S9 were assessed for phenotypic characteristics. The morphological features such as the height of the plantlets, leaf area, leaf length, and leaf width were measured. The *ex-vitro* plantlets, grown under greenhouse conditions were used for comparison. Phenotypic measurements were carried out in five replications. The significance of differences was calculated using the Graph Pad 8.0 software package.

## Genetic fidelity analysis

Genomic DNA was isolated from A9, C9, S9, and an *ex vitro* plantlet (aerial part) using the CTAB method described by Chandra and Tewari (2007) with some minor modifications. The quality and quantity of the isolated genomic DNA were assessed using Nanodrop (Thermo Fisher Scientific Inc. USA). A total of 32 random amplified polymorphic DNA (RAPD) primers were used to assess the genetic fidelity among the selected groups of plants (Supplementary Table S2). The PCR was performed in a total of 25 µl reaction mixture containing 2 µl of genomic DNA (30 ng/ µl), 2.5 µl of 10X PCR buffer, 0.2 mM dNTPs (10 mM), 1.5 mM of MgCl<sub>2</sub>, 1 U *Taq* polymerase and 1 µl of 10 pmol primer. PCR was performed for initial denaturation at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, primer annealing at 37°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The amplified products were resolved in 2.5% agarose gels stained with ethidium bromide (5µl/100ml) in 1X TAE buffer. After electrophoresis, the gel was visualized and documented using the gel documentation system (Gel Doc XR+, Bio-RAD Laboratories, Inc. USA). A 50-base pairs ladder was used as a reference to score the band size. The well-resolved bands were scored based on the presence (1) or absence (0) of the bands. Genetic fidelity status was determined among the DNA samples. The data were pooled in a binary matrix to calculate the dissimilarity matrix using the DICE algorithm by DARwin 6.0 (<http://darwin.cirad.fr/darwin>). The neighbor-joining tree was constructed for phylogenetic analysis.

## RNA extraction and cDNA preparation

Total RNA was extracted from approximately 100 mg plant tissues from selected samples using the Trizol reagent (QIAzol lysis reagent, QIAGEN Inc. Germany) followed by DNase treatment of each sample manufacturer's instructions. The quality and integrity of RNA was assessed using agarose gel electrophoresis. RNA was quantified by NanoDrop (Thermo Fisher Scientific Inc. USA). The RNA was normalized and 1.0 µg of RNA was subjected to first-strand cDNA synthesis using QuantiTect Reverse Transcription kit (QIAGEN Inc. Germany) following the manufacturer's instructions.

## Quantitative real-time polymerase chain reaction (qRT-PCR)

Appropriate dilution of the cDNA was prepared for qRT-PCR analysis. The qRT-PCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-RAD Laboratories, Inc. USA) on CFX connect real-time system (Bio-RAD Laboratories, Inc. USA) according to standard protocol. The ribosomal protein L2 (*rpl2*) gene was used as the internal control for normalization. The gene sequences were retrieved using the basic local alignment search tool (BLAST) search of tomato homologs from the National Centre for

Biotechnology Information (NCBI) database against non-redundant protein sequences. Primers for qRT-PCR were designed from the PrimerQuest™ Tool (Integrated DNA Technologies, Inc. USA). The gene-specific primers for qRT-PCR analysis were synthesized from Eurofins Genomics, India for qRT-PCR analysis were synthesized from Eurofins Genomics, India. The primers used in this study are given in Table 1.

| Table 1<br>List of primers used for expression analysis of redox- and stress-responsive genes in regenerants |                         |                        |         |                      |
|--------------------------------------------------------------------------------------------------------------|-------------------------|------------------------|---------|----------------------|
| Genes                                                                                                        | Forward Primer (5'-3')  | Reverse Primer (5'-3') | Tm (°C) | Amplicon Length (bp) |
| RPL2                                                                                                         | TTAGTGACCCTCCGTAGGAC    | GGTAGGACACAAAGGAGTAACC | 61.5    | 101                  |
| SOD                                                                                                          | ACAGTGCCATCAAATTCAACGG  | TAGTGTCAATAGCCCAGCCAAG | 60      | 126                  |
| CAT                                                                                                          | TCAAGCAACCAGGAGAAAGG    | GTGATACGAGGATCAGACAAGG | 62      | 97                   |
| MYB                                                                                                          | GCGCTAATTATGTGCCAACC    | CCTTCGCACTACTCTTTCTTCT | 62      | 96                   |
| HSP70                                                                                                        | CCGATAACCAACCTGGAGTATTG | GGGAATACCGGAAAGCTCAAA  | 62      | 100                  |

| Table 1<br>Enzyme activities reflecting the oxidative stress and antioxidant defense mechanisms in <i>W. somnifera</i> during <i>in vitro</i> culture. |                    |               |             |
|--------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|---------------|-------------|
| Regenerants                                                                                                                                            | SOD (% inhibition) | CAT (U/min)   | APX (U/min) |
| C6                                                                                                                                                     | 28.31 ± 2.08       | 61.25 ± 12.42 | 0.51 ± 0.13 |
| C7                                                                                                                                                     | 28.16 ± 1.30       | 95.23 ± 17.17 | 0.08 ± 0.01 |
| C8                                                                                                                                                     | 43.61 ± 3.38       | 78.69 ± 2.54  | 0.06 ± 0.00 |
| C9                                                                                                                                                     | 40.19 ± 9.67       | 56.27 ± 9.43  | 0.07 ± 0.00 |
| S6                                                                                                                                                     | 37.35 ± 3.98       | 81.51 ± 7.63  | 0.07 ± 0.03 |
| S7                                                                                                                                                     | 28.32 ± 0.89       | 30.74 ± 6.02  | 0.17 ± 0.01 |
| S8                                                                                                                                                     | 31.78 ± 1.87       | 79.00 ± 27.44 | 0.06 ± 0.01 |
| S9                                                                                                                                                     | 43.04 ± 3.49       | 56.99 ± 4.99  | 0.05 ± 0.00 |

## Withanolide A extraction and high-performance liquid chromatography (HPLC) analysis

Callus were dried and ground into a fine powder and extracted with methanol (Sivanandhan et al., 2012). HPLC was performed with Agilent 1269 infinity quaternary LC system (Agilent Technologies Inc. USA) consisting of diode array detector G42124 (DAD) and 1260 evaporative light scattering detector (ELSD) with auto-injector using NUCLEOSIL C18 (4.6x250 mm) (MACHEREY-NAGEL GmbH & Co. KG). The

wavelength scan range of the PDA detector was 190–400 nm and the chromatograms were recorded at 230nm. Separations were carried out with reverse-phase UltiMate 3000, 2500x4.6 columns (Thermo Fisher Scientific Inc. USA) and isocratic elution with acetonitrile and 0.1% aqueous solution of acetic acid (40:60 ratio) at 35°C and 120 bars with a flow rate of 1 ml/min. Analytical grade withanolide A was used as a standard, procured from Natural Remedies Pvt. Ltd. (Bengaluru, Karnataka, India).

## Statistical analysis

Each experiment for biochemical and gene expression analysis was performed in triplicates. The presented values indicate the mean  $\pm$  standard error (SE). Statistical analysis was performed using Graph Pad Prism 8.0. One-way analysis of variance (ANOVA) was performed for comparing means and significant variations ( $p \leq 0.05$ ) among means were determined using Duncan's multiple range test (DMRT).

## Results

### Establishment of *in vitro* culture of *W. somnifera*

We started our investigation by *in vitro* propagation of *W. somnifera*. The fresh seeds were surface sterilized and inoculated on semi-solid full-strength MS media supplemented with sucrose. Imbibition of seeds was observed after three days of inoculation in the media. We found reduced seed germination. Approximately, 75% of the seeds were germinated which subsequently proliferated into shoots. Roots were developed after eight days. Forty-five days post-inoculation, fully developed plants were observed. These plants were used as mother plants.

## Callus, shoot, and root induction

This study demonstrates the establishment of an efficient protocol for *in vitro* propagation of callus and shoots from the nodal explants. For callus induction, nodes were inoculated on MS media supplemented with 0.5 mg/L 2,4-D. White opaque calluses were initiated seven days after inoculation, primarily at the wound site. Callus was regenerated to multiple adventitious shoots by transferring them to the shoot induction medium containing 2.0 mg/L BAP with 0.1 mg/L IAA. The white callus exhibited a color change from white to green, signaling successful shoot induction. Subsequently, shoot development ensued, leading to the complete development of both shoots and roots in the plants. On the contrary, direct shoot formation from nodal explants was also achieved by inoculating nodes from mother plants on the shoot induction medium. Multiple shoots appeared after seven days post-inoculation. The shoots were grown on root root-inducing medium until further use.

## Estimation of total soluble proteins

Total soluble protein was estimated from plants derived from callus and direct shoot culture. Across both groups, a consistent decline in protein content was observed with each successive subculture (Fig. 2, Supplementary Table S3). The most striking result emerged from shoot-derived plants, where the

highest protein content was recorded in S6 at 265.3 µg/ml. However, further sub-culturing to a fresh media each time progressively reduces total soluble protein content. The lowest protein content in this group was measured in S9 (84.6 µg/ml). Similarly, the notable soluble protein content was 195.5 µg/ml in plants C6 derived from callus. However, the subsequent sub-culturing gradually reduced the total soluble protein content. The lowest protein content in this group was noted in C9 at 110.8 µg/ml, representing an 89.36% variation from the mother plant.

## **Contrasting trends of H<sub>2</sub>O<sub>2</sub> production observed between callus- and shoot-derived plants**

Further, the impact of subculture on H<sub>2</sub>O<sub>2</sub> was investigated in callus and shoot-derived plants of *W. somnifera* (Fig. 2, Supplementary Table S3). Interestingly, contrasting trends were observed between callus- and shoot-derived plants. There was a gradual increase in H<sub>2</sub>O<sub>2</sub> content after each subculture in callus-derived plants. The highest H<sub>2</sub>O<sub>2</sub> content was observed in C9 (0.09 mM). In contrast, direct shoot-derived plants exhibited an opposite trend. In this group, the highest H<sub>2</sub>O<sub>2</sub> was observed in S6 (0.12 mM).

## **Significant variations in the phenolics and flavonoids content among different regenerants**

The total phenolics and flavonoids content in callus and shoot-derived plants of *W. somnifera* were assessed to understand their potential role in stress response and adaptation (Fig. 2, Supplementary Table S3). The results revealed significant variations in the phenolics and flavonoid content among different regenerants. In callus-derived regenerants (C6-C9), the total phenolics content ranged from 329.12 to 453.48 mg/g, with the highest content observed in C8. Similarly, the total flavonoid content ranged from 310.37 to 793.56 mg/g, with the highest content also observed in C8. In contrast, shoot-derived regenerants (S6-S9) showed different trends in phenolics and flavonoid content. While the total phenolics content ranged from 285.09 to 490.91 mg/g, with the highest content observed in S7, the total flavonoids content ranged from 249.09 to 1029.63 mg/g, with the highest content observed in S6.

## **Complex interplay of oxidative stress and antioxidant defense mechanisms in *W. somnifera* during in-vitro culture**

To understand the level of oxidative stress in callus and shoot-derived plants, the activity of reactive oxygen species (ROS) scavenging enzymes (SOD, CAT, and APX) was assessed (Table 1). The results revealed distinct patterns of enzyme activity changes across different stages, highlighting the complex interplay of oxidative stress and antioxidant defense mechanisms in *W. somnifera* during in-vitro culture. Our results provide a better insight into the correlation between the prolonged subculture of plant tissue and antioxidant enzyme activities. SOD is an important enzyme that catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide. The results showed varying levels of SOD activity among the regenerants. SOD activity was substantially low in the early subculture stage of plants

of both groups. However, the SOD activity increased with the number of subcultures. The highest activity of SOD was observed in C8 (43.61%) and S9 (43.04%) among the callus-derived and shoot-derived plants. CAT is another key enzyme involved in the decomposition of hydrogen peroxide into water and oxygen. The regenerants exhibited different CAT activity levels. The lowest activity of CAT was observed in C9 (56.27 U/min) and S7 (30.74 U/min). The highest activity was observed in C7 (95.23 U/min) and S6 (81.51 U/min). This suggests that certain regenerants may have a more efficient system for detoxifying hydrogen peroxide, which is a byproduct of various metabolic processes. APX is an enzyme that plays a crucial role in the ascorbate-glutathione cycle, which is involved in scavenging hydrogen peroxide and protecting cells from oxidative damage. The results showed varying levels of APX activity among the regenerants, with the highest activity observed in C6 (0.51 U/min) and S7 ( $0.17 \pm 0.01$  U/min). This indicates that certain regenerants may have a more efficient system for scavenging hydrogen peroxide and protecting against oxidative stress.

## Correlation of biochemical status with antioxidant compounds

The correlation matrix reveals intricate relationships among various biochemical parameters in *W. somnifera*, shedding light on the plant's response to oxidative stress (Fig. 3, Supplementary Table S4). Total soluble protein (TSP) shows a strong positive correlation with all antioxidant markers except total phenolic content ( $r = -0.01$ ,  $p < 0.05$ ) and SOD ( $r = -0.39$ ), suggesting a potential link between protein content and oxidative stress levels. This indicates that plants producing higher total soluble protein have developed a more efficient ROS scavenging system. The total soluble protein content showed the strongest correlation with total flavonoid content ( $r = 0.88$ ) followed by  $H_2O_2$  ( $r = 0.58$ ). In contrast, TSP showed very weak correlations with CAT ( $r = 0.31$ ), and APX ( $r = 0.33$ ).  $H_2O_2$  showed a positive correlation with total flavonoid content ( $r = 0.62$ ). It suggests that the production of high  $H_2O_2$  levels induces the levels of total flavonoid content in cells, suggesting that this antioxidant compound may be involved in scavenging  $H_2O_2$  and reducing oxidative damage. However,  $H_2O_2$  exhibited a weak correlation with other antioxidants. For example, SOD ( $r = 0.20$ ) and CAT ( $r = 0.08$ ). Interestingly,  $H_2O_2$  showed a negative correlation with total phenolic content ( $r = -0.22$ ) and APX ( $r = -0.15$ ), indicating a complex relationship between  $H_2O_2$  levels and the accumulation of these compounds. Interestingly, total phenolic content showed no significant correlation with other metabolites, antioxidant compounds, and enzymes. SOD was weakly correlated with  $H_2O_2$  ( $r = 0.20$ ) and CAT ( $r = 0.05$ ) while negatively correlated with other parameters in this matrix. Similarly, CAT showed a weak positive correlation with protein,  $H_2O_2$ , flavonoids, and SOD while showing a negative correlation with total phenolic and APX. APX is another enzyme involved in ROS scavenging showed a weak positive correlation with total protein content and total flavonoids, suggesting a potential interaction between APX activity and flavonoid content in ROS detoxification. APX showed a weak negative correlation with  $H_2O_2$ , phenolic, SOD, and CAT, indicating a potential regulatory relationship between these enzymes in ROS scavenging pathways.

## Morphological analysis of in vitro-grown plants

Variations in morphology were observed among regenerated plantlets derived from direct (S) and indirect (C) regeneration propagation methods. Significant differences in plant height and leaf characteristics were noted among *in vitro*-grown plantlets when compared to the *ex-vitro*-grown plants. The plant height of regenerants was significantly lower than *ex vitro* plantlets. The average height of A9, C9, and S9 was 36.8, 104.4, and 108.0 mm, respectively compared to *ex-vitro*-grown plants (251.2 mm). The leaf shape of *in vitro*-grown plants appeared narrower and more elongated (Fig. 4). The mean leaf area recorded for A9, C9, and S9 was 19.0, 53.0, and 28.0 mm<sup>2</sup> respectively (Fig. 5).

## Genetic fidelity assessment using RAPD markers

Genetic fidelity analysis of *in vitro*-grown plantlets was assessed using 25 RAPD markers, which generated a total of 151 scorable bands with an average of 6.04 bands per primer. Among these, 32 bands were found polymorphic with a polymorphism rate of 21.19% and mean polymorphic bands per marker of 1.28. The total number of amplicons ranged from 2 (OPA20 and OPB4) to 11 (OPA2 and OPJ12). The percentage of polymorphism varied from 14.28% to 75.0%. Nine primers were monomorphic and were excluded from further analysis.

Interestingly, *in vitro*-propagated plants exhibited lower levels of polymorphism compared to their *ex-vitro* counterparts. The lower level of polymorphism was further supported by the dissimilarity matrix analysis which indicates higher genetic similarity among *in vitro*-propagated plantlets and exhibits genetic divergence when compared to *ex vitro* counterparts (Fig. 6a). The dissimilarity matrix revealed that the *ex vitro*-grown plants were the most genetically distinct compared to all the *in vitro* regenerated plants. Among the *in vitro*-propagated lines, S9 was the most genetically dissimilar. The result shows greater dissimilarity with both C9 and A9 with a 0.07 dissimilarity index of 0.070 and 0.089, respectively. In contrast, C9 and A9 maintained higher similarity to each other with a 0.048 dissimilarity index.

The weighted Neighbor-Joining tree analysis further supported these findings. The phylogenetic tree revealed two major clusters (Fig. 6b). The *ex-vitro* plantlets formed distinct clusters that were separated from the *in vitro*-grown plantlets. The *in vitro* plantlets were grouped in a separate cluster, which was further divided into two sub-clusters. Among the *in vitro* lines, S9 displayed the greatest genetic diversity relative to C9 and A9. This observed genetic distinctiveness of S9 as compared to C9 and A9 is likely due to variations induced by prolonged exposure to tissue culture environment or repeated sub-culturing during *in vitro* propagation.

## Gene expression profiling of redox- and stress-responsive genes

In this study, we investigated the relative expression of redox-responsive and stress-related genes, namely SOD, CAT, MYB, and HSP70. We observed significantly lower transcript abundance for all genes in plants maintained in the early subculture stage. The 45-day-old shoots derived from nodal segments were taken as a control for normalization of transcript accumulation and comparison with the *in vitro*-grown callus- and shoot-derived plants. Our results reveal that the relative transcript abundance of redox-

responsive genes SOD and CAT in C9 was 3.31- and 3.34-fold higher, respectively, while it was 3.49- and 1.65-fold higher, respectively, in S9 than in the control plant (Fig. 7b-e, Supplementary table S5). Due to the higher accumulation of transcripts for ROS-scavenging enzymes SOD and CAT, we further investigated the stress-responsive genes in C9 and S9. We found that the relative expression of MYB and HSP70 in C9 and S9 was 1.57- and 1.76-fold higher, respectively than in the control. Additionally, the relative expression of HSP70 in C9 and S9 was 4.43- and 6.93-fold higher, respectively than A1. These findings suggest that plants grown in tissue culture with prolonged subcultures experience more stress than those in the early stages of plant tissue culture under controlled conditions.

## Quantification of withanolide A content

Many abiotic stresses positively regulate the production of secondary metabolites. Withanolide is the main constituent of *W. somnifera*. Our previous experiment showed that HSP70 was highly induced in C9 and S9 compared to A1, providing a strong candidate for further study. To understand high-temperature stress as an abiotic elicitor, we investigated the accumulation of withanolide A content in the callus upon exposure to high-temperature stress. Using HPLC, we quantified withanolide A content in the callus of *W. somnifera* when treated with varying degrees of temperature stress. Our results revealed a significant increase in withanolide A content in callus under temperature stress. Compared to the control sample (279.764 ppm), withanolide A content increased to 544.007 ppm at 45°C stress, indicating a moderate but notable effect of heat stress on withanolide production. Furthermore, withanolide A content was increased substantially to 1197.586 ppm at 50°C stress, demonstrating a clear correlation between temperature stress intensity and withanolide A accumulation. The highest withanolide A content was observed at 55°C stress, reaching 1505.34 ppm, highlighting the remarkable capacity of plants to enhance withanolide A production under extreme heat stress conditions (Fig. 7f). These findings shed light on the regulatory mechanisms governing withanolide biosynthesis in *W. somnifera* under temperature stress and may have commercial implications for the cultivation and processing of high-value bioactive compounds from this medicinal plant.

## Discussion

### In vitro propagation and analysis of morphological variations

Ashwagandha is a valuable medicinal plant used in various herbal formulations. However, conventional methods of propagation are inadequate to meet the increasing demand due to low seed viability and poor germination rates (Khanna et al., 2013). Furthermore, biotic stress caused by major pathogens significantly hampers productivity (Shasmita et al., 2018). Therefore, *in vitro* propagation has emerged as a promising alternative for large-scale production. Several studies have developed efficient protocols for micropropagation of Ashwagandha (Dewir et al., 2010; Furmanowa et al., 2001; Kaur et al., 2021; Misra, 2015; Nayak et al., 2013; Rani et al., 2014; Tata et al., 2019). In the present study, we established efficient protocols for both direct and indirect regeneration of plantlets through *in vitro* propagation in *W. somnifera*. Clonal multiplication through micropropagation of *W. somnifera* allows rapid production of raw materials. However, we observed that persistent and prolonged subculture, especially in media

supplemented with high concentrations of plant growth regulators can lead to morphological and genetic changes that adversely affect plant growth and development and metabolite content.

### **Metabolic response in in vitro propagated plants**

In our study, we compared the metabolic responses of plantlets derived from callus cultures (C6) and shoot cultures (S6) of *W. somnifera*. While C6 and S6 plants showed comparable total protein content, their ROS profile differed, particularly in H<sub>2</sub>O<sub>2</sub> accumulation. Excessive H<sub>2</sub>O<sub>2</sub> can be harmful to plants if not properly detoxified. In tissue culture plants, the production of ROS is one of the major reasons that induce morphological, genetic, and epigenetic changes (Ghosh et al., 2021). Plants possess well-developed enzymatic and non-enzymatic antioxidant mechanisms to counter ROS and maintain cellular homeostasis (Mir et al., 2015; Mishra et al., 2019). C6 plants had higher total soluble protein content but relatively low H<sub>2</sub>O<sub>2</sub> levels and further subculturing of C6 to fresh media reduced the protein levels and led to the accumulation of higher H<sub>2</sub>O<sub>2</sub> levels. This suggests that higher protein content in C6 plants may lead to more efficient ROS scavenging or minimal oxidative stress, while plants with low protein content may experience higher oxidative stress. On the other hand, S6 plants had higher total soluble protein content and higher H<sub>2</sub>O<sub>2</sub> levels, which decreased with subsequent subculturing. Additionally, higher H<sub>2</sub>O<sub>2</sub> levels in S6 plants were positively correlated with higher levels of total soluble protein, flavonoids, superoxide dismutase (SOD), and catalase (CAT), but negatively correlated with phenolics and ascorbate peroxidase (APX). These patterns suggest dynamic modulation of antioxidant responses depending on the culture type and subculturing stage.

Biochemical constituents such as proteins, phenolics, and flavonoids, together with enzymatic antioxidants (e.g., SOD, CAT, APX), are known to be upregulated under abiotic stresses such as drought, heat, and salinity. These molecules contribute to stress tolerance by maintaining redox balance and regulating stress signaling pathways (Sanchita et al., 2015; Sharma et al., 2023a).

Among antioxidant enzymes, SOD plays a crucial role in dismutating superoxide radicals into H<sub>2</sub>O<sub>2</sub> which is then further detoxified by CAT and APX. The time-dependent accumulation of SOD was reported in *W. somnifera* during the acclimatization (Fatima et al., 2013). Increased accumulation of H<sub>2</sub>O<sub>2</sub> and reduced SOD levels accompanied the decrease in the withanolide content in *W. somnifera* under heavy metal stress (Mishra et al., 2019). Further, high amounts of phenolics and flavonoids reduce the harmful effect of ROS under Cd stress in *W. somnifera* (Mishra and Singh Sangwan, 2019; Mishra et al., 2014). Salt-induced increments of ROS scavenging enzymes SOD and CAT and non-enzymatic anti-oxidants were reported in *W. somnifera* (Sharma et al., 2023b).

## **Tissue culture-induced morphological variations and assessment of genetic fidelity**

In the present study, the *in vitro*-grown plantlets were maintained up to the 9th subculture stage. Several morphological variations were observed in shoot length and leaf characteristics, including variations in

leaf length, width, and area. These variations can be attributed to several factors, including the transition from a macro- to a micro-environment, the age of the culture, the hormonal composition of the media, culture conditions, the production of a high amount of reactive oxygen species (ROS), the accumulation of mutations over time, the activation of retrotransposons or epigenetic alterations, and the source of the explants (Bairu et al., 2011; Duta-Cornescu et al., 2023; Gao et al., 2009; Orłowska, 2021; Rodríguez López et al., 2010; Sahijram et al., 2003; Smulders and de Klerk, 2011). *In vitro* cultivation restricts plants to a confined environment, limiting their growth and development compared to plants grown in natural field conditions, often resulting in smaller plant sizes (Gantait et al., 2023). Moreover, prolonged *in vitro* culture can induce somaclonal variation, leading to diverse alterations in growth habits, stem structures, and leaf morphology (Bradaï et al., 2016). Morphological variations observed in plants during tissue culture are often linked to genetic instability. Characterizing these genetic instabilities and assessing genetic fidelity is crucial for determining the genetic uniformity of *in vitro* propagated plants. This can be achieved using one or a combination of molecular markers, such as RAPD, ISSRs, SSRs, SCoT, and others (Jogam et al., 2020; Kaur et al., 2021; Rai et al., 2012; Rathore et al., 2016; Rohela et al., 2019; Tikendra et al., 2019). While SCoT and SSRs are highly specific, reproducible, and reliable, RAPD and ISSR are equally useful in crops without available genome sequences (Tikendra et al., 2021). Our results demonstrated long-term maintenance of plantlets in tissue culture media supplemented with a high concentration of plant growth regulators induces morphological variations, which we further verified by 25 RAPD markers. The phylogenetic tree based on RAPD markers revealed direct and indirect regenerated plantlets A9, C9, and S9 were genetically dissimilar to *ex vitro*-grown plantlets.

## Up-regulation of stress-related genes

The variability observed in micropropagated plants can often be attributed to oxidative stress-induced damage incurred by plant tissues during *in vitro* culture (Bednarek and Orłowska, 2020; Cassells and Curry, 2001). Oxidative stress accompanying plant regeneration affects the proper functioning of many vital cell organelles. To counteract this, an anti-oxidant defense system is activated to scavenge the harmful ROS. SOD, CAT, and APX are the major enzymes expressed in response to oxidative stress. SOD transcripts were reported to be highly expressed in the presence of copper stress in *W. somnifera* (Rout and Sahoo, 2013). The expression of MYB, WRKY, and HSP70 was shown to up-regulate under drought and salt stress in *W. somnifera* (Sanchita et al., 2015; Sharma et al., 2023a). However, our results showed no significant change in the relative expression of the MYB gene in response to tissue culture-induced stress. In contrast, HSP70 was significantly expressed in A9, C9, and S9 plantlets. HSP70 has been shown to be involved in protecting proteins in stressed cells. The observed upregulation of HSP70 in our study may be attributed to osmotic or redox stress resulting from prolonged exposure to the tissue culture microenvironment (Sanchita et al., 2015). The role of HSP70 in improving the viability of cryopreserved pollen was highlighted by orchestrating the regulation of oxidative stress and programmed cell death (Ren et al., 2019).

## High-temperature stress elicits withanolide A content in callus

Plants are constantly exposed to various environmental stresses in their lifetime. To survive under adverse conditions, plants have developed several adaptive mechanisms. Under these conditions, the production of secondary metabolites has up-regulated many folds. Abiotic stresses such as drought, salt, cold, low light, UV and heavy metals have been documented to act as elicitors for producing enhanced withanolide content in *W. somnifera* (Jacob et al., 2014; Mir et al., 2015; Mishra and Singh Sangwan, 2019; Mishra et al., 2019; Sabir et al., 2012; Sanchita et al., 2015; Sharma et al., 2023b; Singh et al., 2018; Takshak and Agrawal, 2017).

Under normal conditions, callus showed very low amounts of withanolide A compared to suspension cultures (Sabir et al., 2008). The production of secondary metabolites such as withanolide A and withaferin A in an *in vitro* system is influenced by plant growth regulators such as 2,4-D and kinetin (Chakraborty et al., 2013). Heat stress has also been shown to affect total phytochemical content, flavonoids, and antioxidant enzymes (Singh and Mina 2025). Elevated temperatures (~ 5°C above ambient) have been shown to increase root ginsenoside concentrations in *Panax quinquefolius*, despite a reduction in overall root biomass (Jochum et al., 2007).

Exposure of *W. somnifera* to high temperatures (48°C and 58°C) for 120 days significantly increased withanolide, phenolic, and flavonoid contents in leaves, stems, and roots, compared to control plants at 22°C. In contrast, lower temperatures (8°C and 18°C) led to a decline in these compounds (Sharma and Puri, 2017). Similarly, cold stress at 4°C for 15 days enhanced withanolide accumulation in the leaves and roots of genotypes AGB002 and AGB025, indicating that both high and low-temperature stresses can modulate secondary metabolite production in *W. somnifera* (Mir et al., 2015).

Our results reveal a significant increase in withanolide A content in callus exposed to temperature stress, highlighting the ability of plants to enhance withanolide A production under adverse environmental conditions. Temperature-induced elicitation of high-value compounds is likely mediated by the activation of specific transcription factors and biosynthetic pathway genes. For instance, the WRKY transcription factor WsWRKY1 has been shown to regulate triterpenoid withanolide accumulation by modulating phytosterol and defense pathways. Additionally, heat stress may upregulate genes involved in the mevalonate pathway, such as 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), leading to increased precursor availability for withanolide biosynthesis (Singh et al., 2017). Similarly, the expression of WsMYB34 triggers the accumulation of withanolide and flavonoid content under salinity stress (Sharma et al., 2023a). The quantification of withanolide A content in *W. somnifera* under high-temperature stress provides valuable insights into the regulatory mechanisms of secondary metabolite production in response to abiotic stresses. Understanding these mechanisms can inform strategies for the industrial-scale production of high-value compounds.

### **The commercial implication of producing high-value compounds through in vitro culture**

*In vitro* propagation provides a rapid method for producing true-to-type clones of the mother plant. Therefore, these plants grown under controlled conditions exhibit a season-independent and uniform profile of secondary metabolites, rendering them suitable for the commercial production of high-value

bioactive compounds on an industrial scale (Krishna et al., 2016). However, somaclonal variations pose a significant challenge in the production of secondary metabolites in tissue culture-raised plants (Bairu et al., 2011). While some beneficial variations have been utilized in crop improvement, variations that affect secondary metabolites or are associated with undesirable effects may limit their applicability in the pharmaceutical industry. Callus and suspension cultures are the primary choices for producing pharmaceutically important secondary metabolites (Yue et al., 2016). Callus culture can be grown year-round without influencing metabolite status (Isah et al., 2018). Moreover, large-scale, automated production of callus cultures can be achieved in bioreactors, offering optimized production of withanolide A and scalability to meet the increasing demand in pharmaceuticals, cosmetics, and nutraceuticals (Isah et al., 2018). In our study, we demonstrated that high-temperature treatment in callus culture enhances the production of withanolide A seven-fold compared to untreated. Previously, Bonfill et al. (2002) reported the successful production of ginsenoside from *Panax ginseng* using callus culture. This approach could lead to the development of new products and formulations that harness the therapeutic properties of withanolide A to benefit both the industry and consumers.

## Conclusion

Propagation of *W. somnifera* through seeds is very difficult due to seed dormancy, seed viability, and pathogen infection. Therefore, clonal propagation by tissue culture offers a rapid means to propagate a large number of pathogen-free, true-to-type plants within a short span of time. Here we have shown that  $H_2O_2$  is substantially expressed as the number of subcultures is increased. We examined the influence of oxidative stress and ROS-scavenging enzymes including SOD, CAT, and APX on the age of the tissue-cultured plants. However, long-term maintenance of plantlets through tissue culture led to morphological variations. The genetic fidelity of tissue culture-grown plantlets was determined by RAPD markers. The transcript accumulations were quantified for oxidative stress-responsive genes and abiotic stress-responsive genes revealed upregulation than early sub-cultured plants. Our result demonstrated that the expression of HSP70 was 4.4–6.9 fold higher. Furthermore, exposure to elevated temperatures led to a marked enhancement of withanolide A content in the callus, suggesting heat stress as an elicitor of secondary metabolite in *W. somnifera*. These findings highlight the commercial implication of stress-induced secondary metabolite production for optimizing cultivation practices and improving the yield of bioactive compounds in medicinal plants.

## Declarations

## Competing interest

The authors have no relevant financial or non-financial interests to disclose.

## Ethics declaration

Not applicable

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## Author contribution

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were carried out by Adrija Banerjee, Titiryu Chakraborty, and Alakesh Pal. The first draft of the manuscript was prepared by Adrija Banerjee, and Kishor Kumar. Proofreading of the manuscript with critical suggestions was provided by Dipak Manna. All authors read and approved the final manuscript.

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## Data availability

The data presented in this study are available in the manuscript as supplementary materials.

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## Figures

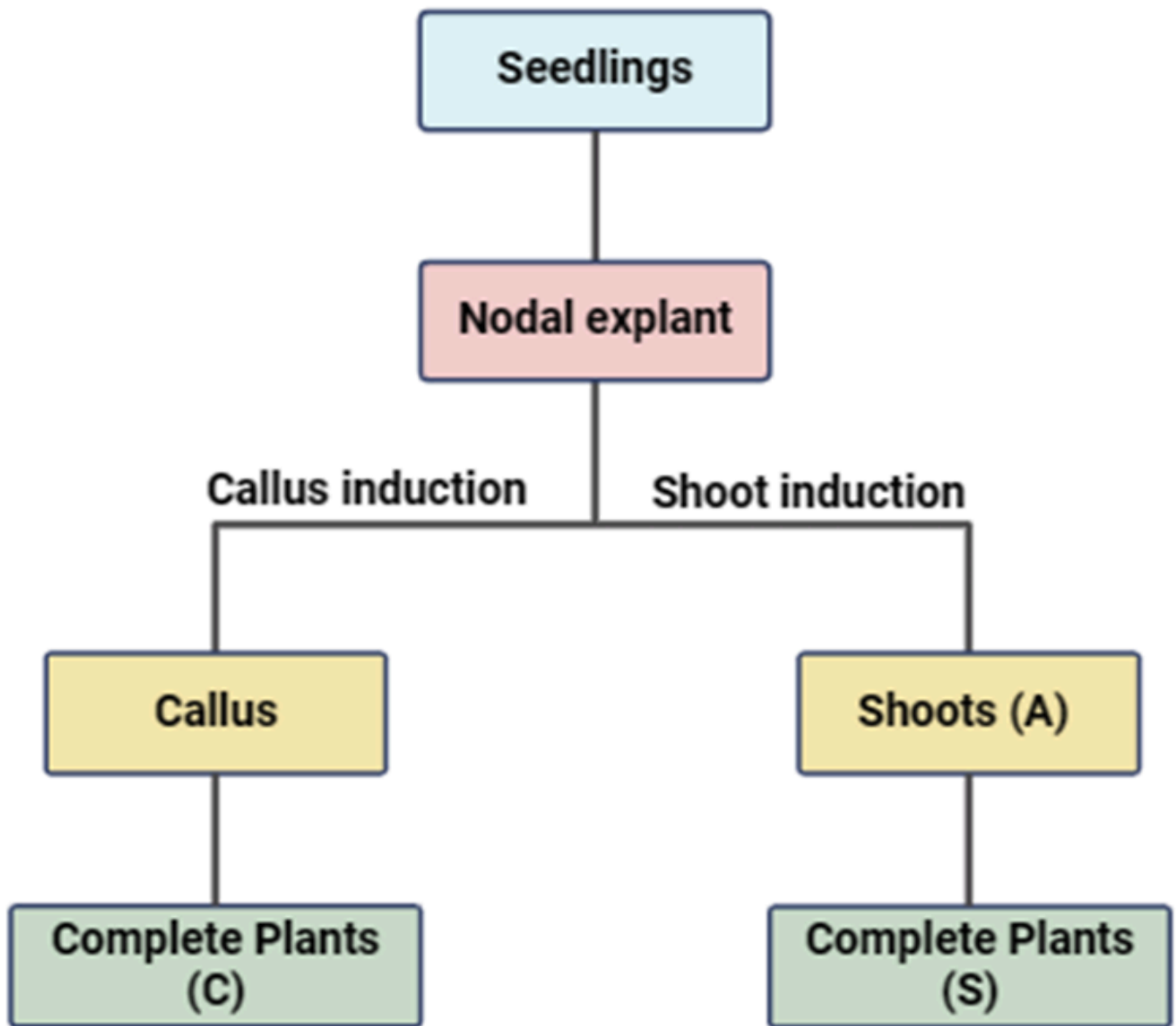


**Figure 1**

Schematic representation of the production of *in vitro* plantlets through direct and indirect regeneration in *W. somnifera*.

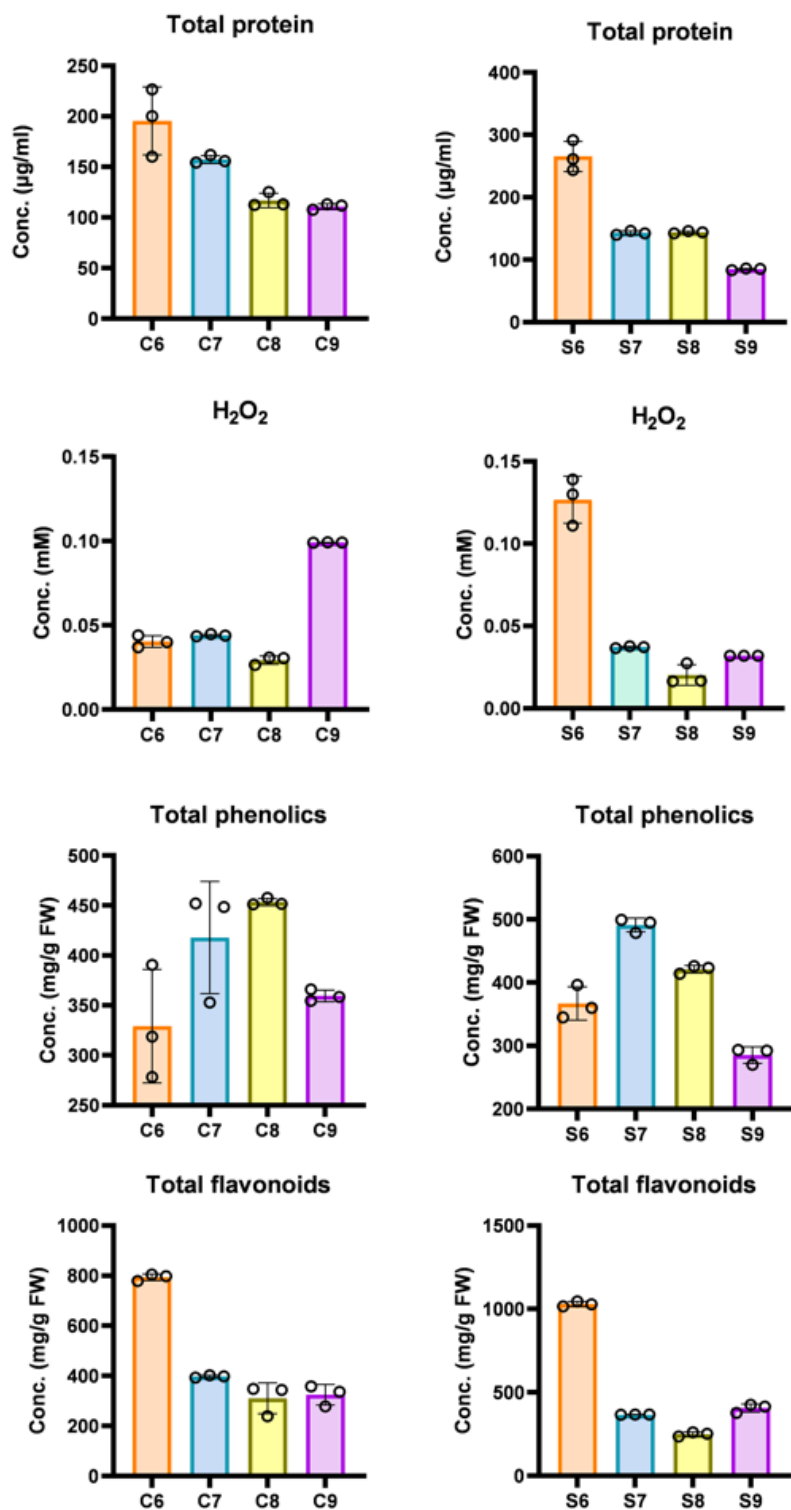


Figure 2

Bar graphs showing biochemical attributes in callus and shoot-derived regenerants of *W. somnifera* across successive subcultures, highlighting variations in total protein content,  $H_2O_2$ , total phenolics, and total flavonoid content.

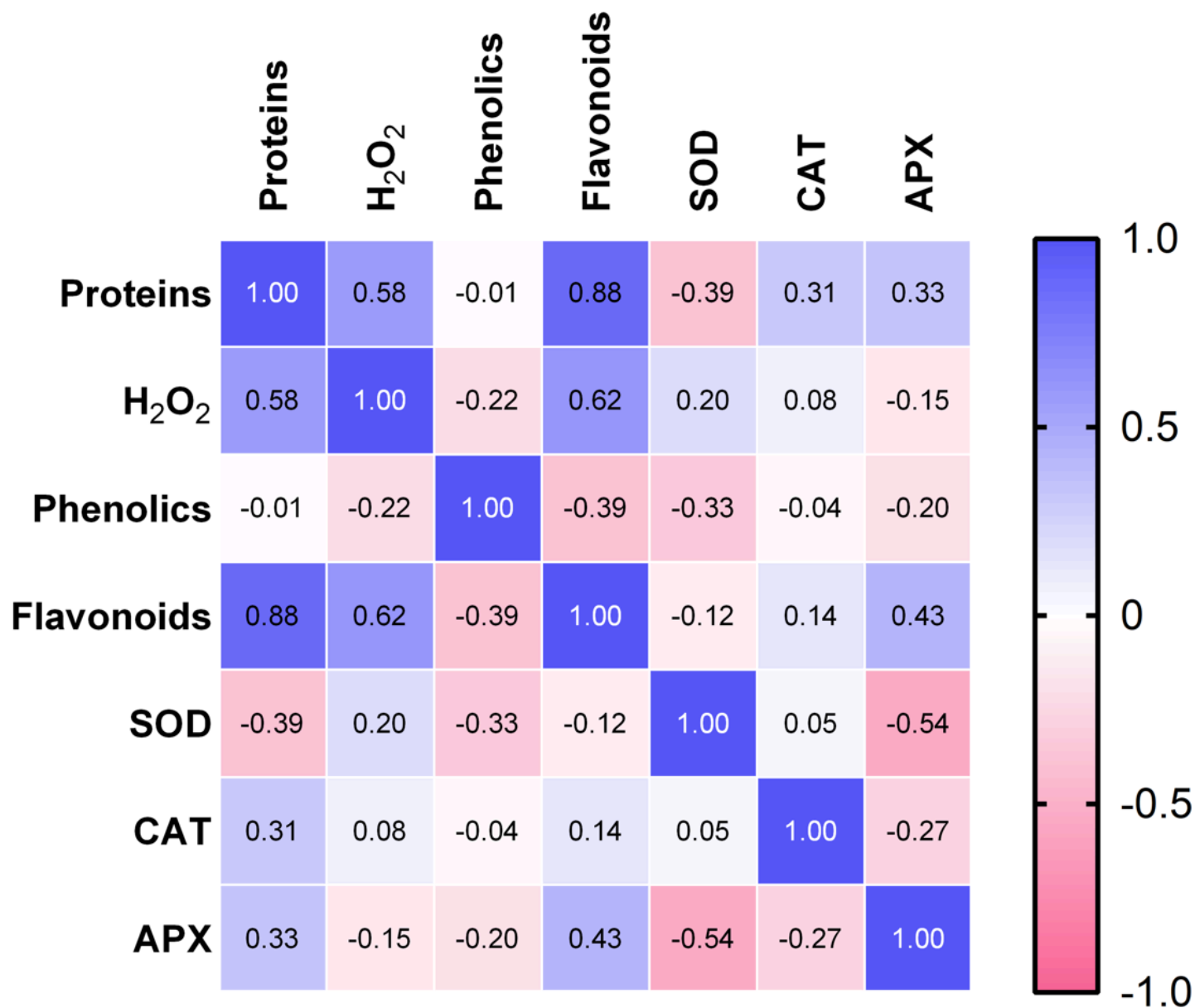


**Figure 3**

Correlation matrix of biochemical parameters and antioxidant compounds in *in vitro* regenerants of *W. somnifera*

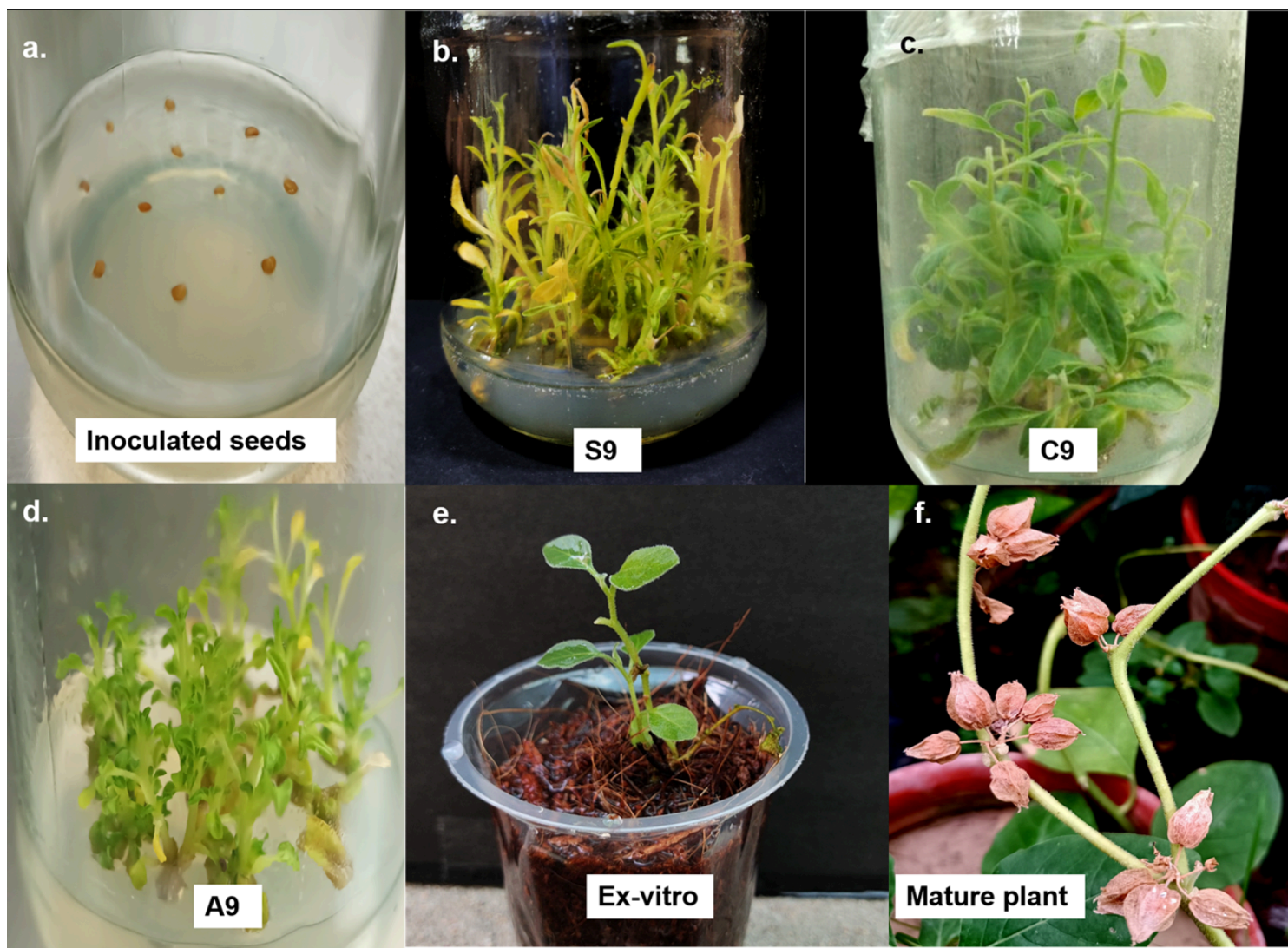


**Figure 4**

*In vitro* culturing and regeneration of plantlets. (a) *In vitro* germination of surface-sterilized *W. somnifera* seeds on MS medium. (b) Highly proliferated shoot cultures after nine subcultures (S9), showing morphological abnormalities and signs of stress. (c) Callus-derived regenerants (C9) exhibiting normal shoot morphology with elongated leaves. (d) Regenerants derived from direct shoot cultures (A9), showing normal growth characteristics. (e) Acclimatization of plantlet displaying successful establishment under greenhouse conditions. (f) Mature *W. somnifera* plantlet bearing fully developed berries under field conditions

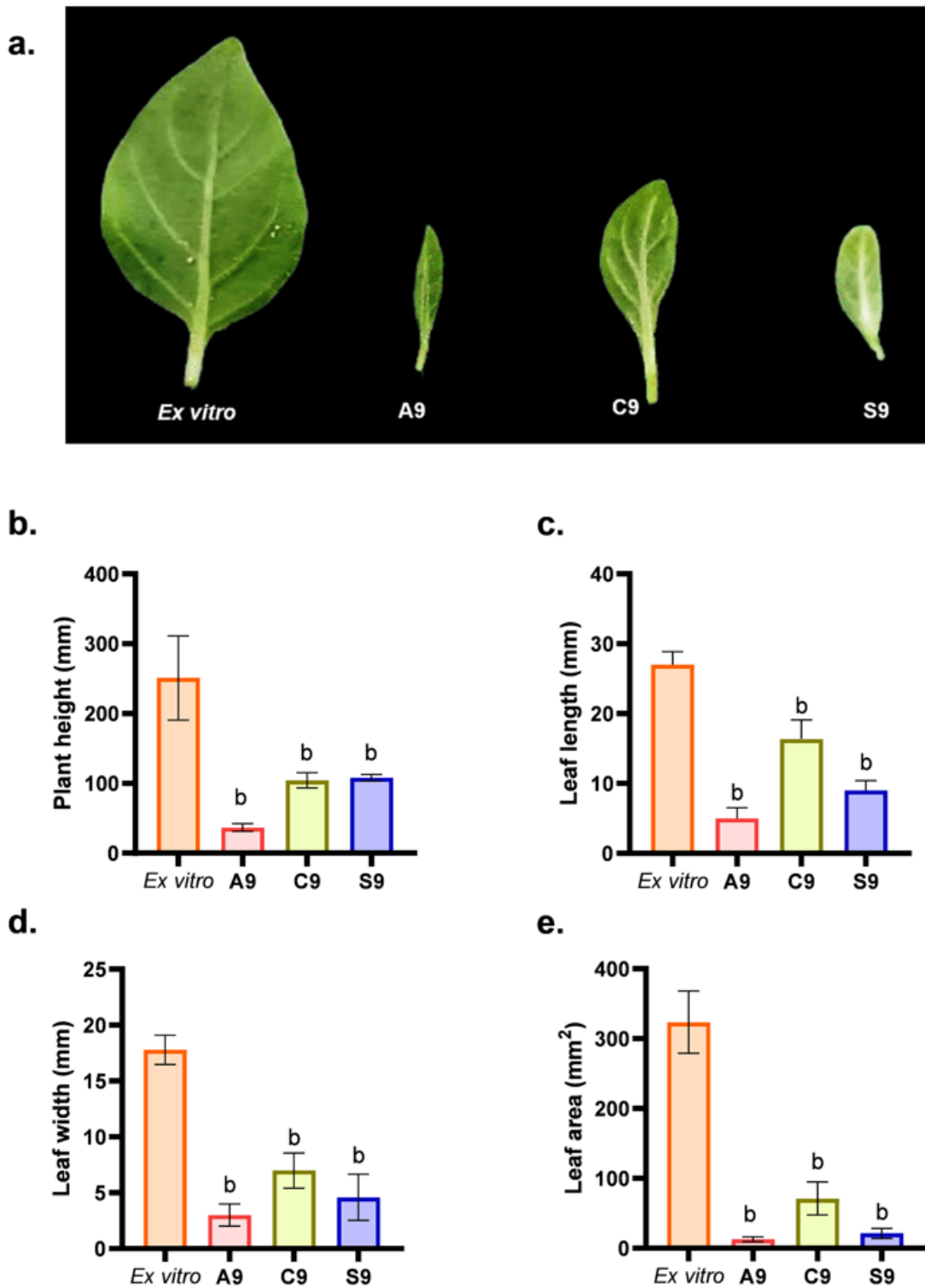


**Figure 5**

Morphological features of *in vitro*-grown and *ex-vitro* plantlets. (a) Leaf morphology of *ex-vitro* and *in vitro* regenerants A9, C9 and S9. (b-e) Bar graph representing variations in morphological parameters including plant height, leaf length, leaf width, and leaf area. Alphabets denote significant differences among *ex vitro*-grown plantlets, A9, C9, and S9 (a:  $P < 0.05$ , b:  $P < 0.01$ ; Student's t-test).

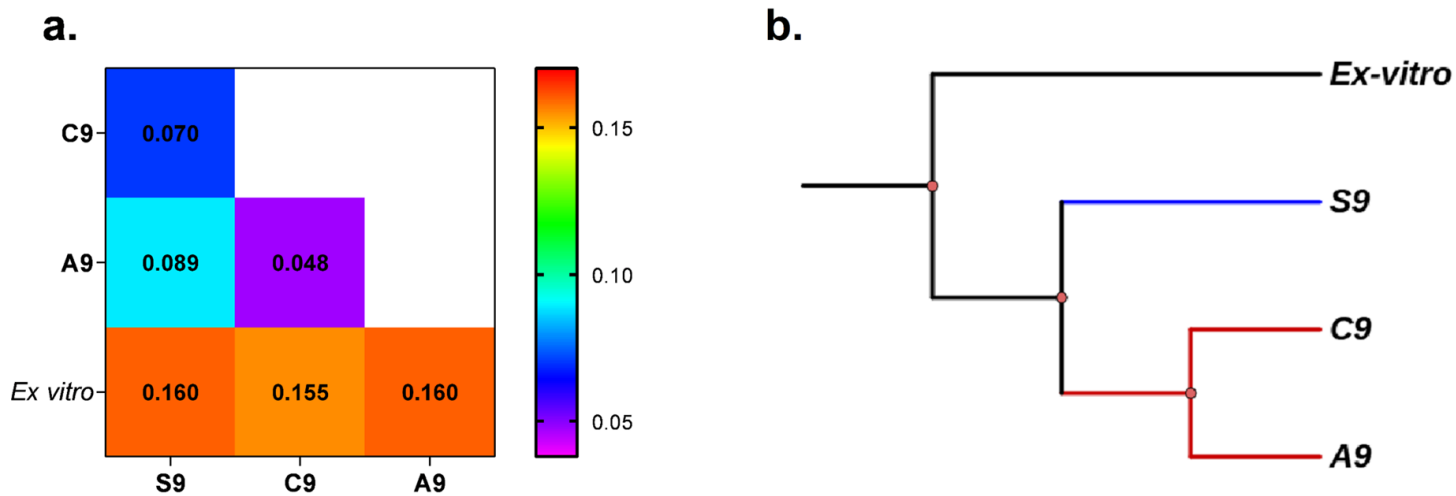


**Figure 6**

(a) Dissimilarity matrix based on presence (1) and absence (0) data produced by 25 RAPD markers using the DICE coefficient. (b) The weighted neighbor-joining tree representing clustering of A9, C9, S9, and *ex vitro* plantlets via DICE coefficient based on the presence and absence of 151 pooled bands produced by 25 RAPD markers.

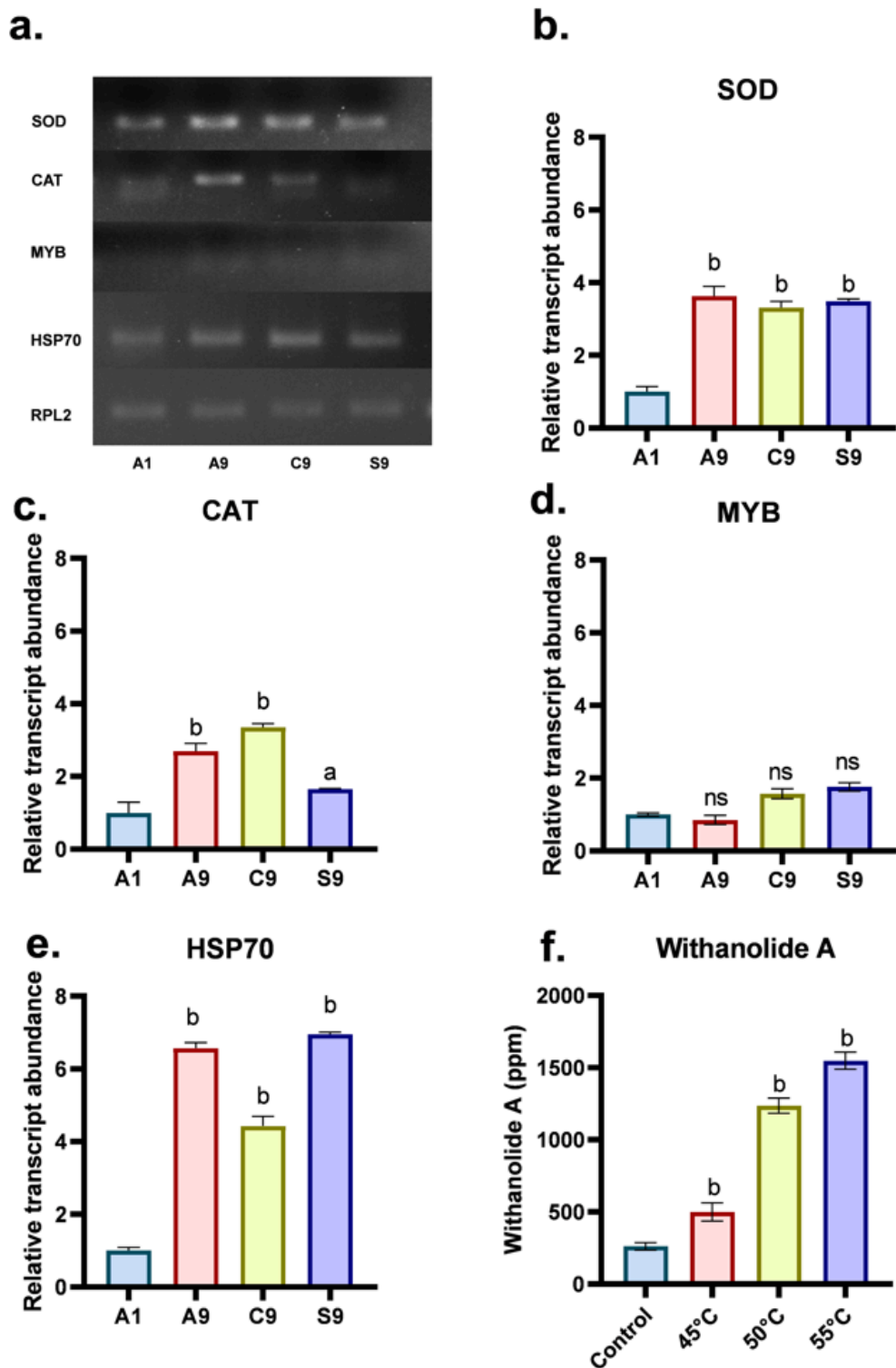


**Figure 7**

(a) Expression analysis of redox- and stress-responsive using semi-quantitative PCR analysis in the *in vitro* regenerated plantlets A1, A9, C9, and S9. (b-e) RT-qPCR analysis displaying the relative abundance of SOD, CAT, MYB, and HSP70 genes in the *in vitro* regenerated plantlets A1, A9, C9, and S9. Transcript abundance was calculated relative to the expression of RPL2 mRNA. (f) Bar graph illustrating quantitative estimation of withanolide A content under heat stress in the callus tissues of *W. somnifera*.

Alphabets denote significant differences among A1, A9, C9, and S9 (a:  $p < 0.05$ , b:  $p < 0.01$ ; Student's t-test).

## Supplementary Files

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