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In Vitro influence of drought on some biochemical, metabolic, and physiological reactions in callus induced from *Vitex agnus-castus*

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In Vitro influence of drought on some biochemical, metabolic, and physiological reactions in callus induced from *Vitex agnus-castus*

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Drought, one of the most largely experienced stresses causing dehydration of cells, affects the acres of agricultural lands and individual production, especially in agriculture and forest land. Plants develop resistance mechanisms to cope with water scarcity. However, rising reactive oxygen species (ROS) production coincides with rising stress-specific secondary metabolite accumulation in response to abiotic stimuli. Upon abiotic stress, the plant elite produces reactive oxygen, which triggers the transcription of defense gene processes, bringing about an improved production of transcripts for some transcription reducers and protective genes as a part of the primary defense system. During these events, the primary abundant antioxidants (enzymes that include CAT, SOD, APX, DHAR, and GR) raise the activity of the defense processes to quickly trigger the plant's secondary metabolic pathways. This study focuses on the positive effects of inducing drought stress using PEG on *Vitex agnus-castus* callus culture. Nodule shoot explants were used to establish callus tissue, using 2,4-D or NAA in addition to BA for inducing callus. The highest callus weight was seen at 3 mg/1 2,4-D and 0.5 mg/1 BA. The production of secondary metabo1ites in response to stress was achieved with MS medium containing 4% polyethylene glycol (PEG 6000), showing improvements in growth and pharmaceutical compounds. Maximum rutin and chlorogenic acid contents were obtained at 2% and 3% PEG concentrations. Drought stress negatively affected various traits, but tolerance to PEG improved, with enhanced PAL and CAT activity regulating ROS levels. This study suggests that bioreactors can increase under stress conditions the content of essential compounds in the pharmaceutical market.

Keywords: Callus Induction; Successive drought stress; enzyme activity; Climate change

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INTRODUCTION

Drought is the most important environmental stress factor that reduces plant growth, metabolism, and productivity. It affects antioxidant enzyme activity and ROS-antioxidant balance in the organism. Drought also increases hexose-level sugar production and antioxidant enzyme activity, affecting carbohydrate metabolism (Laxa et al., 2019; Hassan et al., 2021; Loutfy et al., 2022; Zaki et al., 2023). Understanding the regulation of these pathways is important under drought-induced oxidative stress. Deficit stress negatively impacts plant processes and agricultural production, leading to excessive ROS formation. The accumulation of ROS further affects plants in dry conditions. (Gurrieri *et al*., 2020)

Drought is the most significant factor for the wild plants' survival in their habitats. Changes in gene expression result in a series of reactions consisting of biochemical, metabolic, and physiological processes. Plants, among which biotechnological methods are studied for the culture technique, are mostly aromatic, dye, and pharmaceutical plants. These species used as culture materials by plant producers are becoming extinct rapidly in nature. The studies conducted demonstrate that bioactive compound amounts have changed as a response to plant stress. Abid *et al*., 2018.

Flavonoids are bioactive compounds found in vegetables, fruit, and herbs. Chlorogenic acid, a common polyphenol in the human diet, has antioxidant properties and protects against oxidative stress and brain diseases. Flavonoids have a common structure and are known for their health benefits, including vasoprotective and antiatherogenic effects. They also possess antioxidant and anti-inflammatory properties and play a role in preventing chronic diseases. The main identified compounds include quercetin, kaempferol, rutin, and catechins. (Han *et al*., 2007; Shashank & Pandey 2013). The usage of medicinal plant material accumulation should be limited/enriched based on the active ingredient amounts affected by harvest conditions. This can be achieved through studies on metabolite biosynthesis and accumulation *in vitro* culture systems under stressful conditions.

Vitex agnus-castus L. is a lesser tree from the Lamiaceae family. Its habitat is found in tropical and subtropical areas including India, China, and Sri Lanka. It is known for its medicinal properties in treating fibroid cystosis and female reproductive disorders Kamal *et al*., 2022. Adding PEG as a stress factor can enhance secondary metabolite production in plant cell cultures. This study aimed to evaluate the influence of osmotic stress on *Vitex agnus* callus. Water scarcity is a global challenge and using plants with medicinal properties can be an effective

solution. Drought-exposed plants show adaptations including molecular and biochemical changes.

MATERIALS AND METHODS

Plant materials

The plant *Vitex agnus-castus* was collected from King Abdul Aziz University's Botanical Garden during March and April (2023).

Plant sterilization: Apical and nodule segments of 0.5-1cm in size were taken from a healthy mature *Vitex agnus-castus*. Segments were washed thoroughly for thirty minutes with continuous tap water followed by ten minutes of sterile distilled water containing two to three drops of Tween-20. Then the segments were rinsed three times with sterile water after being disinfected for one minute with 70% ethanol, followed by an immersion with stirring for 30 minutes in a 5 % sodium hypochlorite containing 3% (w/v) Tween 20.

Callus culture establishment and PEG treatment

Finally, the nodule segments were transversely divided into four or five segments (l.0 cm × l.0 cm) based on the segment size. The explants were cultured on Murashige and Skoog (1962) (MS) media complemented with different auxins concentrations in combination with 0.5 mg/l BA, as described in Table 1, in addition to 88 µM sucrose and 2.0 g/l of gelrite (Duchefa Biochemie, The Netherlands). The pH was adjusted to 5.7–5.8 with 0.l N sodium hydroxide (NaOH) and 0. l N HCl (hydrochloric acid) before autoclaving at l2l °C for 20 min. All cultures were incubated in darkness at 25 °C. After that, callus induction percentage, fresh weight of callus, and dry weight of callus were evaluated using a randomized design method.

After 28 days, homogenous calli (3 g) grown on a medium with 3 mg/l 2,4-D supplemented with 0.5 mg/l BA were transferred into 40 ml of prepared MS media by adding different concentrations of PEG 6000 (0%, 0.5%, 1%, 2%, and 4% w/v) supplemented with 1 mg/l 2,4-D and 0.1 mg/l BA. The callus medium without any treatment was used as a control in baby food jars. All jars were incubated in darkness at 25 °C. Every baby food jar was an experimental unit, each treatment consisted of 40-50 polypropylene jars (5 cm high) with 1 explant per jar. Each treatment was tested in triplicate. After 21 days, the calli were harvested and examined.

Determination of biochemical factors

Determination of the total phenolic compounds

The amount of phenol in *V. agnus-castus* extracts was measured by applying the Folin-Ciocalteu method, detailed in Singleton *et al*., 1965. In the short term, 0.1 ml of each trial (extract=10 mg/mL) was thoroughly mixed with 2.8 ml of ddH_2O and 2 ml of Na_2CO_3 (2% w/v).

Stress biomarker estimation

Measurement of lipid peroxidation and hydrogen peroxide content

After extracting the callus samples with trichloroacetic acid (TCA), the samples were centrifuged at 11,500 × g. The lipid peroxidation level was measured using spectrophotometric absorbance at 532 and 600 nm by mixing the supernatant with thiobarbituric acid (TBA) reagent (Heath and Packer, 1968). Adhering to the (Yu *et al*., 2005) method. When callus specimens were homogenized with TCA and centrifuged at 11,500 × g in 2003, the H_2O_2 content was measured. Supernatants were combined with potassium iodide (KI) and potassium-phosphate (K-P) buffer (pH 7.0), and the content of H_2O_2 was assessed by measuring the optical absorbance at 390 nm.

Proline content

A homogenate of 2 g of fresh callus and 10 ml of 5% (v/v) acetic acid was diluted to 50 mL using distilled water to measure the proline content. As previously stated, (Troll and Lindsley 1955), the proline concentration was ascertained.

Determination of metabolites and enzymes of the antioxidant system

The extraction of antioxidant enzymes was performed using homogenized fresh calli (0.05 g) in 50 mM K-phosphate buffer (pH 7) supplemented with l mM EDTA and l% polyvinylpyrrolidone (PVP), following the Gapińska *et al*., 2008 protocol.

Phenylalanine ammonialyase (PAL) activity

The PAL assessments were performed using the reaction solution, which included 1000 µl of the extraction buffer, 500 µl of 10 mM L-phenylalanine, 400 µl of double-distilled water, and 100 µl of enzyme solution. Following a 60-minute incubation period at 37 °C, 500 µl of HCl (6 M) was added to the mixtures to terminate the reaction. Next, the mixtures were measured with the absorbance at 290 nm (D'Cunha *et al*., l996).

Catalase (CAT) activity

In Catalase (CAT) Assay: The decrease in H_2O_2 concentration was measured by the method described by Li with some modifications (Li and Schellhorn, 2007). First, the callus cells were homogenized with 3 mL of 50 mM phosphate buffer (pH 7.0) and then centrifuged at 15,000× g for 20 min. Then, the mixture containing 3 mL of 0.1 M phosphate buffer (pH 7.0) and 1 mL of enzyme extract was prepared, followed by the addition of 2 mL of 0.19 mM H_2O_2. Then, the decrease in H_2O_2 was monitored at 240 nm, and a standard curve prepared with H_2O_2 was used as the reference. The CAT activity was expressed as the change in OD (Choudhary *et al*., 2012).

Analysis of chlorogenic acid and rutin by HPLC

HPLC was used to quantitatively analyze the ethanolic extracts from three biological replicates for each experimental collection and standard (rutin and chlorogenic acid). The mobile phase for HPLC was prepared using 0.2% formic acid in water (solvent A) and 100% acetonitrile (solvent B). A multistep gradient program was used for better resolution of chlorogenic acid and rutin. Column temperature set at 27 °C with a flow rate of 1 ml/min. The injection volume was 20 µl with a total running time of 55 min. Optimization of column and temperature is crucial for retention time and resolution. Detection was done using Agilent 1100 series diode array UV Detector DAD at 330 nm. The HPLC system was equipped with an Agilent 1100 series controller, Model G1322A degasser, Model G1312A binary pump, autosampler, and Model G1315A diode array detector. The system was controlled by a computer and a complete graphic display of the chromatograms was obtained by using the software ChemStation, which was also used for data handling and calculations. Agilent columns (NuSil ODS, 4.6 mm × 25 cm, 5 µm) were used for separation. Determinations were carried out at 322 nm for chlorogenic acid and 330 nm for rutin. The separation was carried out by reversed-phase mode (RP). All samples were analyzed in triplicate (Scarano *et al*., 2020). The standard curve for the corresponding standard in each PEG concentration was used to calculate the concentrations of each compound in mg/kg.

Statistical Analysis

The findings of three separate analyses, each involving three technical reads (n = 3), are displayed as mean values ± standard error (SE) for the antioxidant activity investigations. Microsoft Excel 2013 software was used for the raw data analysis process. With comparison within columns according to Tukey's Honestly Significant Difference Test., which was done at $p<0.05$, the significance of the difference between means ± SE (standard error) values was acquired.

RESULTS

Evaluation of callus growth and development
Influence of auxins on the induction of callus

In this study, different types of auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthaleneacetic acid (NAA), with various concentrations, were used for inducing callus in *V. agnus-castus*. After culture initiation, segment explants initiated swelling up, and callus formation was observed one month later. The nodule segment explants cultured on media containing 2,4-D formed callus irrespective of the auxin concentration used. After 28 days, callus induction percentage, fresh weight, and dry weight of callus in the nodule segment explants were measured. Table 1 and Figure 2 illustrate the effects of various combinations of plant growth regulators. While no callus developed on growth regulator-free MS media, callus tissues were successfully generated in explants by all hormonal combinations and combinations of 2, 4-D, and BA (Table l). Thus, callus induction from *V. agnus-castus* nodule segment explants can be concluded. Hormonal balance is the primary factor affecting *V. agnus-castus*. The maximum percentage of callus induction was noted in explants on all NAA and 0.5 mg/l BA combinations and in explants on 3 mg/l 2,4-D and 0.5 mg/l BA (Figure 1). On hormone-free MS media (control), no callus induction was seen. The highest callus fresh weight was recorded with 3mg/l 2,4-D and 0.5 mg/l BA from nodule segments and 2 mg/l NAA and 0.5 mg/l BA in segment explants (Table 1). In addition, the calli produced were white and transparent (Figure 1).

Total phenol content and nonenzymatic antioxidant activity

PEG is added to *V. agnus-castus* callus at 0.5%, 1%, 2% and 4% concentrations. The total phenol content and non-enzymatic antioxidant activity were determined by *V. agnus-castus*. With *V. agnus-castus*, the calli's total phenol content was noticeably lower *V. agnus-castus* callus culture than with the control; nevertheless, there was no discernible difference in the total phenol content between the

Table 1. Effect of different auxins on callus induction of nodule explants of *Vitex agnus-castus*.

Auxin Type	Concentration mg/l (µM)	Fresh Weight (g/explants)	Dry Weight (g/explants)
	0	1.02±0.02d	0.098±0.02cd
	1(4.5)	3.23±0.08bc	0.31±0.02c
2,4-D	2(9)	4.56±0.1b	0.42±0.06ab
	3(13.57)	6.3±0.04a	0.53±0.09a
	4(18.09)	5.6±1.08ab	0.54±0.07a
	0	1.02±0.02	0.098±0.02d
	1(5.37)	5.45±0.52ab	0.72±0.087b
NAA	2(10.74)	6.77±0.4a	0.52±0.01a
	3(16.1)	5.34±0.42ab	0.62±0.74b
	4(21.48)	4.67±0.31b	0.5±0.041bc

Means ±SE within columns followed by the same letter is not significantly different according to Tukey's Honestly Significant Difference Test. at $P \leq 0.05$.

Figure 1. Callus induced on Ms medium supplemented with 3 mg/l 2, 4-D + 0.5 mg/l BA) after 8 weeks from nodule explants of *Vitex agnus-castus.*

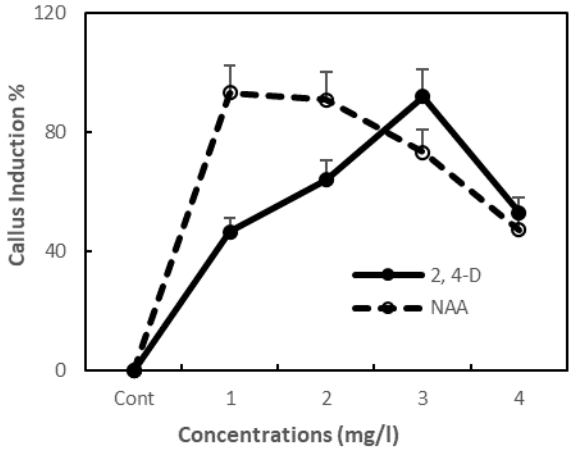

Figure 2. Effect of adding different concentrations of plant growth hormones (2, 4-D, and NAA + 0.5 mg/l BA) on callus induction % after 8 weeks derived from nodule explants of *Vitex agnus-castus*. Values are the means (±S.E.) of five replicates followed by the same letter are not significantly different according to Tukey's Honestly Significant Difference Test. at $P \leq 0.05$.

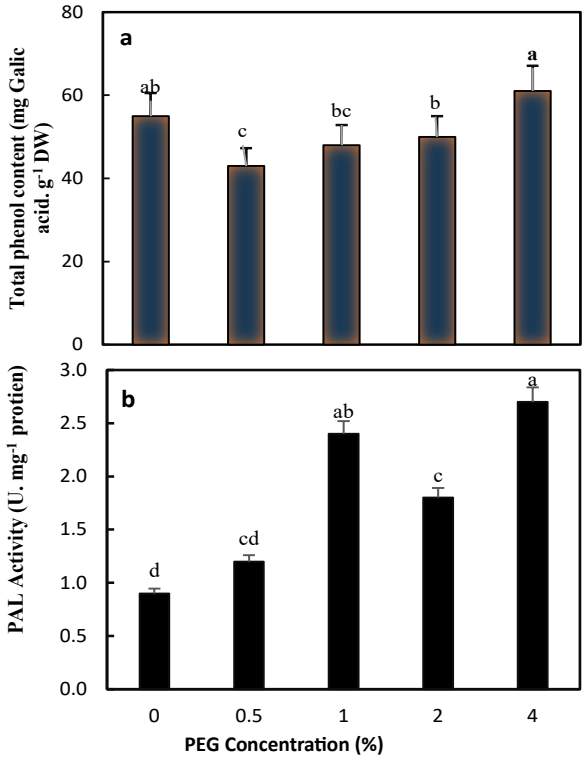

Figure 3. Total phenol content (a) and non-enzymatic antioxidants PAL (b) activity under PEG treatment in *Vitex agnus-castus* calli after 21 days. Values represent the mean ± S.E followed by the same letter are not significantly different according to Tukey's Honestly Significant Difference Test. at $P \leq 0.05$.

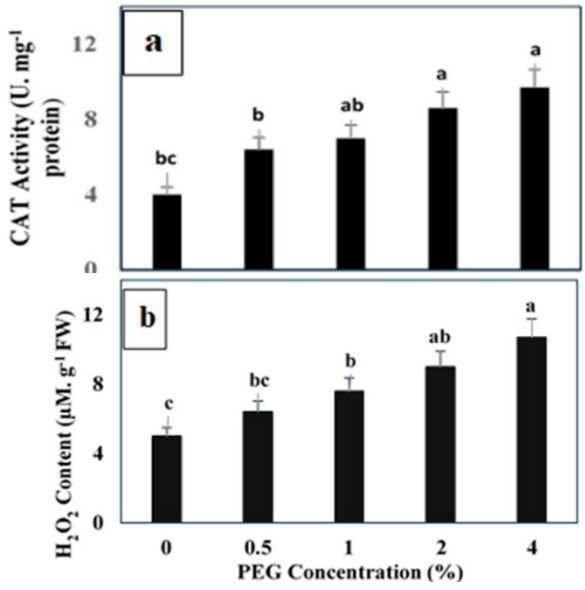

Figure 4. Content and antioxidant enzymes CAT (a) and H_2O_2 content (b) in activity under PEG treatment in *Vitex agnus-castus* calli after 21 days. According to the Duncan test, values represent mean ± S.E followed by the same letter are not significantly different according to Tukey's Honestly Significant Difference Test. at $P \leq 0.05$.

two groups at 2 and 4 % PEG concentrations. The PEG at a concentration of 0.05 % reduced the total phenol content by 5.9% when compared to the control (Figure 3a). The most significant enzyme in the phenylpropanoid pathway and the biosynthesis of phenolic compounds in *V. agnus-castus* is phenylalanine ammonia-lyase (PAL). *V. agnus-castus* calli was also examined about different PEG concentrations (Figure 3b).

H_2O_2 content

The H_2O_2 content in the *V. agnus-castus* calli was increased by increasing the PEG concentration, but only PEG concentrations of 2%, 3% and 4% significantly increased H_2O_2 content when compared to the control. At 4% of PEG concentration, the H_2O_2 content was higher than at any other PEG concentration, reaching 57.50% compared to the control (Figure 4b).

Antioxidant enzyme activity

A 4 % PEG treatment resulted in the highest level of CAT enzyme activity, which increased as PEG concentration in the culture medium increased. There was no significant difference in CAT activity between the two treatments involving 3 and 4% PEG concentration (Figure 4a).

Proline content

Significant increases in proline content during drought were found in the 1% PEG (1.7 times) and 2% PEG (1.8 times) treatments, respectively, in which these increases were significant compared with 0.33-fold (Figure 5).

Analysis of chlorogenic acid and rutin by HPLC

The polyphenol contents of plants and callus cultures were investigated using high-performance liquid chromatography (HPLC). The HPLC fingerprints of phenolic acids profiles of extracts from the callus culture of *V. agnus-castus* showed significant differences in the distribution of investigated compounds. Twenty-one days after being exposed to drought stress, the samples were taken. To find out how phenolic and flavonoid compounds, such as rutin and chlorogenic acid, changed under stressful circumstances, high-performance liquid chromatography (HPLC) analysis was used. When the concentration of PEG increased, so did the concentration of most of the compounds. While flavonoids sharply dropped on day 21 of stress, most phenolic acids kept building up as stress concentrations increased. At 4% PEG,

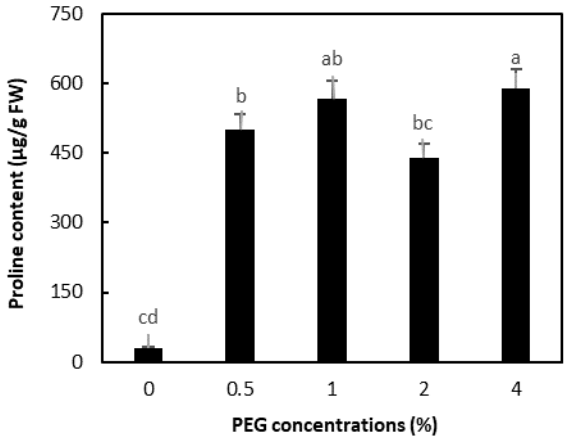

Figure 5. Proline content under PEG treatment in *Vitex agnus-castus* fresh weight calli after 21 days. Values represent mean ± S.E followed by the similar letter are not significantly different according to Tukey's Honestly Significant Difference Test. at P ≤ 0.05.

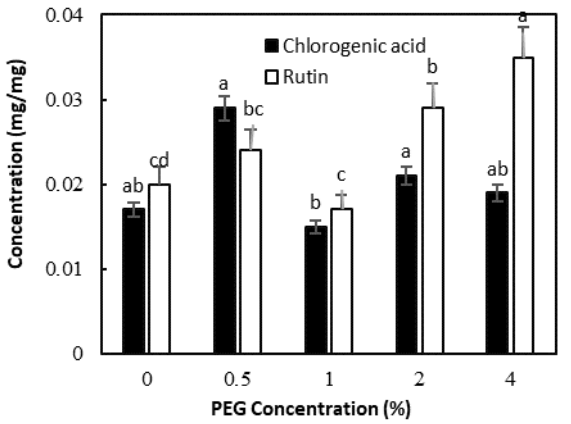

Figure 6. Comparison of chlorogenic acid and rutin as detected by HPLC activity under PEG treatment in *Vitex agnus-castus* fresh weight calli after 21 days. Values representing mean ± S.E followed by a similar letter are not significantly different according to Tukey's Honestly Significant Difference Test. at *P* ≤ 0.05.

chlorogenic acid (3.77 mg/100 g dry weight [DW]) was the most prevalent phenolic acid; at 0.5 %, it dropped, and at 3 %, it increased (Figure 6).

DISCUSSION

Biotechnological methods, especially callus culture, are essential for substitutes for plant-based secondary metabolite production (Karuppusamy 2009). Induction, differentiation, and cell development are the three stages of callus formation (George, 2008). The application of callus in the business sector and for fundamental research is common (Ikeuchi *et al*., 2013). Plant growth regulators are the most crucial elements influencing cell division, growth, and metabolite formation. Auxin is required to induce callus and form explants.

To start cell division, elongation, development, and growth, auxin is very effective (Gaspar *et al*., 1996; George 2008). Following the dedifferentiation stage, cells divide once more in response to auxin (George 2008). Comparing 2,4-D to other auxins, either alone or in combination, the application of 2,4-D for callus induction was more common among different plants. This experiment aimed to find the best kind and concentration of auxins to produce callus from *V. agnus-castus*. Despite displaying a 93.3% response rate on medium enhanced with 2 mg/l NAA, the callus on the explants was a mixed type, crispy, and nodular, and it turned brown in the following subcultures. Reddy *et al*., 2001 published identical observations as stated in *P. barbarus*. In the same way, *Aloe vera* has been shown to possess the capacity of 2,4-D to augment callus replacement. (Rathore 2011).

It is generally recognized that osmotic stress induces reactive oxygen species (ROS), which are involved in different signaling processes. While levels of ROS increase significantly under water stress, this signal can sometimes induce adaptive responses that may be realized more quickly or efficiently than traditional chemical signals. Furthermore, the level of oxidative stress is less severe when trehalose is accumulated normally in *Arabidopsis thaliana* under osmotic stress. Comparative examination of transcriptomes in plants with two different growth conditions suggested that trehalose does not inhibit 1-aminocyclopropane-1-carboxylate expression induced by sodium chloride, which may be involved in ROS production by influencing the level of nitric oxide and cytochrome c oxidase and regulated antioxidase gene expression. It is also noted that increased levels of ethylene might increase the level of excessive ROS by reducing antioxidase gene expression. Additionally, ROS is not the most direct signal for abscisic acid synthesis, even though ROS transcriptionally regulates enzymes related to abscisic acid synthesis, which may function as activators between cis-elements and cis-acting elements of the gene in the abscisic acid signal transduction pathway (Devireddy *et al*., 2021).

The two defense mechanisms plants possess are increased activity of antioxidant enzymes to eliminate free radicals and lower oxidative stress (Mishra *et al*., 2023). Higher PEG concentration in the medium in the study above increased the contents of H_2O_2 in the treated calli, indicating the presence of oxidative stress brought on by water shortage stresses in the calli. Among the many defense strategies plants have been a rise in antioxidant enzymes activity, which eliminate free radicals and lessens oxidative stress. (Blokhina *et al*., 2003). Reducing the damaging effects of oxidative stress was achieved by increasing the activity of CAT enzymes, especially at high PEG concentrations. This strategy is known as antioxidant defense. Antioxidant response to free radicals in wheat has been documented to increase total phenol, flavonoids, and PAL levels under water deficit conditions (Gill and Tuteja 2010). Miliauskas *et al*. 2004 state that phenolic compounds have antioxidative properties and work by snaring free radicals, among other mechanisms. In addition to being a crucial enzyme in synthesizing phenolic compounds, PAL is a biomarker of a plant's ability to defend itself against environmental stressors and an indicator of ecological susceptibility (Solecka, 1997; Isah, 2019) due to the high concentration of phenolic compounds in the *V. agnus-castus* cells. When most phenolic compounds build up in the culture medium, *V. agnus-castus* calli becomes extremely sensitive (Berrani *et al*., 2021).

Polyphenols have garnered attention due to epidemiological studies demonstrating links between eating high in polyphenols and preventing diseases like osteoporosis, cancer, and coronary heart disease. Owing to their diverse biological actions, including metal chelation, scavenging of free radicals, alterations in signal transduction pathways, and modulation of enzymatic activity, dietary polyphenols are believed to be advantageous for human health (Rathod *et al*., 2023). Due to their potential interactions with numerous enzymes and antioxidant qualities, phenolic compounds have been extensively demonstrated to protect against specific diseases. (Dai and Mumper 2010)

Proline content increased significantly in the 1% PEG (1.7 times) and 2% PEG (1.8 times) treatments during the drought, respectively. These increases were significant when compared to a 0.33-fold increase (Figure 5). In plants under drought stress, proline functions as a major osmoregulatory, scavenges ROS, and acts as a cellular redox buffer (Dawood *et al*., 2019). Proline helps conform protein structure, scavenges reactive oxygen species, preserves thylakoid membranes, and stabilizes membrane structure in higher plants (Hayat *et al*., 2012). Plants' antioxidant defenses against environmental stressors are strengthened by proline in conjunction with catalase, ascorbate peroxidase, and superoxide dismutase (Slabbert and Kruger, 2014). Proline also

lessens oxidative damage when under stress and serves as a compound for the storage of nitrogen and carbon. Additionally, it is essential for mitochondrial regulation and signal transduction (Ozturk *et al*., 2021). It has been reported that phenols, glycine betaine, and proline contents in carrot plant leaves increase underwater deficit (Razzaq *et al*., 2017). The formation of proline in plants has been attributed to the breakdown of arginine and increased Ornithine δ-aminotransferase (OAT) activity (Verslues and Sharma, 2010). The increase in activities of biosynthesis enzymes such as P5CS in *Vitex* callus under PEG stress could be possible, leading to changes in proline biosynthesis.

This study found that increased PEG levels in the culture medium significantly increased the activity of the PAL enzyme (Figure 3b), which is responsible for the biosynthesis of phenolic compounds, even though the total phenol content was lower in the calli treated with low to moderate PEG concentrations than in the control. PEG is composed of synthetic polymers. The concentration of most polyphenols started to rise at 2%, most of which were noticeably higher than 2 % (Figure 3a). Hodaei et al., 2018 showed that *Chrysanthemum morifolium* can have a higher concentration of chlorogenic acid when under drought stress. This may be explained by lignin precursors that accumulate under drought stress, including ferulic, caffeic, and chlorogenic acids (Hodaei et al., 2018).

CONCLUSION

This study analyzed the impact of auxin concentrations on callus culture enhancement using *Vitex agnus-castus* nodule segment explants. The most effective combination for callus production was MS media with 3mg/l 2,4-D + 0.5 mg/l BA. Drought stress negatively affected various traits, but tolerance to PEG improved, with enhanced PAL and CAT activity regulating ROS levels. The study highlights the protective role of bioactive compounds in preventing macromolecular damage. These findings will aid in secondary compound synthesis and improve plant resistance to drought, potentially reducing the attrition of wild types through tissue culture techniques.

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CONFLICT OF INTEREST

The author pronounces that there is no conflict of interest.

DATA AVAILABILITY

Data will be made available on request.

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