Physiological behavior of in vitro cultures of Clerodendrum indicum (L.) O. Kuntze under polyethylene glycol (PEG)-induced osmotic stress

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Abstract

The present study aims to elucidate the physio-biochemical changes in the in vitro polyethylene glycol (PEG)-induced drought-stressed organogenic callus of *Clerodendrum indicum* (L.) O. Kuntze (Verbenaceae) and its drought mitigation strategies. The callus was cultured in different organogenic media under drought stress (2%, 4%, 8%, and 12% PEG 400, v/v) (Ψw = -0.12, -0.21, -0.46, and -0.71 MPa, respectively) and assessed for physiological and biochemical parameters. The study revealed a significant reduction in biomass and in shoot and root development after 30 days of incubation under 2% and 4% PEG stress (Ψw = -0.12 and -0.21 MPa, respectively) compared to 0% PEG-stressed conditions (Ψw = -0.012 MPa). However, a considerable decline in all physio-biochemical parameters under the 8% (v/v) PEG-stressed (Ψw = -0.46 MPa) condition, and no organogenic development was observed in Ψw = -0.71 MPa. Proteins, proline, flavonoids, phenolic compounds, hydrogen peroxide (H_2O_2), and lipid peroxidation (MDA) were increased in 4% PEG-induced drought stress (Ψw = -0.21 MPa) sets. HPLC analysis showed that PEG stress induced shoots and roots to produce compounds like vanillic acid, sinapic acid, ferulic acids, naringenin, and kaempferol, which were absent in 0% PEG-stressed (control) plantlets. Antioxidant enzymes (Ascorbate peroxidase, Catalase, Glutathione reductase, and Superoxide dismutase) were also maximally enhanced in Ψw = -0.12 and -0.21 MPa than drought unstressed sets. The callus exhibited the maximum POX, H_2O_2, and $O_2^{\bullet-}$ localization at a 4% PEG concentration (Ψw = -0.21 MPa) in the chlorophyllous tissue and root tip regions. Therefore, it is assumed that drought stress at Ψw = -0.12 and -0.21 MPa significantly impairs the physio-biochemical parameters and triggers ROS-mediated stress, which interferes with the differentiation of new shoot buds from the callus. However, this stress is quenched by several potent ROS-scavenging enzymes and secondary metabolites, thereby enabling drought stress tolerance. The study concludes that drought stress up to 4% PEG (Ψw = -0.21 MPa) significantly alters the physio-biochemical behavior of *C. indicum* callus, with enhanced flavonoid and phenolic contents that can be utilized to achieve higher recovery of secondary metabolites from in vitro plantlets for pharmaceutical demand without affecting the habitat.

Introduction

Clerodendrum indicum (L.) O. Kuntze (Verbenaceae), commonly known as Bamunhati in West Bengal, India, is a threatened medicinal plant that grows as a sparsely branched shrub throughout India (Kundu et al., 2024). The entire plant, especially the root, possesses several highly valued secondary metabolites, such as cleroindicins, hispidulin, taraxerol, lupeol, scutellarein, dehydroclerosterol, oleanolic acid-3-acetate, scutellarein-7-O-β-D-glucuronide, dehydroclerosterol-3-O-β-D-glucopyranoside, and pectolinergenin that are active against several diseases, such as cough, asthma, scrofulous infections, diarrhoea, jaundice, leprosy, syphilitic rheumatism, and septic wounds (Ghosh and Pal, 2012; Somwong et al., 2015; Soundalgekar et al., 2021; Mitra et al., 2022; Kundu et al., 2024). Therefore, an *in vitro* micropropagation method is crucial for enabling large-scale biomass regeneration and the recovery of value-added metabolites for pharmaceutical purposes. In this context, Kundu et al. (2024) reported a

cost-effective micropropagation technique for this plant, which could be further extended to enhance secondary metabolites to meet pharmaceutical demand.

Several studies show that drought stress can affect plant growth and productivity, and increase the synthesis of secondary metabolites (Zahir et al., 2014; Kapoor et al., 2020; Jan et al., 2021; Yadav et al., 2021; La Scala et al., 2024). Mannitol, sorbitol, and polyethylene glycol (PEG) are three osmotic agents used to mimic the effects of drought *in vitro*. PEG is the most commonly used agent to induce water stress by increasing the solute potential of the culture medium, thereby impeding the root system's ability to absorb water (Shehab et al., 2010). However, the mechanisms by which these processes enable the plant to undergo adaptive changes in growth and physiological behaviour vary across species (Lei et al., 2006; Yang et al., 2021). In this direction, no such studies have been conducted on *C. indicum*. So, assessing the physiological and biochemical processes involved in *C.indicum* to drought response might provide insight into the drought tolerance strategies in this plant. This will also offer an attractive promise for the synthesis of secondary metabolites with pharmacological properties (Putalun et al., 2007; Zahir et al., 2014; Jan et al., 2021; La Scala et al., 2024). When plants are exposed to drought stress, an excessive buildup of reactive oxygen species (ROS) occurs, leading to oxidative damage to lipids, proteins, and nucleic acids (Hussain et al., 2019). This ROS accumulates under drought stress, serving as a signaling molecule that coordinates the activation of many genes and various stress-responsive pathways (Zandi and Schnug, 2022). Several enzymatic and non-enzymatic antioxidant systems, osmolytes, and phytohormones are produced to mitigate the detrimental effects of ROS and control their levels. Superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR) act along with redox and non-enzymatic antioxidants such as ascorbic acid, reduced glutathione (GR), phenols, flavonoids, and tocopherol, which are produced in higher quantities to mitigate oxidative stress (Soares et al., 2019; Rajput et al., 2021).

Thus, the objectives of this study are to determine the extent of drought stress tolerance and to examine the physicochemical mechanisms and tissue differentiation in *C. indicum* that mitigate drought stress in vitro under different PEG-stress levels.

Materials and Methods

Plant material and in vitro culture conditions

Clerodendrum indicum (L.) O. Kuntze plant was collected from wild populations in Malda, West Bengal, India (25°0' 39.0276" N and 88° 8' 27.9528" E). The voucher specimen number BS/Mld/Clero – 01 was identified by the Botanical Survey of India (BSI), Howrah, West Bengal. Different explants, such as nodes and leaves, were surface-sterilized with 70% (v/v) ethanol for 30 sec, followed by 0.1% Mercuric Chloride ($HgCl_2$) for 15 mins, and washed several times. A callus was cultured in different organogenic media on Murashige and Skoog medium (MS) supplemented with varying concentrations of BAP and NAA, and maintained under a 16:8 h light: dark photoperiod in a plant growth chamber for 30 days (Murashige and Skoog 1962; Kundu et al., 2024).

Callus induction, shoot bud, and root initiation

The in-vitro callus induction, shoot bud, and root initiation were conducted following the method of Kundu et al. (2024). Briefly, two explant types (leaf and node) were used in full-strength or half-strength MS medium supplemented with growth regulators, including BAP (1–5 mg/L) and NAA (0.5 mg/L) for shoot bud and root initiation (Kundu et al., 2024). Cultures were maintained in a plant growth chamber under a 16:8 h light: dark cycle at 25 ± 2°C and 70% humidity. The percentage of shoot and root induction, the time to bud initiation, and the growth state of the vegetative buds were recorded every 2 days for 30 days of subculture from callus. Data on average root numbers and length were recorded after 30 days of culturing.

Experimental design for PEG-induced drought stress

After subculture for six cycles, 15-day-old calli were placed in a culture tube containing MS (Murashige and Skoog 1962) basal medium (10 mL), pre-optimized PGRs concentrations for shoot and root induction from callus with 2%, 4%, 8%, and 12% Poly Ethylene Glycol 400 (PEG-400, Merck, India) (v/v) (Ψw = -0.12, -0.21, -0.46, and − 0.71 MPa, respectively) and the pH was adjusted to 5.7–5.8 with 1 M NaOH or 1 N HCl before autoclaving at 121°C under 15 psi for 30 min. Cultures were maintained in a plant growth chamber under a 16:8 h light: dark cycle at 25 ± 2°C and 70% humidity. Each treatment had 10 replicates, with pre-optimized PGR concentrations for large-scale production. After 30 days of PEG treatment, the shoot and root induction from the callus were recorded.

Estimation of total carbohydrate

The total carbohydrate of the in vitro plantlet was estimated using the Anthrone method (Yemm and Willis, 1954). 0.5 g of leaf and root samples (fresh weight) were crushed with 5 mL of 80% ethanol (v/v), then heated in a boiling water bath for 20 min. The extract was centrifuged (RM12C Plus, Remi Mumbai, India) at 4,032 x *g* for 10 min at room temperature. The supernatant was collected, and the pellet was again crushed and reextracted. The final volume of the supernatant was adjusted to 5 mL using double-distilled water. To 1 mL of suitably diluted supernatant under ice-cold conditions, 2 mL of Anthrone reagent (200 mg Anthrone/100 mL ice-chilled conc H_2SO_4) was added, and the mixture was gently mixed. Then, it was placed in a boiling water bath for 10 minutes. The solution's blue-green absorbance was measured at 620 nm in a UV-Vis spectrophotometer (Cary 60, Agilent Technologies, USA). The experiment was repeated three times. The total carbohydrate content was estimated from the standard curve of D-Glucose (100 µg/mL) using the following formula.

Total carbohydrate content (mg/g of FW tissue) = Total carbohydrate obtained from the standard curve × volume makeup (mL) × dilution factor/weight of sample (g).

Estimation of total protein content

0.5 g of fresh in vitro shoots were homogenized in a mortar-pestle with a 5 mL solution containing 0.1 g of polyvinylpyrrolidone (PVPP) (w/v) in 0.1 M potassium phosphate buffer (pH 7.0) and kept at 4°C. The extract was centrifuged at 12,000 × g and 4°C for 10 min, and the supernatant was collected. The protein content of the extract was determined at 750 nm using a UV-Vis spectrophotometer (Cary 60, Agilent Technologies, USA) using BSA (100 µg/ml) as a standard, following the method of Lowry et al. (1951).

Total protein content (mg/g of FW tissue) = Total protein obtained from the standard curve × volume makeup (mL) × dilution factor/weight of sample (g).

Estimation of total phenol content

The phenol content in the shoot and root was determined by the Folin-Ciocalteu method (Mandal et al., 2021). 1 g of tissue samples was homogenized in a mortar and pestle with 10 mL of 80% methanol (v/v). The homogenate was then centrifuged (RM12C Plus, Remi Mumbai, India) at 7000 × *g* for 10 min, and the supernatant was collected. The 1 mL of supernatant was mixed with 0.5 mL of 10% Folin-Ciocalteau reagent. After 3 min, 2 mL of 20% Na_2CO_3 solution (w/v) was added. The solution was mixed thoroughly and incubated for 1 hr at room temperature in dark conditions. The absorbance was measured in a UV-Vis spectrophotometer (Cary 60, Agilent, USA) at 765 nm. The phenol concentration (mg/mL) was determined from the calibration curve of gallic acid (10–100 µg/mL), and the phenolic content was expressed as gallic acid equivalents (mg of GA/g of extract).

Total phenol content (µg/g of tissue) = Total phenol obtained from the standard curve × volume makeup (mL) × dilution factor/weight of sample (g).

Estimation of total flavonoid content

The total flavonoid content in the shoot and root was measured by aluminum chloride ($AlCl_3$) colorimetric assay (Rajak et al., 2024). 0.1 mL of 80% methanolic extract (1 mg/mL) was taken, and 0.15 mL of 5% $NaNO_2$ (w/v) was added. After 5 min, 0.15 mL of 10% $AlCl_3$ (w/v in 100% methanol) was added to the solution, which was then mixed well and left for 6 min. 1.0 mL of 1(M) NaOH solution was then added to it. Afterward, it was incubated for 1 hr, and the absorbance was measured at 510 nm using a UV-Vis spectrophotometer (Cary 60, Agilent, USA). The flavonoid content was estimated from the standard of quercetin (10–100 µg/mL).

Total flavonoid content (µg/g of tissue) = Total phenol obtained from the standard curve × volume makeup (mL) × dilution factor/weight of sample (g).

Estimation of proline content

The fresh tissue (0.5 g) was homogenized in a mortar and pestle with 5 mL of 3% sulfosalicylic acid (w/v), and the homogenate was centrifuged at 10,000 × g for 10 min. After that, 1 mL of supernatant was

combined with 1 mL of glacial acetic acid, and 1 mL of acid ninhydrin reagent (1.25 g ninhydrin (1,2,3-indantrione monohydrate), 30 mL glacial acetic acid, 20 mL of 6 M orthophosphoric acid, dissolved by vortexing and gentle warming) was incubated in a boiling water bath for 1 hour. The reaction setup was immediately cooled in an ice bath for 15 minutes to stop it. Then, 2 mL of toluene was added, the mixture was vortexed, and the top toluene layer was separated. The absorbance of the top chromophore was determined in a UV-Vis spectrophotometer (Cary 60, Agilent Technologies, USA) at 520 nm, and the proline concentration was determined from the standard curve of Proline (Bates et al., 1973; Kundu et al., 2026). The proline content was expressed as μmol g^{-1} FW using the following equation to calculate the amount of proline in the extracts:

Proline (μmol/g FW) = (Abs extract – blank)/slope*Vol extract/Vol aliquot*1/FW

Where: Abs extract is the absorbance determined with the extract, blank (expressed as absorbance), and slope (expressed as absorbance•$nmol^{-1}$) are determined by linear regression. Vol extract is the total volume of the extract, Vol aliquot is the volume used in the assay, and FW (expressed in mg) is the amount of plant material extracted.

HPLC profiling of the phenolic acids and flavonoids of in vitro organ

The ethyl acetate extracts of in vitro shoots and roots were prepared using a microwave-assisted extraction protocol with a Panasonic Microwave Oven (20L Solo Microwave Oven (NN-SM25JBFDG), Mechanical Knob, Panasonic Life Solutions India Pvt. Ltd., Haryana, India) at 360 Watts for 15 min, with 5 min run and 5 min rest, and the extract was filtered through Whatman No. 1 Filter paper, and made into a stock solution at 1 g/mL. 20 μl of the respective extract was analyzed on an HPLC system using a Dionex Ultimate 3000 liquid chromatograph equipped with a reversed-phase Acclaim C_{18} column (5 μm particle size, 250 × 4.6 mm) and detection at 280 nm. The system was run with a mobile solvent phase consisting of methanol (Solvent A) and a 0.5% aqueous acetic acid solution (Solvent B), maintained at 25°C. Phenolic acids and flavonoid contents in the EA extracts of plant organs were quantified by comparing them to standard references of 13 phenolic and 5 flavonoid compounds (Sigma Aldrich, USA). The identification and quantification of these compounds were done based on their retention times, concentrations (μg/mL), and peak areas (%) relative to the standards (Kundu et al., 2024).

Assay of the antioxidant defense system

The assay of different antioxidants was done as follows:

i) Superoxide dismutase activity assay

The method of Giannotolitis and Ries (1977) was used to determine superoxide dismutase (SOD, EC 1.15.1.11) activity. The fresh shoot tissue (0.5 g) was homogenized in a mortar and pestle with 50 mM sodium phosphate buffer (pH 7.6), centrifuged at 10,000 × g for 10 min at 4°C using a cold centrifuge (RM12C Plus, Remi Mumbai, India). 100 μL of the extracts was added to a mixture of 50 mM sodium

phosphate buffer (pH 7.6) containing 200 mM L-methionine, 1.5 M Na_2CO_3, 60 µM riboflavin, 3 mM ethylene diamine tetraacetic acid (EDTA, Sisco Research Laboratory, India), and 2.25 mM p-nitro blue tetrazolium chloride (Sisco Research Laboratory, India) in a dark environment. The reaction was carried out under a fluorescent lamp at a light intensity of 45 µmol m^{-2} s^{-1}. The absorbance was measured at 560 nm using a UV-Vis spectrophotometer (Cary 60, Agilent Technologies, USA). The enzyme units (EU) for SOD activity were measured as the amount of protein that produced a 50% reduction in SOD-inhibitable NBT reduction (Beyer and Fridovich 1987).

ii) Catalase activity assay

The catalase (CAT, EC 1.11.1.6) activity was examined using Aebi's (1984) method, which relies on the principle that the rate of H_2O_2 breakdown (extinction coefficient 36 mM^{-1} cm^{-1}) is measured by absorbance at 240 nm. The reaction mixture included 50 mM sodium phosphate buffer (pH 7.0), 0.5 mL H_2O_2(37%) in 100 mL Phosphate buffer (pH 7.0), and 100 µl of enzyme extract in a 2 mL volume cuvette, and absorption was recorded in a UV-Vis spectrophotometer (Cary 60, Agilent Technologies, USA) every 30 sec. interval. Enzyme activity was measured as units per gram of fresh weight per minute.

iii) Ascorbate peroxidase activity assay

The ascorbate peroxidase activity (APX, EC 1.11.1.11) was measured using the method described by Nakano and Asada (1981). 0.1 mL of enzyme extract was added to 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA (w/v), 0.5 mM ascorbate (w/v), and 0.1 mM H_2O_2. The absorbance of ascorbate reduction was measured in a UV-Vis spectrophotometer (Cary 60, Agilent Technologies, USA) at 290 nm. Enzyme activity was measured as units per gram of protein.

iv) Glutathione reductase activity assay

Glutathione reductase (GR, EC 1.6.4.2) activity was measured according to the protocol of Carlberg and Mannervik (1985). 100 µL of the extracts was added to a mixture of 50 mM sodium phosphate buffer (pH 7.6) containing 0.01 mM NADPH, 0.1 M EDTA, and 6 mM glutathione (w/v) in the dark. The activity of GR was measured by monitoring the decrease in absorbance at 340 nm for 3 minutes using a UV-Vis spectrophotometer (Cary 60, Agilent Technologies, USA). Enzyme activity was measured as units per gram of protein.

Estimation of lipid peroxidation content

Malondialdehyde (MDA), a specific product of lipid peroxidation, was quantified using the method of Heath and Packer (1968). Fresh in vitro shoots and roots (0.5 g) were homogenized in 1% trichloroacetic acid (TCA, w/v) at 11,200 x g for 5 minutes (RM12C Plus, Remi Mumbai, India). After adding 4.0 mL of 0.5% (w/v) thiobarbituric acid (TBA) to the supernatant (1.0 mL), the mixture was heated to 95°C for 30 min, cooled in an ice bath, and centrifuged at 3000 x *g* for 5 min. The absorbance of supernatant was measured at 532 and 600 nm using a UV-Vis spectrophotometer (Cary 60, Agilent Technologies, USA). The following formula was used to determine the MDA content:

MDA (µmol/ g FW) = [(A532- A600)/156] $\times 10^3$ × dilution factor

Estimation of H_2O_2 content

Fresh in vitro shoots and roots (0.5 g) were homogenized with 5 mL of 0.1% trichloroacetic acid (w/v) in a mortar pestle, and the homogenate was centrifuged (RM12C Plus, Remi Mumbai, India) at 12,000×g for 10 min. An equal volume of potassium phosphate buffer (0.1 M, pH 7.0) and potassium iodide (0.1 M) was mixed with the supernatant (0.5 mL). The samples were gently vortexed, and absorbance was measured at 390 nm using a UV-Vis spectrophotometer (Cary 60, Agilent Technologies, USA). All these preparations were taken in amber containers or under light-controlled conditions. H_2O_2 content was expressed as µmol/ g FW (Velikova et al., 2000).

Histochemical Localization of ROS species in the organs

i) Localization of O2 •− in callus

The transverse section of the callus from different treatment sets was stained with 0.5 mM NBT (nitroblue tetrazolium chloride, Sisco Research Laboratory, India) dissolved in 50 mM sodium phosphate buffer (pH 6.8) for 1 hour at 25°C to localize $O_2^{\bullet -}$ (modified from Liszkay et al., 2004). A detectable bluish-violet colour indicated the accumulation of $O_2^{\bullet -}$ in the tissue. The properly stained sections were examined under a phase-contrast microscope (Leica DM750, Germany) equipped with LAS EZ camera software, and photomicrographs were taken.

ii) Localization of H_2O_2 in callus

The transverse sections of callus were incubated in a staining solution composed of 1 mM TMB (3,3',5,5'-tetramethyl benzidine dihydrochloride hydrate, Sisco Research Laboratory, India) dissolved in 10 mM potassium-citrate buffer, pH 6.0 (modified from Liszkay et al. 2004), for 1 hour at 25°C to localize H_2O_2. A detectable blue colour indicated the accumulation of H_2O_2 in the tissue.

iii) Localization of POX in callus

POX enzyme was localized using TMB and exogenous H_2O_2, as described by Linkies et al. (2010). The staining solution consisted of 1 mM TMB, 10 mM potassium citrate buffer (pH 6.0), and 10 mM H_2O_2. Live transverse slices of callus were stained by incubating them in this solution for 10 minutes at 25°C. Live sections were cleaned in distilled water both before and after staining and examined under a phase-contrast microscope (Leica DM 750, Germany) fitted with LAS EZ camera software.

iv) H_2O_2 accumulation in roots

Hydrogen peroxide (H_2O_2) accumulation in the roots of both normal and PEG-stress-grown plants was localized using the H_2O_2-specific stain TMB (3,3', 5,5'-tetramethylbenzidine dihydrochloride hydrate). Control and stress-grown sapling roots were dipped in a 1 mM TMB solution for 30 minutes, washed with distilled water, and photographed as previously described.

Statistical analysis

Statistical analysis was performed with SPSS 21. All results were presented as the mean ± standard error (SE) of triplicate trials, and the data were analyzed using one-way analysis of variance (ANOVA) at a 0.05 significance level (p). Tukey's different letters indicated significant differences. A two-way ANOVA was performed in RStudio (4.1.1).

Results

Effects of water stress on shoot and root induction of C. indicum

Shoot and root induction of *C. indicum* was established in the MS medium + 5 mg/L BAP + 0.5 mg/L NAA and MS medium + 1 mg/L BAP + 0.5 mg/L NAA, respectively (Kundu et al., 2024). The effect of different concentrations of PEG (2%, 4%, and 8%, v/v) induced drought stressed conditions after 30 days showed the maximum height (6.5 ± 0.11 cm) and number (4.5 ± 0.05 per callus head) of the shoot were found in 0% PEG (Ψw = -0.012 Mpa) stressed condition and as the drought-stressed increased height and a number of the shoot also decreased (Fig. 1, Table 1). No growth of shoots was observed in the 12% PEG-stressed condition (Ψw= -0.71 Mpa). Similarly, in the 0% PEG-stressed condition, the highest number of roots and their lengths were observed. However, both root length and number decreased as stress concentration increased (Fig. 1, Table 1). Furthermore, total shoot and root biomass decreased as the PEG concentration increased, and the lowest shoot biomass (0.66 ± 0.023 g) and the lowest root biomass (0.16 ± 0.005 g) were observed at an 8% PEG (v/v) (Ψ_w= -0.46 Mpa) concentration (Table 1).

Table 1
Effects of different concentrations of PEG on shoot and root induction of *C. indicum* cultured on MS medium after 30 days.

Organogenic parts	Media composition (mg/L)	Height (cm)	Number	Biomass for fresh weight(gm)
Shoot	MS + 5 BAP + 0.5 NAA + 0% PEG	6.5 ± 0.11^{a}	4.5 ± 0.05^{c}	1.81 ± 0.01^{a}
	MS + 5 BAP + 0.5 NAA + 2% PEG	5.3 ± 0.02^{a}	2.1 ± 0.08^{g}	1.24 ± 0.02^{b}
	MS + 5 BAP + 0.5 NAA + 4% PEG	4.6 ± 0.02^{b}	3.2 ± 0.02^{e}	0.72 ± 0.017^{c}
	MS + 5 BAP + 0.5 NAA + 8% PEG	4.26 ± 0.05^{c}	2.4 ± 0.01^{f}	0.66 ± 0.023^{d}
	MS + 5 BAP + 0.5 NAA + 12% PEG	0.0 ± 0.00	0.0 ± 0.0^{d}	0.0 ± 0.0
Root	½ MS+ 1BAP + 0.5 NAA + 0% PEG	3.3 ± 0.04^{c}	6.6 ± 0.04^{a}	0.64 ± 0.04^{d}
	½MS + 1 BAP + 0.5 NAA + 2% PEG	2.9 ± 0.05^{d}	5.8 ± 0.02^{b}	0.52 ± 0.017^{e}
	½MS+ 1BAP + 0.5 NAA + 4% PEG	2.8 ± 0.01^{d}	3.8 ± 0.11^{d}	0.4 ± 0.005^{f}
	½MS+ 1BAP + 0.5 NAA + 8% PEG	2.1 ± 0.02^{e}	1.5 ± 0.03^{h}	0.16 ± 0.005^{g}
	½MS+ 1BAP + 0.5 NAA + 12% PEG	0.0 ± 0.00	0.0 ± 0.0	0.0 ± 0.0

Values with the same letter within each column indicate no significant difference among treatments ($p < 0.05$) by the Tukey test. Data represent the means ± standard error (SE) ($n = 5$).

Changes in the biochemical parameters

Total carbohydrate content

The total carbohydrate content of *C. indicum in vitro* shoot and root is shown in Table 2. It was observed that the total carbohydrate content in both shoot and root regions decreased remarkably under different PEG-induced drought stress conditions compared to the 0% PEG-stressed plant (Ψw = -0.012 Mpa), except at 2% PEG stress in the shoot (Ψw = -0.21 Mpa), where it was observed that carbohydrate content increased by 4.7% compared to the control set. However, total carbohydrate content decreased by

18.09% and 35.27% in 4% and 8% PEG-treated shoots, respectively, compared with the PEG-untreated plant (Table 2).

Total protein content

The protein content of PEG-treated shoots increased with drought stress and was 8.02, 19.51, 20.39, and 9.27 mg/g fresh weight for 0, 2, 4, and 8% (v/v) PEG, respectively (Ψw = -0.12, -0.21, -0.46, and − 0.71 MPa, respectively) (Fig. 2a). Similar to this, the amount of protein in PEG-treated roots also increased with drought stress and was 4.69, 9.21, 12.3, and 3.36 mg/g fresh weight for 0, 2, 4, and 8% (v/v) PEG, respectively. 8% (v/v) PEG stress resulted in lower protein content than 2% and 4%, but the levels were still higher than the control (Fig. 2a). Statistical analysis shows a significant difference in protein content among different levels of PEG in the shoot and root of *C. indicum* (Fig. 2a).

Total proline content

PEG stress led to a noticeable increase in shoot and root proline content up to 4% PEG concentration (Ψw = -0.46, MPa) in a similar fashion (Fig. 2b). Proline accumulation was found in 4% of the PEG concentration in the shoot (1.71 mg/g fresh weight) and in the root (2.09 µmol/g FW). After that, proline accumulation decreased to 0.589 µmol/g FW in the shoot and 0.446 µmol/g FW in the root at an 8% (v/v) PEG stress concentration (Fig. 2b).

Total phenol and flavonoid content

The total phenol content (TPC) in the *in vitro* shoots and roots is shown in Table 2. It was observed that the TPC in both shoot and root regions decreased remarkably under 8% PEG-induced drought stress (Ψw = -0.71 Mpa) compared with the 0% PEG-stressed plant. However, in shoot and root, TPC increased by 16.71% and 35.65%, and by 17.90% and 31.08% in 2% and 4% PEG-stressed plants, respectively, compared with the PEG-untreated plant (Table 2).

The total flavonoid content (TFC) of in vitro shoots and roots is shown in Table 2. It was observed that the TFC in both shoot and root regions decreased remarkably under 8% PEG-induced drought stress (Ψw = -0.71 Mpa) compared with the 0% PEG-stressed plant. However, TFC in shoots increased by 10.76% and 24.61% in 2% and 4% PEG-stressed plants, respectively; in roots, TFC increased by 10.52% and 26.31% in 2% and 4% PEG-stressed plants, respectively, compared with the PEG-untreated plant (Table 2).

Table 2
Effects of carbohydrate content, TPC, and TFC of different concentrations of PEG on shoot and root samples of *C. indicum* cultured on MS medium after 30 days.

Sample	Total Carbohydrate content (mg/100 g)	Total Phenol content (mg/100 g)	Total Flavonoid content (mg/100 g)
Shoot (0% PEG)	239.8 ± 4.6^{b}	67.6 ± 0.8^{c}	6.5 ± 0.2^{c}
Shoot (2% PEG)	251.2 ± 3.5^{a}	78.9 ± 0.6^{b}	7.2 ± 0.2^{b}
Shoot (4% PEG)	226.4 ± 2.8^{c}	91.7 ± 0.6^{a}	8.1 ± 0.3^{a}
Shoot (8% PEG)	195.2 ± 2.6^{d}	52.8 ± 0.5^{d}	5.1 ± 0.3^{d}
Root (0% PEG)	128 ± 1.8^{e}	29.6 ± 0.5^{g}	3.8 ± 0.1^{g}
Root (2% PEG)	113 ± 2.2^{f}	34.9 ± 0.6^{f}	4.2 ± 0.2^{f}
Root (4% PEG)	97 ± 1.9^{g}	38.8 ± 0.7^{e}	$4.8 \pm 0.2d^{e}$
Root (8% PEG)	73 ± 1.5^{h}	25.2 ± 0.2^{h}	2.1 ± 0.08^{h}

Values with the same letter within each column indicate no significant difference among treatments ($p < 0.05$) by the Tukey test. Data represent the means ± standard error (SE) ($n = 5$).

HPLC-based phenolic acids and flavonoids analysis

The HPLC analysis of the root ethyl acetate extracts demonstrated that 0% PEG stressed root extracts showed the presence of the least number of phenolic acids and flavonoids. However, 2%, 4%, and 8% PEG-stressed root extracts contain 12, 14, and 13 distinct phenolic acids and flavonoids, respectively (Fig. 3). Similarly, shoot ethyl acetate extracts show the presence of 11 different phenolic acids and flavonoids in the 0% PEG stressed condition, and 15, 12, and 15 were recorded in 2%, 4%, and 8% PEG stressed shoots, respectively (Table 3). Kaempferol, catechin, ferulic acid, naringin, p-Hydroxy benzoic acid, and caffeic acid were found in different PEG-stressed conditions in the root, but not in 0% PEG stressed root (Table 3). Gallic acid and apigenin were the most important compounds of *C.indicum* root found in all stressed conditions, but the amount of compounds increased with the PEG stress (Fig. 3b). Similarly, in the shoot, p-Hydroxy benzoic acid, Catechin, Sinapic acid, Rutin, Naringenin, Naringin, and Vanillic acid were present in different PEG-stressed conditions but were absent in 0% PEG stressed shoot (Table 3). Here, the amount of gallic acid increased simultaneously with PEG stress. Elagic acid was found to be highest in the 4% PEG-stressed shoot. After that, as the stress increased up to 8%, the elagic acid content decreased. The same observation was found in Sinapic acid, Syringic acid, p-

Coumaric acid, and Kampeferol. It was found that the content of myricetin, naringenin, quercetin, and ellagic acid was highest in the 2% PEG (v/v) stressed condition in roots.

Antioxidant and oxidative enzyme contents

The expression of antioxidants in *C. indicum* under different PEG (0%, 2%, 4%, and 8%) treatments is shown in Fig. 2(a–h). Moreover, CAT, APOX activity (U/mg protein), and GR content were increased by up to 4% at a 4% PEG (v/v) concentration compared to the control (0% PEG (v/v). After that, CAT, APOX, and GR activity decreased by 8% (v/v) under PEG-stressed conditions in both shoots and roots (Fig. 2d, c, e). The highest CAT, APOX, and GR activity was observed at 4% (v/v) PEG concentration. SOD content (U/mg protein) also increased in both shoots and roots with the increase of PEG concentrations, except 8% PEG stress, where a sharp decline was observed in SOD content (Fig. 2f). In comparison to the control, 2% PEG (v/v) induced a substantial increase in SOD activity about 29.15% and 36.60% in shoot and root, respectively. In contrast, SOD activity decreased by approximately 26.29% in 8% PEG (v/v) compared to 4% PEG (v/v) in the shoot and decreased by about 41.81% at 8%PEG (v/v) compared to 4%PEG (v/v) in the root (Fig. 2f).

The MDA contents varied considerably between the 0% and 8% (v/v) PEG treatments and rose with the increase of PEG concentration (Fig. 2g). In contrast to the 0% PEG (v/v) treatment value of 1.62 µmol/ g fresh weight in shoots, the 8% PEG (v/v) treatment showed the highest MDA value of 2.60 µmol/g fresh weight in shoots (Fig. 2g). Similar outcomes were also seen in roots, where the highest MDA value was 2.67 µmol/g fresh weight callus (Fig. 2g) after an 8% PEG (v/v) treatment. H_2O_2 activity was also increased with the increase of PEG concentration, and the highest H_2O_2 content (µmol/ g FW) was found in 8% PEG (v/v) concentration, which was 18.12 µmol/g fresh weight in the shoot and 19.26 µmol/g fresh weight in the root (Fig. 2h).

Table 3
HPLC analysis of phenolics from in vitro shoot and root extracts of *C.indicum* in different PEG (v/v) stressed conditions.

Plant parts	**Root**				**Shoot**			
Stress conditions (%)	**0**	**2**	**4**	**8**	**0**	**2**	**4**	**8**
Phenolic compounds and their contents (µg/g of tissue)								
Apigenin	18.129	26.871	77.044	60.358	14.5	11.305	36.887	31.391
Caffeic acid	0	0	0.351	0	0.15	0.21	0	0
Catechin	0	2.971	0	1.471	0	0.711	1.612	0.097
Ellagic acid	**10.553**	**40.318**	**23.792**	**18.447**	**0.57**	**0.845**	**26.969**	**14.942**
Ferulic acid	1.67	0.695	1.057	0.482	12.82	0.303	2.542	0.483
Gallic acid	**10.691**	**21.25**	**55.485**	**59.898**	**4.19**	**22.547**	**42.928**	**45.395**
Kaempferol	0	13.97	14.483	9.669	6.09	9.676	11.36	1.366
Myricetin	**0.0238**	**1.724**	**0.281**	**1.749**	**2.1**	**9.371**	**2.902**	**0.622**
Naringenin	0.199	44.326	0.491	31.419	0	25.008	0.318	0.297
Naringin	**0**	**0**	**0.77**	**0**	**0**	**0**	**0.664**	**0**
p-Coumaric acid	0.665	0.295	1.182	0.073	0.35	0.107	1.292	0.131
p-Hydroxy benzoic acid	0	0	0.308	0	0	0.024	0.039	0
Protocatechuic acid	**0**	**0**	**0**	**0**	**4.34**	**0**	**0**	**0**
Quercetin	**5.721**	**35.996**	**19.962**	**7.005**	**1.84**	**16.722**	**12.359**	**4.819**
Rutin	16.723	0	0	0.412	0	0.182	0	0
Sinapic acid	5.035	6.187	4.228	2.183	0	0.673	3.04	0.863
Syringic acid	1.894	5.723	0.633	0.384	1.44	0.038	3.298	1.131
Vanillic acid	0	0	0	0	0	0	0.455	0

Tissue localization of ROS in drought stress

Localization of H_2O_2 accumulation in the root

Staining for H_2O_2 in roots using TMB showed that roots took a more intense blue colour in PEG stress-grown plantlets compared to PEG untreated normal-grown plantlets, indicating the H_2O_2 accumulation in

roots under PEG treatment (Fig. 4). The highest coloration developed in 4% PEG stressed roots compared to 2% PEG stressed root. After that, the colouration decreased at 8% PEG (v/v) concentrations (Ψw = -0.46 Mpa). No blue colour developed in 0% PEG-stressed root (Ψw = -0.012 Mpa) (Fig. 4).

Histochemical localization of Hydrogen Peroxide (H_2O_2)

Histochemical localization of H_2O_2 (blue colour) using TMB stain in the callus shows that H_2O_2 accumulated in the apoplastic region of the ventral side of the tissue and gradually increased up to 8% PEG (v/v, Ψ_w= -0.46 MPa) stress (Fig. 5). In the drought non-stressed control sample of callus shows no blue colouration (Fig. 5a). In 2% of PEG (v/v) (Ψ_w= -0.12 Mpa)) stress show that the H_2O_2 accumulated at the apoplastic region of the cortex (Fig. 5b). However, in 4% and 8% PEG stress, the accumulation of H_2O_2 increased in the cortex region and covered more tissue areas (Fig. 5c,d).

Histochemical localization of POX

POX activity using a specific stain was observed by histochemical localization in callus tissue, which was exposed to different concentrations of PEG stress (Fig. 5). In 0% PEG (v/v) (Ψw = -0.012 Mpa) stressed condition, the transverse section of callus shows no localization (Fig. 5e). Similarly, in 2% PEG (Ψ_w= -0.12 MPa) stress condition POX activity was showed highest colour development in the cell wall region (Fig. 5f). In the 4% PEG stressed (Ψ_w= -0.21 MPa) condition showed POX localization on the cell confined to a few cell walls of intact cell region compared to 2% (Ψ_w= -0.12 MPa) and 8% PEG (Ψ_w= -0.46 MPa) stress (Fig. 5g). Furthermore, POX activity declined at 8% PEG (Ψ_w= -0.46 MPa) stress conditions (Fig. 5h).

Histochemical localization of superoxide (O_2•−)

The extracellular production of superoxide (O_2•−) was examined using an NBT stain through the transverse section of *C. indicum* callus, which had different percentages of PEG stress. In 0% PEG stressed condition, the TS of the callus shows localization of superoxide production (Fig. 5i). An initial increase of O2 •− production was observed, with the rise in PEG concentration that is PEG 2% (Ψ_w=-0.12 MPa), PEG 4% (Ψ_w= -0.21 MPa). Still, in PEG 8% (Ψ_w= -0.46 MPa), the superoxide development in cells declined significantly. The level of superoxide accumulation was highest in PEG 4% compared to PEG 2% (Ψ_w= -0.12 MPa) and PEG 8% (Ψ_w= -0.46 MPa) (Fig. 5j,k, and l).

Discussion

The current study observed that PEG-induced osmotic stress (Ψ_w = -0.12, -0.21, -0.46 MPa) modulated the growth and physiological behaviour of *C. indicum* plants in in vitro conditions. It was found that the drought stress had reduced bud and root primordial development under PEG-induced drought stress (Ψ_w= -0.12, -0.21, -0.46 Mpa) (Fig. 1; Table 1). The overall height and fresh weight of the plantlets were

also decreased. These results were consistent with earlier research on other medicinal plants (Razavizadeh et al., 2019; Hosseini et al., 2020; Martínez-Santos et al., 2021). Plants under abiotic stress often exhibit reduced growth metrics, such as length and weight, which may result from a shift in their metabolism (Hund et al., 2009). Studies have reported that plants alter their morphology, physiology, and anatomy to become more tolerant of drought (Seleiman et al., 2021). As found in the current study, *C. indicum*, like other plants, reduced its total biomass under these conditions; however, it increased root biomass to maximize water absorption and reduced shoot biomass to minimize water loss. The loss of total fresh weight in a drought-stressed plant may be associated with a significant decrease in the aerial structure, thereby reducing photosynthesis and plant development under water-deficit stress (Shao et al., 2008; Abid et al., 2016).

The findings revealed that *C. indicum* at the maximum drought level of 8% PEG (v/v) (Ψw = -0.46 MPa) did shorten root length; however, this level of drought stress may be extreme to tolerate and thus may not promote overall development. Therefore, it withstands drought by maintaining a well-developed root system. Furthermore, plants' response to drought stress is linked to metabolic changes that result in the accumulation of several osmolytes, including proline, and the expression of water-stress proteins known as dehydrins (Hanin et al., 2011; Abdul Aziz et al., 2025). In the current investigation, following a 4-week PEG treatment, proline concentration in *C. indicum* plants increased in 4% PEG stress (Ψw = -0.21 Mpa). Subsequently, it decreased in 8% PEG-stressed (Ψ_w = -0.46 MPa) conditions (Fig. 2). Whereas, proline is maintained at high levels to support cell hydration status, scavenge free radicals, and protect membranes and proteins from stress (Ghaffari et al., 2019). The elevated proline content in 4% PEG is due to upregulation of proline biosynthetic pathways, leading to increased proline production. However, the drop might have occurred because proline degradation exceeded synthesis. Severe and persistent drought stress is often accompanied by severe oxidative stress, which can also affect enzymes in the proline biosynthesis pathway, thereby slowing plant growth and metabolism (Kishor et al., 2005). The protein levels at 8% PEG (v/v) (Ψw = -0.46 MPa) were lower than those at 2% (Ψw = -0.12 MPa) and 4% PEG (v/v) (Ψw = -0.21 MPa), although they were still much greater than the protein content of the 0% PEG-stressed sample. At the same time, it is reasonable to assume that plants under stress, such as drought or salinity, synthesize fewer proteins overall (Cohen et al., 2021). However, stress-induced proteins can also be synthesized at higher rates to modify cell osmotic potential and provide nitrogen-based storage material for metabolic processes involved in the plants' response to drought stress (Muktadir et al., 2020). In the current study, the observed rise in sugar content in *C. indicum* shoots was likely a passive effect of stem and leaf dehydration (Wang et al., 1995; Muller et al., 2011). Total carbohydrate accumulation is essential for reducing drought stress, either by osmotic adjustment or by conferring desiccation resistance (Ozturk et al., 2021). At lower water deficits, starch synthesis was already inhibited, while sucrose synthesis remained constant or increased. This change in partitioning was accompanied by an increase in sucrose-phosphate synthase activity (Zrenner and Stitt, 1991). The current experiment on *C. indicum* under PEG treatment found higher levels of phenolic compounds under stress than in unstressed conditions. Phenolic compounds play crucial ecological functions in plants' defense and protection systems (Kumar et al., 2023). It has been proposed that abiotic limitation might

increase the production of these chemicals in response to oxidative stress (Kumar et al., 2023). Additionally, phenolics are employed in a variety of plant species to preserve water homeostasis and scavenge ROS generated in response to abiotic stressors (Kumar et al., 2023).

According to the current findings, under standard development conditions, cells produce fewer ROS. In contrast, PEG-induced in vitro drought stress in *C. indicum* tissue increased ROS production, including hydrogen peroxide and superoxide radicals, which disrupt cellular ROS homeostasis, leading to ROS accumulation{such as O_2 •−, H_2O_2, hydroxyl radical (OH•), and singlet oxygen (1 O_2)} and membrane damage as indicated by MDA levels (He et al., 2017; Garcia-Caparros et al., 2021; Sies et al., 2022). Subsequently, several antioxidant enzymes associated with the ROS scavenging system, such as APOX, CAT, GR, and SOD, were induced in response to enhanced ROS production in drought-stressed callus and plantlets (Fig. 2). SOD converts superoxide to H_2O_2 and O_2, serving as the first line of defense against the production of superoxide radicals (Birben et al., 2012). SOD activity increased in response to 2% and 4% PEG (v/v) treatments compared to the PEG-untreated control to eliminate the toxicity of superoxide radicals during oxidative stress. Similarly, CAT, GR, and APOX activities were also increased, thereby minimizing plant protein breakdown and detoxifying and decomposing H_2O_2. On the other hand, under severe drought stress (8% PEG, v/v, Ψw = -0.46 MPa), SOD, CAT, GR, and APOX activities decreased significantly. This indicates that, under extreme drought stress, cells fail to maintain essential stress-defense proteins. Subsequently, they proceed to the senescence program and shut down all developmental programs. MDA, one of the final byproducts of oxidative lipid modification, damages cell membranes by altering their intrinsic properties, including fluidity, ion transport, protein cross-linking, and enzyme function, decreasing the activity of photosynthetic electron transport chains and ultimately leading to cellular death (Yaser et al., 2010; Patade et al., 2012; Sharma et al., 2012; Ayala et al., 2014; Garcia-Caparros et al., 2021).

Additionally, it was observed that PEG stress significantly increased phenolic compound accumulation during in vitro cultivation. With 4% PEG (v/v) (Ψ_w= -0.21 MPa) growth, the maximum phenolic content was reached in different tissues implicated in the senescence-associated degeneration process (Van Breusegem and Dat, 2006; Ribeiro et al., 2017). The histochemical localization utilizing superoxide-specific labeling, extracellular O_2 •− buildup appears to be linked to chloroplast and other organelles damage, especially around the chlorophyllous areas close to vascular bundles (Fig. 4). Abiotic constraints have been proposed to augment the production of phenolic compounds (phenols and flavonoids) in response to oxidative stress, as these compounds play crucial ecological roles in plant defense and protection (Sharma et al., 2019). Moreover, phenolics help different plant species preserve water homeostasis and scavenge ROS generated by abiotic stressors (Hajam et al., 2023). Additionally, the levels of gallic acid, catechin, ellagic acid, vanillic acid, syringic acid, and apigenin were significantly elevated by 4% PEG stress, chiefly in root tissues (Table 3; Fig. 5). This suggests an increase in metabolic activity within the lipoxygenase, phenylpropanoid, and mevalonate pathways. These findings suggest that the synthesis of several pharmacological components in this plant can be increased by applying PEG-induced drought stress for large-scale production via in vitro tissue culture, thereby

facilitating the recovery of targeted pharmacological compounds from the endangered medicinal plant C. *indicum*.

Conclusion

The current investigation concludes that *in vitro*-cultured *C. indicum* plants are tolerant to drought stress up to Ψ_w = -0.46 MPa. Despite the remarkable antioxidant activity and solute accumulation, PEG-induced drought treatment increased MDA and H_2O_2 levels and decreased growth and biomass in *C. indicum* plants. Nonetheless, notable differences in shoot and root lengths were observed across PEG concentrations. Drought stress (2%, 4%, 8% and 12% PEG 400 (v/v) (Ψ_w= -0.12, -0.21, -0.46, and – 0.71 Mpa,, respectively) raised the phenol and flavonoid content like gallic acid, catechin, ellagic acid, vanillic acid, syringic acid, and apigenin up to 4% PEG (v/v) stressed conditions that can be exploited for targeted metabolites production and recovery for the pharmaceutical purposes.

Statements & Declarations

Competing Interests: *The authors have no relevant financial or non-financial interests to disclose.*

Data availability statement: Not Applicable

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Author's Contribution

Ashutosh Kundu, and Bikram Sahani did the methodology, investigation, data curation, validation, and writing - the original draft of the manuscript, formal analysis (lead); statistical analysis, validation, and visualization; **Tapan Seal** did the HPLC analysis of the plant samples; **Vivekananda Mandal:** Conceptualization (lead); formal analysis (lead); validation, visualization; funding acquisition**;** resources, writing – review and editing; software; supervision and corrected the manuscript for article submission and communication.

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Figures

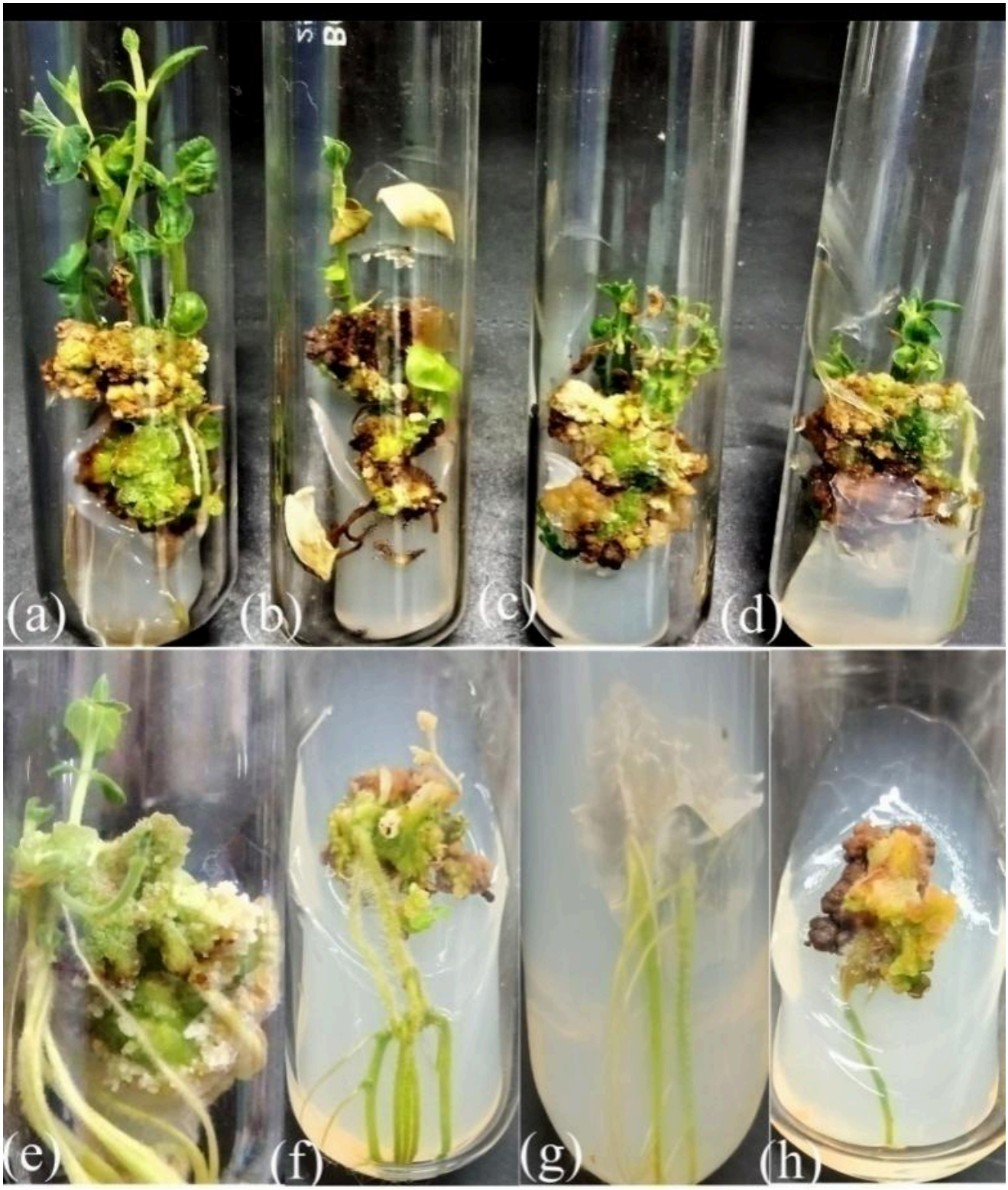

Figure 1

Photographs of the shoot (a-d) and root(e-h) development of *C. indicum* (L.) from callus after 30 days in different PEG stress conditions (0%, 2%, 4% and 8%). (a) Shoot development in 0% PEG; (b) 2% PEG (v/v); (c) 4% PEG (v/v); (d) 8% PEG (v/v); (e) root development in with 0% PEG; (f) 2% PEG (v/v); (g) 4% PEG (v/v), and (h) 8% PEG (v/v).

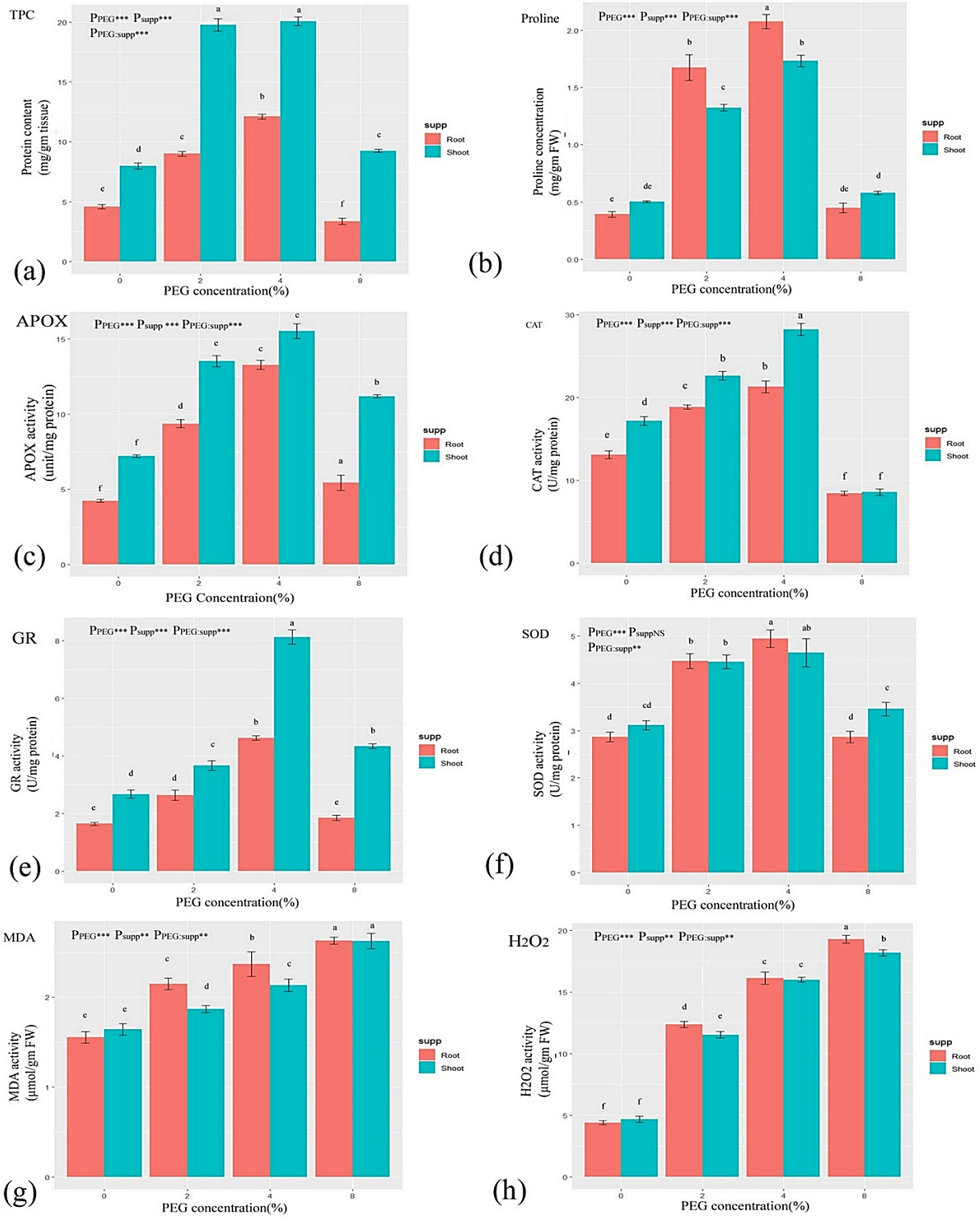

Figure 2

Effect of polyethylene glycol (PEG) stress on biochemical parameters and antioxidant enzyme activity of *C. indicum*. (a) Total protein content (TPC); (b) Proline content; (c) Ascorbate peroxidase (APOX); (d) Catalase (CAT); (e) Gluthione Reductase (GR); (f) Superoxide dismutase (SOD); (g) malondialdehyde (MDA), and (h) hydrogen peroxide (H_2O_2) of shoots and roots measured after 60 days of PEG treatment. Here, the bars represent the mean of three replicates with error bars indicating ± standard deviation, and

dissimilar letters denote significant differences among treatments using two-way ANOVA followed by Tukey's test (P ≤ 0.05). NS: no significant effect; ***: significant effect at P≤0.001; **: significant effect at P ≤ 0.01; *: significant effect at P≤0.05.

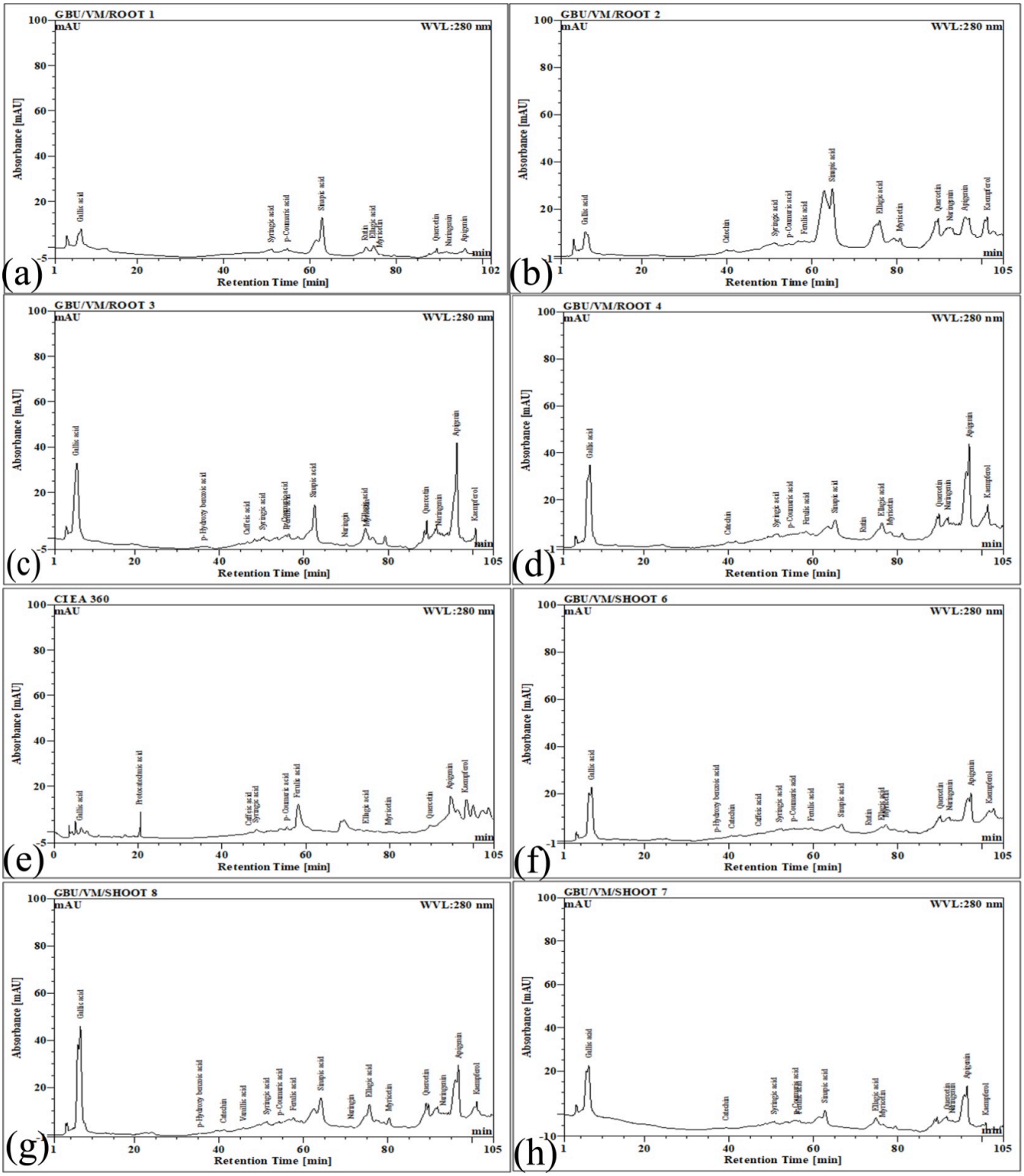

Figure 3

Comparative HPLC-based secondary metabolite (phenolics and flavonoids) profiling from the in-vitro organogenic *C.indicum* shoot and root. (a) Root (0% PEG v/v); (b) Root (2% PEG v/v); (c) Root (4% PEG, v/v), and (d) Root (8% PEG v/v) (e) Shoot (0% PEG, v/v); (f) Shoot (2% PEG, v/v); (g) Shoot (4% PEG, v/v), and (h) Shoot (8% PEG, v/v).

Figure 4

Extracellular production of H_2O_2 in the root in different PEG treatments (v/v), (a) 0 %PEG (v/v); (b) 2 %PEG (v/v); (c) 4 %PEG (v/v), and (d) 8 % PEG (v/v).

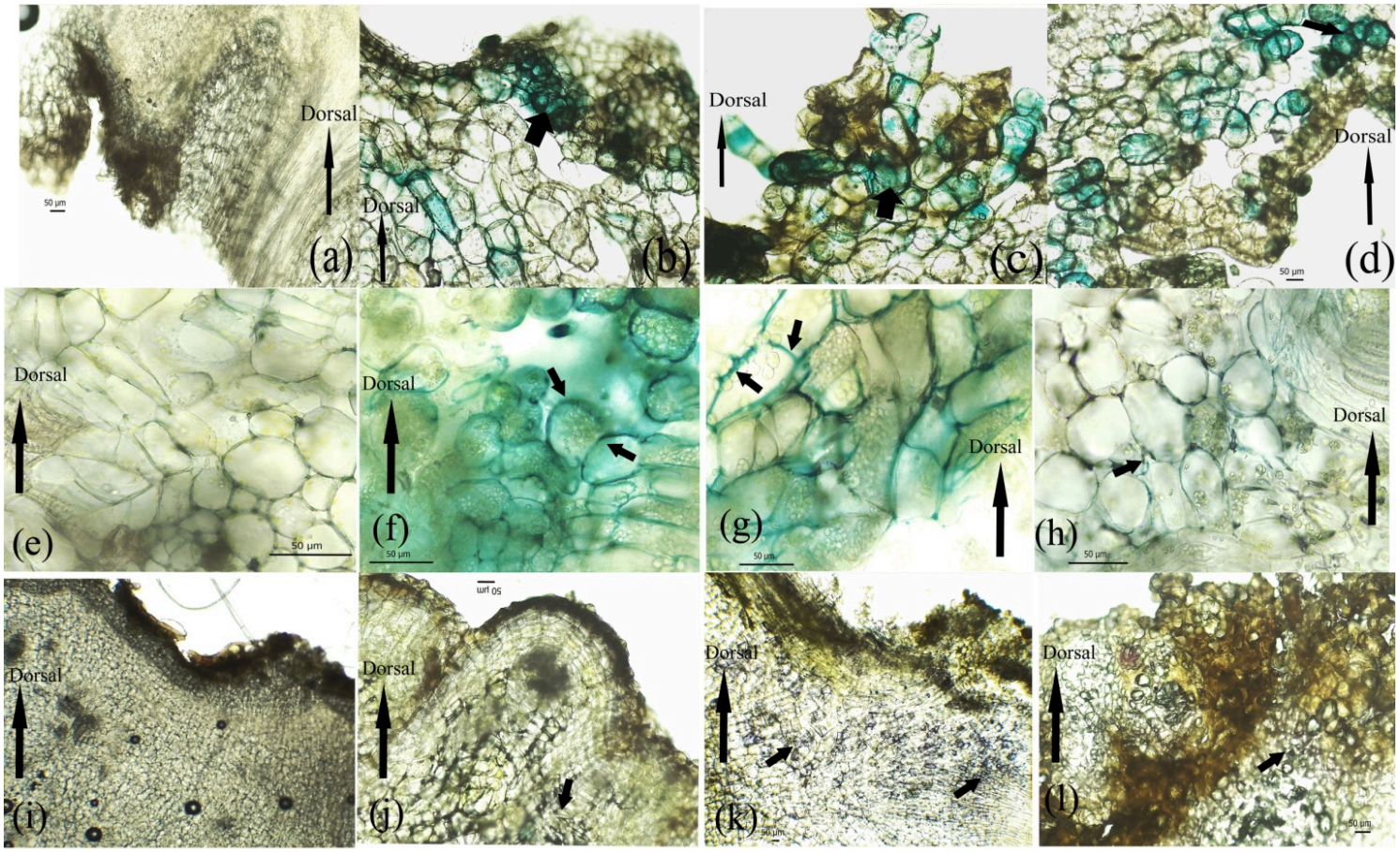

Figure 5

The vertical section of the callus of *C. indicum* showing localization of H_2O_2 (a-d), POX (e-h), and O2 •−(i-l). (a) 0% PEG stressed; (b)2% PEG stressed; (c) 4% PEG stressed;(d) 8% PEG stressed; (e) 0% PEG;(f) 2% PEG; (g) 4% PEG; (h) 8% PEG; (i) 0% PEG; (j) 2% PEG; (k) 4% PEG and (l) 8% PEG. Here, the black arrow indicates the localization of superoxide.