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Research Article

Effect of methyl jasmonate and salicylic acid elicitors on biochemical analysis and antioxidant enzyme activity of *Tylophora indica* (Berm.f.) Merr. callus culture

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Abstract

In the present study, leaf-derived callus of *Tylophora indica* (Berm.f.) Merr. was established on Murashige and Skoog (MS) medium and exposed to methyl jasmonate (MJ) and salicylic acid (SA) at 50, 100, and 200 µM concentrations. The most vigorous callus proliferation among the tested plant growth regulator combinations occurred at 3.0 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) with 0.06 mg L^{-1} Thidiazuron (TDZ). The control group exhibited the highest carbohydrate (102.80±2.24 mg g^{-1} FW) and chlorophyll (0.466±0.033 mg g^{-1} FW) levels. SA at 100 µM produced the strongest coordinated antioxidant response, with 9.62±0.328 mg GAE g^{-1} FW phenols, 6.84±0.17 U mg^{-1} FW ascorbate peroxidase (APX), 0.288±0.023 U mg^{-1} FW guaiacol peroxidase (GPX), and 14.00±0.34 U mg^{-1} FW catalase (CAT); these values were approximately 30%, 101%, 75%, and 128% higher than those of the untreated callus, respectively. In contrast, 200 µM MJ produced the highest malondialdehyde (MDA) (1.98±0.092 nmol g^{-1} FW) and hydrogen peroxide (H_2O_2) (13.85±0.24 µmol g^{-1} FW) values and coincided with reduced carbohydrate, pigment, and APX levels. Across the seven treatments, chlorophyll and carotenoid contents were strongly positively correlated (r=0.883), APX and CAT activity were positively correlated (r=0.821), whereas H_2O_2 and carbohydrate contents were negatively correlated (r=-0.835). The first two principal components explained 70.12% of the total variation. Among the measured treatments, 100 µM SA is found to be the most favourable for antioxidant enzyme activity as well as phenolic content accumulation, whereas higher MJ treatments resulted in greater oxidative damage. These results suggest a well-defined callus culture protocol along with effective range of elicitor treatment for optimization of subsequent metabolite production.

Keywords: Antioxidant enzymes, Callus culture, Methyl jasmonate, Salicylic acid, *Tylophora indica*

Introduction

Tylophora indica (Burm. f.) Merr. is a perennial medicinal climber of the Apocynaceae family with a long history of use in South Asian traditional medicine. Its leaves and roots contain phenanthroindolizidine alkaloids, including tylophorine, tylophorinine, and tylophorinidine, as well as flavonoids, terpenoids, sterols, and other specialized metabolites. Several studies on this species have described the anti-inflammatory, immunomodulatory, antioxidant, and cytotoxic activities of its extracts or isolated constituents, although most of the evidence remains preclinical and should not be interpreted as proof of clinical efficacy (Gururani *et al.,* 2020). Efforts have been driven by the chemical diversity and pharmacological potential of this species to create reliable *in vitro* systems for its propagation, conservation, and regulated production of metabolites (Gupta *et al.,* 2020; Gururani *et al.,* 2020; Mamgain *et al.,* 2023; Mostafa *et al.,* 2023).

Plant tissue culture provides dual advantages for medicinal plants: it reduces dependence on seasonal field materials and serves as a controlled experimental environment for growth, differentiation, and metabolism (Parihar & Dwivedi, 2019, 2020). *In vitro* plant cultures provide a controlled system for manipulating specialized metabolism, while callus and cell-suspension cultures can be subjected to elicitation and other biotechnological interventions to enhance the accumulation of valuable secondary metabolites (Mandal *et al.,* 2023; Chavan *et al.,* 2024). In *T. indica*, diverse explant types have been used to develop callus, organogenic cultures, somatic embryos, and regenerated plants. Auxin-enriched media, particularly those with 2,4-D, frequently succeed in triggering callus formation. In contrast, formulations incorporating TDZ may either enhance substantial callus growth or foster organogenic potential, depending on the explant source and its concentration (Faisal *et al.,* 2005; Thomas & Philip, 2005; Rathore *et al.,* 2014, Gantait & Kundu, 2017; Parihar, 2018, 2020, 2023; Chouhan *et al.,* 2023). Recent studies have confirmed that tissue-culture-derived *T. indica* material retains a broad phytochemical profile and responds to elicitors with altered primary metabolism, antioxidant activity, and accumulation of selected specialized metabolites (Mamgain *et al.,* 2023).

Elicitation uses the plant cell's own defence-signalling network. MJ and SA are widely employed because both can reprogram primary and specialized metabolism through partly distinct and interacting signalling routes (Monte, 2023; Selwal *et al.,* 2025). In cultured plant cells and organs, MJ commonly initiates reactive oxygen species (ROS) production, defense-gene activation, and secondary metabolite biosynthesis; however, when dose or exposure duration becomes excessive, the same response can inhibit growth and intensify oxidative damage (Ho *et al.,* 2020; Sohn *et al.,* 2022). Recent study on holy basil further reveals that MeJA-induced metabolite accumulation is both concentration- and time-dependent, with different metabolite classes exhibiting distinct response patterns

(Chutimanukul *et al.,* 2025). SA is closely associated with maintaining cellular redox balance and antioxidant protection, and its influence on peroxide metabolism varies with its concentration and the type of tissue involved (Saleem *et al.,* 2021; Lam *et al.,* 2024; Tian *et al.,* 2025). In medicinal plant cultures, reactions to both MJ and SA vary widely, shaped by factors such as genotype, tissue type, developmental phase, concentration of treatment, and length of exposure, which require empirical fine-tuning (Jeyasri *et al.,* 2023).

When the objective is to increase metabolite production while preserving the viability of the culture, it is important to consider the biochemical cost of elicitation. H_2O_2, when maintained at specific levels, functions as a redox signal. However, if it accumulates excessively, it can result in the peroxidation of membrane lipids, the degradation of pigments, and the disruption of metabolic functions (Smirnoff & Arnaud, 2019; Qureshi *et al.,* 2022). CAT, APX, and GPX provide alternative enzymatic routes for breaking down peroxides, whereas phenolic compounds enhance the non-enzymatic antioxidant reserves (Sakihama *et al.,* 2002; Ahmad *et al.,* 2010; Rajput *et al.,* 2021). Evaluating these characteristics alongside carbohydrates and photosynthetic pigments provides a more accurate distinction between a beneficial defense mechanism and a detrimental oxidative condition than analyzing an individual metabolite or enzyme alone (Gill & Tuteja, 2010).

Currently, there is a scarcity of comparative data regarding the redox responses triggered by MJ and SA in *T. indica* callus derived from leaves. In this research, we first optimized the induction of callus from leaf explants and then subjected the proliferating cultures to MJ or SA at concentrations of 50, 100, and 200 µM. Carbohydrate status, chlorophyll and carotenoid contents, total phenols, H_2O_2 and MDA accumulation, and APX, GPX, and CAT activities were evaluated to determine how elicitor identity and concentration alter the balance between primary metabolism, antioxidant defense, and oxidative stress. This study was designed to test whether moderate elicitor concentrations would promote phenolic and antioxidant responses, whereas higher concentrations would impose metabolic and oxidative costs.

Material and methods

Collection of plant sample

Young plantlets of *Tylophora indica* were procured from the Ecology Department Garden, Central Arid Zone Research Institute, Jodhpur, Rajasthan, India (26.258852, 72.994397). The plantlets were established in Soilrite-filled pots and maintained in the departmental nursery for subsequent experiments.

Explant preparation

Fresh young leaves collected from nursery-grown plants were rinsed under running tap water for 10-15 min to remove adhering dust and debris and then washed with detergent for 5 min. The material was first washed with distilled water and then briefly immersed in 70% (v/v) ethanol. In a sterile laminar-air-flow chamber, the explants were treated for surface sterilization with 0.1% mercuric chloride (w/v) for 1-2 minutes, followed by 0.1% Bavistin (w/v) for 4-5 minutes. After this, the explants were rinsed five to six times with autoclaved distilled water to ensure the removal of any sterilant residues. They were then cut down to a size of roughly 0.5-1.0 cm^2 and placed on a culture medium. The cultures were observed regularly for signs of growth, contamination, and callus formation.

Callus induction and culture conditions

Leaf explants were inoculated on MS medium (Murashige & Skoog, 1962), which was prepared with 0.8% agar (w/v) and 3% sucrose (w/v), and the pH was adjusted to 5.8±0.1. To ensure sterility, the medium was autoclaved at 121 °C and 15 psi for 20 minutes before use. The explants were treated with various plant growth regulator combinations: 2,4-D alone at 1.0, 2.0, and 3.0 mg/L; 2,4-D with 6-Benzylaminopurine (BAP) at 1.0+0.1, 2.0+0.2, and 3.0+0.3 mg/L; and 2,4-D with TDZ at 1.0+0.02, 2.0+0.04, 3.0+0.06, 4.0+0.08, and 5.0+0.10 mg/L, along with a hormone-free control. The cultures were maintained in a laboratory environment at 25±2 °C, with a light cycle of 16 hours of illumination and 8 hours of darkness, using cool white fluorescent lights. After achieving initiation and induction of callus, it was maintained by subculturing every 3 weeks. 28-days old callus tissues were used for experimental studies, including growth observations, induction-frequency assessment, morphological documentation, biochemical assays, and antioxidant enzyme analyses. Each treatment was evaluated in triplicates.

Elicitation of Methyl Jasmonate and Salicylic acid

Stock solutions of MJ (prepared in 100% ethanol) and SA (initially dissolved in ethanol and diluted with distilled water) were prepared for treatment. Elicitors were applied to callus cultures at 50, 100, and 200 µM concentrations, and untreated cultures served as the control. All biochemical and antioxidant measurements were performed using the same analytical procedures for the control and elicited calli.

Biochemical estimations

Callus produced on the optimum PGR combination was used for biochemical measurements after MJ or SA elicitation, with untreated leaf-derived callus analyzed in parallel as the control.

Total carbohydrate

The anthrone method (Yemm & Willis, 1954) was used to estimate the carbohydrate content in samples, and glucose was used as a standard curve for evaluation. The optical density of the anthrone-carbohydrate compound

was assessed at 620 nm and is represented as mg glucose equivalents per g of fresh plant tissue.

Total phenol

The Folin-Ciocalteu procedure was followed to estimate the total phenol content (Singleton *et al.,* 1999), and the optical density was measured at 760 nm using gallic acid as a standard curve. Data are represented as mg of gallic acid equivalents per g of fresh plant tissue.

Chlorophyll and carotenoid

After homogenizing in 80% acetone and centrifugation, the supernatants of the callus and leaf extracts were subjected to spectrophotometric analysis, and optical density measurements were taken at 663, 645, and 480 nm for chlorophyll a, b, and carotenoids, respectively. Photosynthetic pigment contents were estimated using the formula provided in the protocol (Arnon, 1949; Kirk & Allen, 1965) and presented as mg per g of fresh plant tissue.

Hydrogen peroxide

The protocol described by Alexieva *et al.* (2001) was used to estimate H_2O_2 (Alexieva *et al.,* 2001). Both tissues were homogenized in trichloroacetic acid (0.1% pre-chilled) and centrifuged. The supernatant was used to prepare a reaction mixture, and the optical density was measured at 390 nm. The H_2O_2 content was evaluated using an extinction coefficient (ε=0.28 μM^{-1} cm^{-1}) and is represented as µmol per g of fresh plant tissue.

Lipid peroxidation

Lipid peroxidation or MDA content estimation was performed using the thiobarbituric acid (TBA) responsive substance assay (Heath & Packer, 1968). Both tissues were homogenized in 0.25% TBA (made in 10% trichloroacetic acid), and the optical density of the MDA-TBA compound was measured at 532 nm and presented as nmol MDA per g of fresh plant tissue, where the extinction coefficient was ε=155 μM^{-1} cm^{-1}.

Antioxidant enzyme activity

A crude extract was prepared for antioxidant enzyme analysis using fresh callus samples homogenized in pre-chilled phosphate buffer (50 mM, pH 7.0). After centrifugation at 4 °C, the clear supernatant was used for further experiments.

Catalase

The method described by Aebi (1984) was followed to measure CAT enzyme in the reaction mixture containing crude extract, phosphate buffer, and hydrogen peroxide was observed at a wavelength of 240 nm (Aebi, 1984). CAT enzyme activity was presented as units per milligram of plant tissue, and one unit is proportional to the quantity of enzyme that decomposes 1 µmol of H_2O_2 per minute.

Ascorbate peroxidase

The Chen and Asada (1989) method was followed to estimate APX activity (Chen & Asada, 1989). It is based on measuring the oxidation of ascorbate at optical density 290 nm over a period of three minutes and the results are presented as units per mg of plant tissue (ε=2.8 μM^{-1} cm^{-1}).

Guaiacol peroxidase

The procedure described by Pütter (1974) was used to estimate GPX enzyme activity by measuring the optical density of the tetraguaiacol compound formed at 436 nm (Pütter, 1974) and presented as units per mg of plant tissue. The crude extract was mixed with phosphate buffer, H_2O_2, and guaiacol to prepare the reaction mixture.

Statistical data analysis

Each experiment was performed in triplicate (n=3) and is represented as the mean±standard error (SE). To calculate these, one-way analysis of variance (ANOVA) was performed in IBM SPSS Statistics 27 with post hoc comparison using Duncan's multiple range test (DMRT), with a default significance level of $p<0.05$ (Duncan, 1955; Ståhle & Wold, 1989). Heatmap, Pearson's correlation analysis, and biplot principal component analysis (PCA) were prepared using Origin Pro 2026b.

Results

Callus induction

Callus initiation was visible 7-10 days after leaf-explant inoculation, and cultures were followed for 4-6 weeks. Among the plant growth regulator (PGR) combinations tested, 2,4-D with TDZ gave the most vigorous callus response (Figure 1), with the highest induction frequency on MS medium containing 3.0 mg L^{-1} 2,4-D and 0.06 mg L^{-1} TDZ. The callus was friable, yellow-green, and proliferative through successive subculture cycles. Hormone-free media and less effective PGR combinations either failed to induce callus or produced lower-yielding, compact, poorly proliferating tissues.

Elicitor treatment

Following a four-week period of treatment using elicitors, it was noted that both SA and MJ around 50 and 100 µM concentrations led to peak levels of chlorophyll, carotenoids, carbohydrates, and phenols, along with antioxidant enzymes in the callus. Conversely, oxidative stress indicators, namely MDA and H_2O_2, rose with elevated treatment concentrations.

Biochemical analysis

Total carbohydrate

Untreated callus had the highest carbohydrate content (102.80±2.24 mg g^{-1} FW). The carbohydrate content decreased

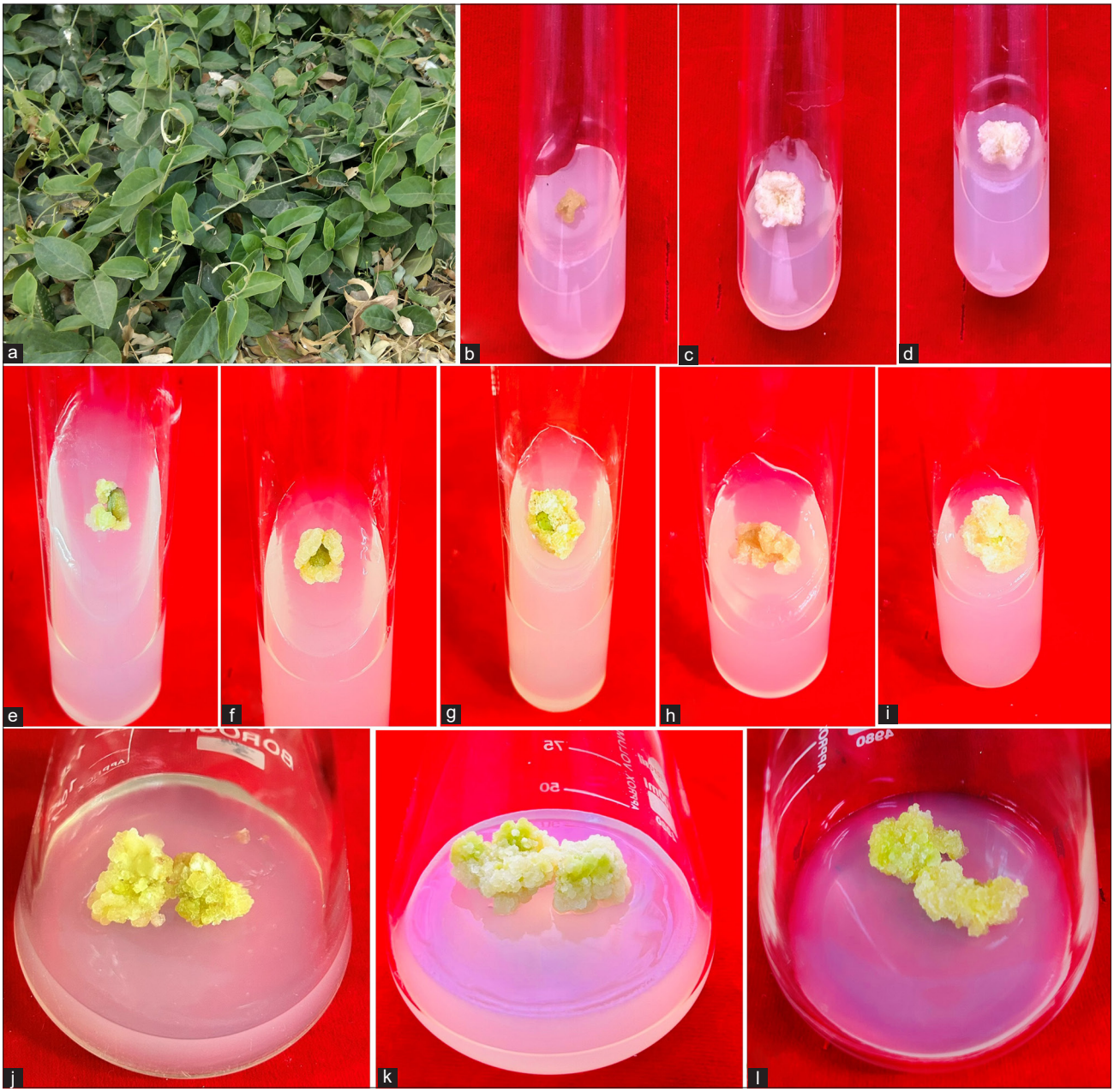


Figure 1: Establishment of callus cultures from leaf explants of *Tylophora indica* Berm.f: a) A plant of *T. indica* in field conditions, b-d) initiation of callus from leaf explant in MS media containing 2,4-D+BAP at 1+0.1, 2+0.2, and 3+0.3 mg L^{-1} respectively, e-i) initiation of callus in MS media containing 2,4-D+TDZ at 1.0+0.02, 2.0+0.04, 3.0+0.06, 4.0+0.08 and 5.0+0.10 mg L^{-1} respectively, j-l) proliferation of callus of *T. indica* in MS media containing 2,4-D+TDZ at 1+0.02, 2+0.04, and 3+0.06 mg L^{-1} respectively

to 92.88±0.60, 89.35±1.76, and 69.50±1.51 mg g^{-1} FW with 50, 100, and 200 µM MJ, respectively, and to 88.72±0.82, 84.28±1.44, and 78.90±1.03 mg g^{-1} FW with the corresponding SA treatments (Figure 2 & Table 1). The strongest numerical reduction relative to the control was observed at 200 µM MJ.

Photosynthetic pigments

Untreated callus also had the highest total chlorophyll content (0.466±0.033 mg g^{-1} FW). Among the elicited cultures, 100 µM MJ and 100 µM SA retained comparatively higher chlorophyll levels (0.378±0.005 and 0.290±0.028 mg g^{-1} FW, respectively) than their 50 or 200 µM counterparts. The carotenoid content at 100 µM MJ (0.044±0.001 mg g^{-1} FW) was close to that of the untreated callus (0.043±0.005 mg g^{-1} FW), whereas 200 µM SA produced the lowest chlorophyll and carotenoid values (0.013±0.002 and 0.013±0.001 mg g^{-1} FW, respectively) (Figure 2 & Table 1).

Total phenol

Total phenol content showed a bell-shaped response to both elicitors. The highest value was recorded with 100 µM SA (9.62±0.328 mg GAE g^{-1} FW), followed by 100 µM MJ (8.35±0.35 mg GAE g^{-1} FW), compared with 7.41±0.64 mg GAE g^{-1} FW in the untreated callus. At 200 µM, the phenol content returned to 7.39±0.59 mg GAE g^{-1} FW for MJ and 7.10±0.33 mg GAE g^{-1} FW for SA (Figure 2 & Table 1).

Table 1: Biochemical traits, oxidative stress markers, and antioxidant enzyme activities in untreated and MJ and SA-treated leaf-derived callus of *T. indica*

Treatment	Carbohydrate (mg/g FW)	Chlorophyll (mg/g FW)	Carotenoid (mg/g FW)	Phenol (mg GAE/g FW)	MDA (nmol/g FW)	H_2O_2 (µmol/g FW)	APX (U/mg FW)	GPX (U/mg FW)	CAT (U/mg FW)
Callus control	**102.80±2.24^{a}**	**0.466±0.033^{b}**	0.043±0.005^{b}	7.41±0.64^{b}	0.95±0.19^{b}	0.44±0.04^{h}	3.41±0.18^{c}	0.165±0.008^{c}	6.15±1.17^{d}
MJ 50 µM	92.88±0.6^{b}	0.117±0.004^{b}	0.027±0.002^{b}	6.70±0.178^{b}	0.96±0.06^{b}	4.75±0.09^{f}	3.49±0.11^{c}	0.253±0.009^{b}	9.27±0.27^{c}
MJ 100 µM	89.35±1.76^{b}	0.378±0.005^{b}	**0.044±0.001^{b}**	8.35±0.35^{b}	1.73±0.06^{a}	12.19±0.314^{b}	2.97±0.09^{d}	0.182±0.008^{c}	8.60±0.29^{c}
MJ 200 µM	69.50±1.51^{c}	0.096±0.006^{b}	0.024±0.001^{b}	7.39±0.59^{b}	**1.98±0.092^{a}**	**13.85±0.24^{a}**	1.34±0.02^{e}	0.155±0.010^{c}	6.89±0.23^{d}
SA 50 µM	88.72±0.82^{bc}	0.074±0.003^{b}	0.027±0.001^{b}	7.78±0.249^{b}	0.69±0.02^{b}	5.61±0.32^{e}	4.82±0.13^{b}	0.164±0.004^{c}	13.86±0.34^{a}
SA 100 µM	84.28±1.44^{c}	0.290±0.028^{b}	0.035±0.002^{b}	**9.62±0.328^{b}**	0.73±0.02^{b}	7.65±0.29^{d}	**6.84±0.17^{a}**	**0.288±0.023^{b}**	**14.00±0.34^{a}**
SA 200 µM	78.90±1.03^{d}	0.013±0.002^{b}	0.013±0.001^{b}	7.10±0.33^{b}	0.89±0.01^{b}	10.85±0.22^{c}	4.44±0.15^{b}	0.090±0.003^{b}	11.32±0.26^{b}

Values are means±SE (n=3); superscript letters are the reported DMRT groupings at $p<0.05$. Bold values are the maximum means observed during elicitor treatments.

Figure 2: Heatmap of MJ and SA elicitor treatments, biochemical tests, and antioxidant analysis of the leaf-derived callus of *T. indica*

Oxidative stress markers: H_2O_2 and MDA

Oxidative stress markers responded differently to MJ and SA. In MJ-treated callus, MDA increased from 0.96±0.06 nmol g^{-1} FW at 50 µM to 1.73±0.06 at 100 µM and 1.98±0.092 at 200 µM, while H_2O_2 increased from 4.75±0.09 to 12.19±0.314 and 13.85±0.24 µmol g^{-1} FW. SA also produced a concentration-dependent rise in H_2O_2 (5.61±0.32, 7.65±0.29, and 10.85±0.22 µmol g^{-1} FW at 50, 100, and 200 µM), but MDA remained numerically below the untreated callus value (0.95±0.19 nmol g^{-1} FW) across all SA treatments (Figure 2 & Table 1).

Antioxidant enzyme activity

The strongest coordinated antioxidant enzyme response occurred with SA. At 100 µM SA, APX, GPX, and CAT reached 6.84±0.17, 0.288±0.023, and 14.00±0.34 U mg^{-1} FW, respectively; CAT was similarly high at 50 µM SA (13.86±0.34 U mg^{-1} FW). In MJ-treated callus, GPX and CAT were highest at 50 µM (0.253±0.009 and 9.27±0.27 U mg^{-1} FW) and declined at higher concentrations, whereas APX decreased to 1.34±0.02 U mg^{-1} FW at 200 µM MJ. These measurements showed that SA maintained greater antioxidant enzyme activity than MJ under these conditions (Figure 2 & Table 1).

Multivariate relationships among measured traits

The treatment heatmap (Figure 2) summarizes the dose-dependent separation evident in the individual measurements: untreated callus was characterized by high carbohydrate and pigment values, 100 µM SA combined high total phenols with high APX, GPX, and CAT activities, and 200 µM MJ was distinguished by the highest MDA and H_2O_2 values.

Pearson correlation analysis of different treatments (Figure 3) displayed a strong positive association among chlorophyll and carotenoids (r=0.883), and among APX and CAT (r=0.821). H_2O_2 showed a strong negative association with carbohydrate content (r=-0.835), whereas

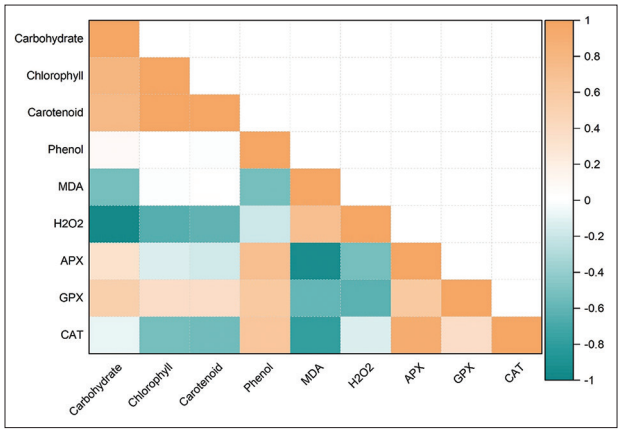


Figure 3: Pearson's correlation analysis of the biochemical, antioxidant tests performed on MJ and SA treated callus (n=21 observations)

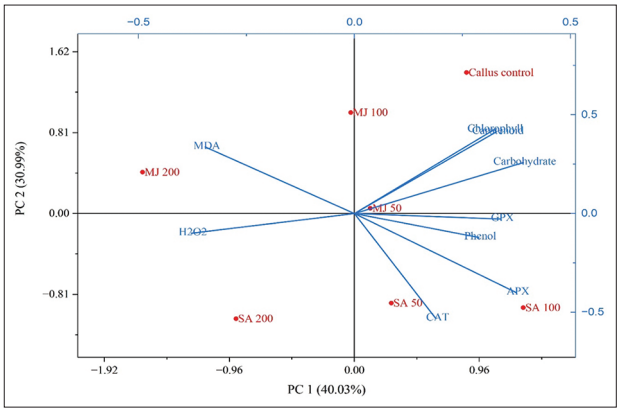


Figure 4: Principal component analysis (PCA) of the control and callus treated with different concentrations MJ and SA

MDA showed negative association with APX (r=-0.793). The correlation between total phenol content and APX was positive (r = 0.526), as was the case with GPX (r=0.479) and CAT (r=0.451), suggesting that enzymatic and non-enzymatic antioxidant components responded in a coordinated manner to elicitor-induced stress (Mishra *et al.,* 2023; Wang *et al.,* 2024). Similar relationships among ROS, lipid peroxidation products, and antioxidant enzymes have been reported in plants exposed to stress and exogenous regulatory treatments (Zarbakhsh *et al.,* 2024). These correlations were determined using data from individual replicates across three repeated experiments for all treatments, thus reflecting relationships among specific experimental observations. This method offers a more appropriate foundation for statistical inference compared to correlations based only on treatment means (Rogers *et al.,* 2021).

The PCA biplot (Figure 4) summarizes the treatment structure, with PC1 explaining 40.03% and PC2 explaining 30.09% of the total variation, respectively (70.12% cumulatively). The untreated control was associated with carbohydrates and photosynthetic pigments, SA at 100 µM aligned more closely with the antioxidant/phenolic trait space, and high MJ separated toward the oxidative stress region of the ordination. Therefore, the multivariate pattern reinforced the contrast between a comparatively balanced SA response and a more oxidative high-MJ response.

Discussion

The present study links callus establishment with a comparative redox assessment of MJ- and SA-elicited *T. indica* callus cultures. Leaf explants produced a friable, proliferating callus most effectively on MS medium containing 3.0 mg L^{-1} 2,4-D and 0.06 mg L^{-1} TDZ. This response is consistent with the broader tissue culture literature for *T. indica*, in which 2,4-D is repeatedly associated with callus induction and TDZ-containing systems have supported vigorous indirect regeneration or organogenic responses (Faisal *et al.,* 2005; Thomas & Philip, 2005; Gantait & Kundu, 2017). Nevertheless, the effective concentration combination is specific to the explant and culture conditions, which can vary experimentally.

Elicitation imposes a measurable cost on primary metabolism. Carbohydrate content declined under every MJ and SA treatment, and the reduction was most pronounced at 200 µM MJ, where the mean was approximately 32% below that of the untreated callus. Pigments were similarly dose-sensitive. Chlorophyll was markedly reduced at 50 µM of either elicitor, partially recovered at 100 µM, and then declined sharply at 200 µM, with 200 µM SA showing the lowest chlorophyll and carotenoid values. This non-linear behavior cautions against assuming that increasing the elicitor concentration simply increases the desired defence response. In cultured plant tissues, signalling, growth inhibition, metabolic reallocation, and cellular injury can occur simultaneously, and their relative contributions change with dose and exposure time (Jeyasri *et al.,* 2023).

Total phenols exhibited a different pattern from carbohydrates and pigments. The phenolic pool increased to 9.62±0.328 mg GAE g^{-1} FW at 100 µM SA, approximately 30% above the untreated callus, and reached 8.35±0.35 mg GAE g^{-1} FW at 100 µM MJ before declining again at 200 µM SA. A mid-dose optimum is compatible with the general concept of elicitation: a moderate defence signal can redirect metabolism toward protective specialized compounds, whereas excessive stress can restrict biosynthetic capacity or impair cell function (Chouhan *et al.,* 2026). MJ and SA are widely used to enhance specialized metabolism in medicinal plant cultures; however, their effective concentrations and metabolite outcomes are highly system-dependent (Ho *et al.,* 2020; Jeyasri *et al.,* 2023). Importantly, the Folin-Ciocalteu assay estimates a complex reducing phenolic pool; the present data do not identify which individual phenolic compound changed and, therefore, cannot be interpreted as direct evidence for increased tylophorine or another specific constituent.

The contrast between H_2O_2 and MDA is particularly informative. MJ caused a steep, concentration-dependent increase in both markers: at 200 µM, MDA was approximately 108% above the control, and H_2O_2 was more than thirty times the untreated value. The concurrent

reduction in carbohydrate, chlorophyll, and APX levels at this dose is consistent with a transition from useful redox signalling to oxidative injury. Exogenous MJ is known to trigger ROS production in plant cells and organ cultures; ROS participate in defense signalling but can damage membranes and suppress growth when their accumulation becomes excessive (Ho *et al.,* 2020). SA also increased H_2O_2 with concentration, yet mean MDA values remained below those of the untreated callus across all three SA treatments. The separation between H_2O_2 accumulation and lipid peroxidation suggests that SA-treated callus protected the membrane from damage more effectively, despite a strong redox signal (Farghaly *et al.,* 2021; Saleem *et al.,* 2021).

The antioxidant enzyme profiles support this interpretation. At 100 µM SA, APX activity was approximately 101% above that of the control, GPX was approximately 75% higher, and CAT was approximately 128% higher. CAT was strongly induced at 50 µM SA, and the coordinated increase in APX, GPX, and CAT at 100 µM coincided with the highest phenolic value. MJ produced a different trajectory, where GPX and CAT were highest at 50 µM and declined as MJ concentration increased, whereas APX was below the control at 100 µM and approximately 61% lower than the control at 200 µM. SA is tightly integrated with redox signalling and antioxidant defense, and its net effect depends on the concentration, tissue, developmental state, and prevailing stress environment (Saleem *et al.,* 2021). Under the conditions tested here, 100 µM SA appeared to generate the most effective coupling between the defence signal and enzymatic peroxide-scavenging capacity.

The exploratory correlation and PCA results integrated these trade-offs across the treatments. Chlorophyll and carotenoids tracked closely, indicating a coordinated pigment response, whereas the negative relationship between H_2O_2 and carbohydrates linked increasing oxidative load with the depletion of primary carbon reserves. MDA was negatively associated with APX, and APX was positively associated with CAT, separating treatments that maintained coordinated peroxide detoxification from those that showed greater membrane oxidative damage. The first two PCA axes accounted for 70.12% of the total variation, with the control positioned with carbohydrate/pigment traits, 100 µM SA closer to the antioxidant/phenolic trait space, and high MJ closer to oxidative stress traits.

These findings also align with the growing evidence that elicitation can be used to alter the metabolism of *T. indica*. Mamgain *et al.* (2023) reported that cadmium chloride and chitosan treatments changed soluble sugars, stress-related biochemistry, and antioxidant enzyme activities in *T. indica in vitro* cultures and enhanced the accumulation of the triterpenoid α-amyrin at selected doses. The elicitors, tissues, exposure periods, and target measurements differ from those of the present study, but both show that *T. indica* cultures are metabolically responsive to external defense signals and that the highest elicitor dose is not necessarily the most favorable for biochemical performance and metabolite accumulation (Mubeen *et al.,* 2025). This dose dependence is especially relevant when developing a production system in which the culture biomass and product concentration must be optimized together. Comparable dose-dependent responses to jasmonate elicitation have been reported in medicinal plant cultures, where the metabolite yield varied substantially with elicitor concentration (Siva *et al.,* 2015; Moola *et al.,* 2024). These signalling events are closely related with the regulation of antioxidant enzymes, including GPX, CAT, and APX, which contribute to ROS homeostasis by coordinating superoxide dismutation and hydrogen peroxide removal (Jiménez *et al.,* 2025).

The present work used different PGRs concentrations but only a single four-week exposure endpoint; shorter time-course sampling would help distinguish an early signalling pulse from later oxidative damage. Biomass gain, dry matter, and metabolite yield per culture vessel were not quantified alongside biochemical concentration; therefore, the treatment that maximizes the concentration cannot yet be assumed to maximize total productivity. In addition, the study measured total phenols, rather than individual *T. indica* marker compounds. Targeted HPLC, LC-MS, or validated HPTLC analysis of phenanthroindolizidine alkaloids and other relevant metabolites could therefore be combined with biomass-normalized productivity, culture viability, and replicate-level multivariate analysis. In this study, 100 µM SA proved to be the most effective treatment for future investigations, whereas 200 µM MJ was associated with a comparatively high oxidative cost. A comparable response has been reported in Corylus avellana cell cultures, where elicitor treatment increased H_2O_2, MDA and antioxidant enzyme activities, while moderate treatment promoted phenolic and taxane accumulation more effectively than higher concentrations (Hazrati *et al.,* 2025).

Conclusion

In this research, a reliable callus system derived from *T. indica* leaves was established, demonstrating that MJ and SA lead to unique biochemical and redox changes. The treatment with 3.0 mg L^{-1} 2,4-D combined with 0.06 mg L^{-1} TDZ resulted in the most significant callus proliferation among the PGR treatments evaluated. During elicitation, 100 µM SA produced the most favorable combination of measured defence traits: total phenols reached 9.62±0.328 mg GAE g^{-1} FW and APX, GPX, and CAT reached 6.84±0.17, 0.288±0.023, and 14.00±0.34 U mg^{-1} FW, respectively, whereas MDA remained below the untreated callus mean. In contrast, 200 µM MJ produced the highest MDA and H_2O_2 values and coincided with substantial losses in carbohydrates, pigments, and APX activity. Correlation and PCA patterns further separated primary metabolism/pigment traits from oxidative stress traits and placed 100 µM SA closer to the antioxidant/phenolic response. Thus, moderate SA elicitation is the most promising treatment among those tested for subsequent optimizations. Prior to suggesting any expansion, it is essential to validate results by conducting time-course experiments, evaluating productivity normalized to biomass, and accurately measuring the pharmacologically important metabolites of *T. indica*.

Author contributions

Gourav Chouhan: experimental work, investigation, data curation, and writing of original draft. Neelam Solanki and Poonam Jodha: review and editing. Suman Parihar: conceptualization, methodology, review and editing. All authors approved the final manuscript.

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References

Aebi, H. (1984). Catalase *in vitro*. *Methods in Enzymology, 105*, 121-126. https://doi.org/10.1016/S0076-6879(84)05016-3

Ahmad, P., Jaleel, C. A., Salem, M. A., Nabi, G., & Sharma, S. (2010). Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. *Critical Reviews in Biotechnology, 30*(3), 161-175. https://doi.org/10.3109/07388550903524243

Alexieva, V., Sergiev, I., Mapelli, S., & Karanov, E. (2001). The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant, Cell & Environment, 24*(12), 1337-1344. https://doi.org/10.1046/j.1365-3040.2001.00778.x

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology, 24*(1), 1-15. https://doi.org/10.1104/pp.24.1.1

Chavan, J., Patil, P., Patil, A., Deshmukh, A., Panari, P., Mohite, A., Lawand, P., Yadav, P., Bodhe, M., Kadam, A., Namdas, D., Pawar, B., Jadhav, A., Shekhawat, M., & Santa-Catarina, C. (2024). *Salacia* spp.: Recent insights on biotechnological interventions and future perspectives. *Applied Microbiology and Biotechnology, 108*, 200. https://doi.org/10.1007/s00253-023-12998-z

Chen, G.-X., & Asada, K. (1989). Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. *Plant and Cell Physiology, 30*(7), 987-998. https://doi.org/10.1093/oxfordjournals.pcp.a077844

Chouhan, G., Solanki, N., Jodha, P., Parihar, S., & Shekhawat, G. S. (2026). Influence of environmental stress on secondary metabolite production in plants. In A. Srivastava & A. K. Mishra (Eds.), *Secondary metabolites in stress and disease management* (pp. 315-328). Academic Press. https://doi.org/10.1016/B978-0-443-33959-2.00009-5

Chouhan, G., Solanki, N., Shekhawat, G. S., & Parihar, S. (2023). *Convolvulus* prostratus Forssk: a memory boosting herb. *Plant Science Today, 10*(3), 86-93. https://doi.org/10.14719/pst.2110

Chutimanukul, P., Thongtip, A., Panya, A., Phonsatta, N., Thangvichien, S., Mosaleeyanon, K., Wanichananan, P., Sae-Tang, W., & Chutimanukul, P. (2025). Enhancing the biochemical potential of holy basil through methyl jasmonate elicitation with insights into physiological responses in a plant factory. *Scientific Reports, 15*, 27518. https://doi.org/10.1038/s41598-025-13463-9

Duncan, D. B. (1955). Multiple range and multiple F tests. *Biometrics, 11*(1), 1-42. https://doi.org/10.2307/3001478

Faisal, M., Singh, S., & Anis, M. (2005). *In vitro* regeneration and plant establishment of *Tylophora indica* (Burm. f.) Merrill: petiole callus culture. *In vitro Cellular & Developmental Biology-Plant, 41*, 511-515. https://doi.org/10.1079/IVP2005674

Farghaly, F. A., Salam, H. K., Hamada, A. M., & Radi, A. A. (2021). The role of benzoic acid, gallic acid and salicylic acid in protecting tomato callus cells from excessive boron stress. *Scientia Horticulturae, 278*, 109867. https://doi.org/10.1016/j.scienta.2020.109867

Gantait, S., & Kundu, S. (2017). Neoteric trends in tissue culture-mediated biotechnology of Indian ipecac [*Tylophora indica* (Burm. f.) Merrill]. *3 Biotech, 7*, 231. https://doi.org/10.1007/s13205-017-0865-8

Gill, S. S., & Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry, 48*(12), 909-930. https://doi.org/10.1016/j.plaphy.2010.08.016

Gupta, V., Guleri, R., Gupta, M., Kaur, N., Kaur, K., Kumar, P., Anand, M., Kaur, G., & Pati, P. K. (2020). Anti-neuroinflammatory potential of *Tylophora indica* (Burm. f.) Merrill and development of an efficient *in vitro* propagation system for its clinical use. *Plos One, 15*(3), e0230142. https://doi.org/10.1371/journal.pone.0230142

Gururani, R., Patel, S., Yaduvanshi, N., Dwivedi, J., Paliwal, S., & Sharma, S. (2020). *Tylophora indica* (Burm. f.) merr: An insight into phytochemistry and pharmacology. *Journal of Ethnopharmacology, 262*, 113122. https://doi.org/10.1016/j.jep.2020.113122

Hazrati, R., Zare, N., Asghari-Zakaria, R., Sheikhzadeh, P., Shekhawat, M. S., & Singh, R. K. (2025). Enhancing taxane production in *Corylus avellana* L. cell cultures through elicitation using molybdenum oxide and iron oxide nanoparticles. *Industrial Crops and Products, 238*, 122269. https://doi.org/10.1016/j.indcrop.2025.122269

Heath, R. L., & Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics, 125*(1), 189-198. https://doi.org/10.1016/0003-9861(68)90654-1

Ho, T.-T., Murthy, H. N., & Park, S.-Y. (2020). Methyl jasmonate induced oxidative stress and accumulation of secondary metabolites in plant cell and organ cultures. *International Journal of Molecular Sciences, 21*(3), 716. https://doi.org/10.3390/ijms21030716

Jeyasri, R., Muthuramalingam, P., Karthick, K., Shin, H., Choi, S. H., & Ramesh, M. (2023). Methyl jasmonate and salicylic acid as powerful elicitors for enhancing the production of secondary metabolites in medicinal plants: an updated review. *Plant Cell, Tissue and Organ Culture (PCTOC), 153*, 447-458. https://doi.org/10.1007/s11240-023-02485-8

Jiménez, A., Martí, M. C., & Sevilla, F. (2025). Oxidative post-translational modifications of plant antioxidant systems under environmental stress. *Physiologia Plantarum, 177*(1), e70118. https://doi.org/10.1111/ppl.70118

Kirk, J. T. O., & Allen, R. L. (1965). Dependence of chloroplast pigment synthesis on protein synthesis: effect of actidione. *Biochemical and Biophysical Research Communications, 21*(6), 523-530. https://doi.org/10.1016/0006-291X(65)90516-4

Lam, V. P., Loi, D. N., Shin, J., Lee, K. M., & Park, J. (2024). Optimization of salicylic acid concentrations for increasing antioxidant enzymes and bioactive compounds of *Agastache rugosa* in a plant factory. *Plos One, 19*(7), e0306340. https://doi.org/10.1371/journal.pone.0306340

Mamgain, J., Mujib, A., Bansal, Y., Gulzar, B., Zafar, N., Syeed, R., Alsughayyir, A., & Dewir, Y. H. (2023). Elicitation induced α-amyrin synthesis in *Tylophora indica in vitro* cultures and comparative phytochemical analyses of *in vivo* and micropropagated plants. *Plants, 13*(1), 122. https://doi.org/10.3390/plants13010122

Mandal, S., Das, T., Nandy, S., Ghorai, M., Chatterjee Saha, S., Gopalakrishnan, A. V., Kumar, M., Radha, Ghosh, A., Mukerjee, N., Shekhawat, M. S., Pandey, D. K., & Dey, A. (2023). Biotechnological and endophytic-mediated production of centellosides in *Centella asiatica*. *Applied Microbiology and Biotechnology, 107*, 473-489. https://doi.org/10.1007/s00253-022-12316-z

Mishra, N., Jiang, C., Chen, L., Paul, A., Chatterjee, A., & Shen, G. (2023). Achieving abiotic stress tolerance in plants through antioxidative defense mechanisms. *Frontiers in Plant Science, 14*, 1110622. https://doi.org/10.3389/fpls.2023.1110622

Monte, I. (2023). Jasmonates and salicylic acid: Evolution of defense hormones in land plants. *Current Opinion in Plant Biology, 76*, 102470. https://doi.org/10.1016/j.pbi.2023.102470

Moola, A. K., Seenivasan, H. K., Satish, L., Balasubramanian, P., Sathish, S., Indukuri, H., Nandhitha, C. C., Kumar, T. S., & Kumari, B. D. R. (2024). Experimental strategies to enhance in vitro production of plant secondary metabolites. In P. B. Kavi Kishor, T. Pullaiah, Penna Suprasanna, A. Ranga Rao & Anabela Romano (Eds.), *Biotechnological production of bioactive phytochemicals of medicinal value: A comprehensive treatise* (pp. 245-276). Elsevier. https://doi.org/10.1016/B978-0-443-21818-7.00021-6

Mostafa, E. M., Musa, A., Mohammed, H. A., Alzarea, A. I., Abdelgawad, M. A., Al-Sanea, M. M., Ismail, A., Zafar, A., Elmowafy, M., Selim, S., & Khan, R. A. (2023). Phenanthroindolizidine alkaloids secondary metabolites diversity in medicinally viable plants of the genus *Tylophora*. *Plants, 12*(5), 1143. https://doi.org/10.3390/plants12051143

Mubeen, B., Murtaza, S., Yaqoob, S., Aregbe, A. Y., Boasiako, T. A., Xiong, Y., & Ma, Y. (2025). Harnessing plant defense: Elicitors, hormones and immunity-driven production of medicinally valuable secondary metabolites. *South African Journal of Botany, 179*, 280-292. https://doi.org/10.1016/j.sajb.2025.02.018

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum, 15*(3), 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Parihar, S. (2018). *In vitro* biochemical characterization of *Caralluma edulis* (Edgew.) Benth. & Hook. f. and *Caralluma adscendens* (Roxb.) R. Br.: medicinally potent Indian plant species. *Vegetos, 31*(special), 132-136. https://doi.org/10.5958/2229-4473.2018

Parihar, S. (2020). Protein profiling of regenerative and non regenerative callus cultures of *Glossonema varians*: A rare, endemic and edible plant of Indian Thar Desert. *Vegetos, 33*, 385-389. https://doi.org/10.1007/s42535-020-00120-x

Parihar, S. (2023). *In vitro* regeneration of *Caralluma stalagmifera* var. stalagmifera through LCT and ex vitro rooting: a cost effective approach for conservation of succulents. *Vegetos, 36*, 1535-1543. https://doi.org/10.1007/s42535-023-00567-8

Parihar, S., & Dwivedi, N. K. (2019). Comprehensive analysis of liquid and semisolid culture system for in vitro propagation and conservation of *Caralluma edulis*: an appetite suppressant medicinal succulent of the Indian Thar Desert. *Plant Cell Biotechnology and Molecular Biology, 20*(21-22), 1020-1031.

Parihar, S., & Dwivedi, N. K. (2020). A note on an important edible, rare and the famine food plant of Indian Thar Desert: *Glossonema varians* (Stocks) Benth. ex Hook.f. *Genetic Resources and Crop Evolution, 67*, 1929-1934. https://doi.org/10.1007/s10722-020-00995-4

Pütter, J. (1974). Peroxidases. In H. U. Bergmeyer (Ed.), *Methods of Enzymatic Analysis* (2nd Edition, pp. 685-690). Academic Press. https://doi.org/10.1016/B978-0-12-091302-2.50033-5

Qureshi, M. K., Gawroński, P., Munir, S., Jindal, S., & Kerchev, P. (2022). Hydrogen peroxide-induced stress acclimation in plants. *Cellular and Molecular Life Sciences, 79*, 129. https://doi.org/10.1007/s00018-022-04156-x

Rajput, V. D., Harish, Singh, R. K., Verma, K. K., Sharma, L., Quiroz-Figueroa, F. R., Meena, M., Gour, V. S., Minkina, T., Sushkova, S., & Mandzhieva, S. (2021). Recent developments in enzymatic antioxidant defence mechanism in plants with special reference to abiotic stress. *Biology, 10*(4), 267. https://doi.org/10.3390/biology10040267

Rathore, M. S., Paliwal, N., Anand, K. G. V., & Agarwal, P. K. (2014). Somatic embryogenesis and *in vitro* plantlet regeneration in *Salicornia brachiata* Roxb. *Plant Cell, Tissue and Organ Culture (PCTOC), 120*, 355-360. https://doi.org/10.1007/s11240-014-0571-8

Rogers, A., Dietz, K.-J., Gifford, M. L., & Lunn, J. E. (2021). The importance of independent replication of treatments in plant science. *Journal of Experimental Botany, 72*(15), 5270-5274. https://doi.org/10.1093/jxb/erab268

Sakihama, Y., Cohen, M. F., Grace, S. C., & Yamasaki, H. (2002). Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. *Toxicology, 177*(1), 67-80. https://doi.org/10.1016/S0300-483X(02)00196-8

Saleem, M., Fariduddin, Q., & Castroverde, C. D. M. (2021). Salicylic acid: A key regulator of redox signalling and plant immunity. *Plant Physiology and Biochemistry, 168*, 381-397. https://doi.org/10.1016/j.plaphy.2021.10.011

Selwal, N., Supriadi, K., Rahayu, F., Sukmadjaja, D., Khamidah, A., Budiaarto, K., Antarlina, S. S., Tripatmasari, M., & Wani, A. K. (2025). Elicitation strategies for enhanced secondary metabolite synthesis in plant cell cultures and its role in plant defense mechanism. *Plant Gene, 41*, 100485. https://doi.org/10.1016/j.plgene.2024.100485

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology, 299*, 152-178. https://doi.org/10.1016/S0076-6879(99)99017-1

Siva, G., Sivakumar, S., Prem Kumar, G., Vigneswaran, M., Vinoth, S., Muthamil Selvan, A., Parveez Ahamed, A., Manivannan, K., Rajesh Kumar, R., Thajuddin, N., Senthil Kumar, T., & Jayabalan, N. (2015). Optimization of elicitation condition with jasmonic acid, characterization and antimicrobial activity of psoralen from direct regenerated plants of *Psoralea corylifolia* L. *Biocatalysis and Agricultural Biotechnology, 4*(4), 624-631. https://doi.org/10.1016/j.bcab.2015.10.012

Smirnoff, N., & Arnaud, D. (2019). Hydrogen peroxide metabolism and functions in plants. *New Phytologist, 221*(3), 1197-1214. https://doi.org/10.1111/nph.15488

Sohn, S.-I., Pandian, S., Rakkammal, K., Largia, M. J. V., Thamilarasan, S. K., Balaji, S., Zoclanclounon, Y. A. B., Shilpha, J., & Ramesh, M. (2022). Jasmonates in plant growth and development and elicitation of secondary metabolites: An updated overview. *Frontiers in Plant Science, 13*, 942789. https://doi.org/10.3389/fpls.2022.942789

Ståhle, L., & Wold, S. (1989). Analysis of variance (ANOVA). *Chemometrics and Intelligent Laboratory Systems, 6*(4), 259-272. https://doi.org/10.1016/0169-7439(89)80095-4

Thomas, T. D., & Philip, B. (2005). Thidiazuron-induced high-frequency shoot organogenesis from leaf-derived callus of ia medicinal climber, *Tylophora Indica* (Burm. F.) merrill.

In vitro Cellular & Developmental Biology-Plant, 41, 124-128. https://doi.org/10.1079/IVP2004575

Tian, H., Xu, L., Li, X., & Zhang, Y. (2025). Salicylic acid: The roles in plant immunity and crosstalk with other hormones. *Journal of Integrative Plant Biology, 67*(3), 773-785. https://doi.org/10.1111/jipb.13820

Wang, P., Liu, W.-C., Han, C., Wang, S., Bai, M.-Y., & Song, C.-P. (2024). Reactive oxygen species: Multidimensional regulators of plant adaptation to abiotic stress and development. *Journal of Integrative Plant Biology, 66*(3), 330-367. https://doi.org/10.1111/jipb.13601

Yemm, E. W., & Willis, A. (1954). The estimation of carbohydrates in plant extracts by anthrone. *Biochemical Journal, 57*(3), 508-514. https://doi.org/10.1042/bj0570508

Zarbakhsh, S., Shahsavar, A. R., Afaghi, A., & Hasanuzzaman, M. (2024). Predicting and optimizing reactive oxygen species metabolism in Punica granatum L. through machine learning: role of exogenous GABA on antioxidant enzyme activity under drought and salinity stress. *BMC Plant Biology, 24*, 65. https://doi.org/10.1186/s12870-024-04740-2