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Agni Patel
Department of Botany, Ramniranjan Jhunjhunwala (Autonomous) College, Ghatkopar (West), Mumbai, Maharashtra, India

Anil Avhad
Department of Botany, Ramniranjan Jhunjhunwala (Autonomous) College, Ghatkopar (West), Mumbai, Maharashtra, India

Corresponding Author:
Agni Patel
Department of Botany, Ramniranjan Jhunjhunwala (Autonomous) College, Ghatkopar (West), Mumbai, Maharashtra, India

Browning suppression and yield improvement in *Solanum diphyllum* L. callus cultures

Agni Patel and Anil Avhad

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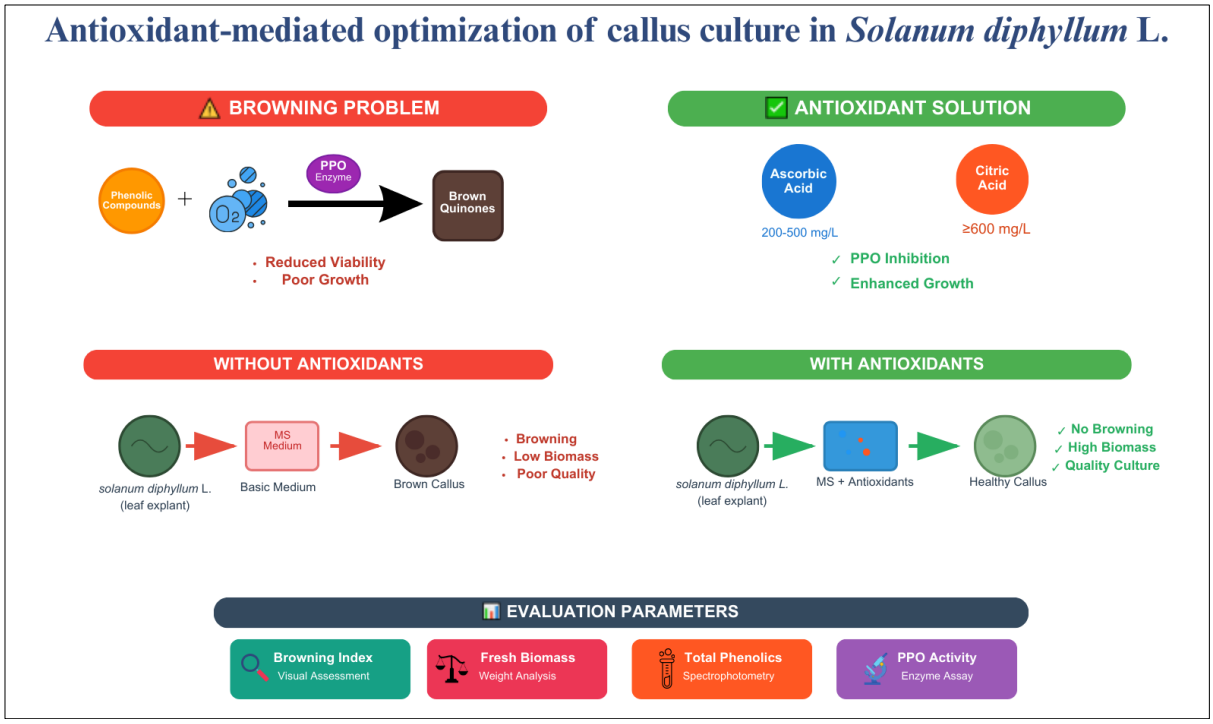
Abstract
PPO-mediated phenolic compound oxidation causes callus browning, a major plant tissue culture limitation. The medicinal shrub *Solanum diphyllum* L. has little tissue culture research, and browning destroys its culture. Ascorbic acid and citric acid (0-600 mg/L) were tested for 30 days to reduce oxidative discoloration and improve biomass, TPC, and PPO activity in *Solanum diphyllum* L. callus. In leaf explants on MS medium with antioxidants, both acids dose-dependently reduced browning and PPO activity, with citric acid (600 mg/L) inhibiting PPO the most. At 200 mg/L ascorbic acid, biomass peaked, while TPC peaked at 500 mg/L and 600 mg/L citric acid. Due to phytotoxicity, high antioxidant levels reduced growth but avoided browning. Citric acid inhibited PPO with moderate biomass gains, while ascorbic acid promoted phenolic biosynthesis. These results show that antioxidant supplementation reduces oxidative stress, improves callus viability, and increases metabolite accumulation. This is the first optimized antioxidant-assisted *Solanum diphyllum* L. callus culture protocol, applicable to other phenolic-rich medicinal plants.

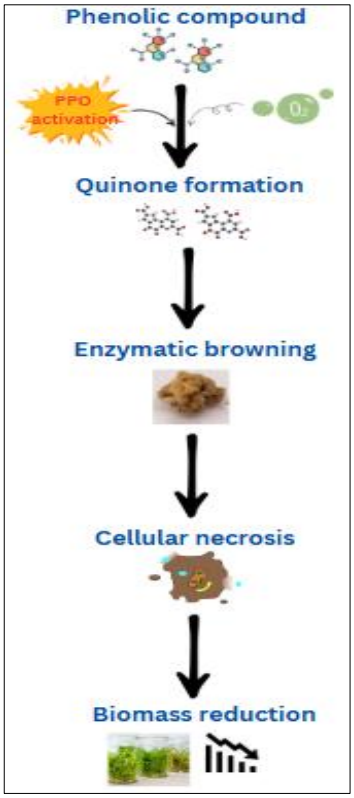
Keywords: *Solanum diphyllum* L., tissue culture, callus browning, antioxidants, PPO activity, phenolic content, biomass

Introduction
Solanum diphyllum L., a member of the Solanaceae family, is a medicinal shrub valued for its pharmacological properties, including antioxidant, antimicrobial, anti-inflammatory, and anticancer activities (SJ Hossain *et.al*., 2009 & El-Sayed *et.al*., 2009) [6, 4]. In India, *Solanum diphyllum* L. is traditionally used by the Kanikkar tribe to treat intestinal worms, while in Uttar Pradesh, its fruits serve as a tonic, laxative, appetite stimulant, and remedy for asthma, skin diseases, and ringworm, with dried seeds fried in oil consumed as a vermifuge., the plant has gained attention due to its rich phytochemical profile comprising alkaloids, flavonoids, and steroidal compounds (P Singh *et.al*., 2019) [13].

Its natural propagation is limited by low seed viability and slow growth, posing challenges for conservation and commercial use. In vitro culture offers an efficient alternative for mass propagation and metabolite production. The production of secondary metabolites, biomass accumulation, and in vitro propagation are all seriously hampered by the browning phenomenon in plant tissue cultures, especially in callus cultures. The main cause of this discolouration is the enzymatic oxidation of phenolic compounds, which is facilitated by the copper-containing enzyme PPO, which is found in many plants. PPO causes tissue browning and cellular necrosis by catalysing the conversion of phenolics to quinones, which then polymerise to form brown pigments (N Permadi *et.al*., 2024) [10]. The viability and regenerative potential of callus cultures are severely compromised by this oxidative browning, which reduces their usefulness for secondary metabolite biosynthesis and biotechnological applications (KD Gandhi *et.al*., 2018) [5].

Graphical abstract





While several studies have explored antioxidant-based browning control in other plant species, no published work has systematically evaluated such strategies in *Solanum diphyllum* L. Moreover, there has been no direct comparison of citric acid and ascorbic acid in relation to both browning suppression and enhancement of secondary metabolite production in this species. Addressing this gap is important, as *Solanum diphyllum* L. is a phenolic-rich medicinal plant, and optimizing its callus culture could not only improve propagation and conservation efforts but also serve as a transferable protocol for other medicinal species prone to oxidative browning. Extensive research has been conducted on antioxidants such as citric and ascorbic acids as possible agents to scavenge ROS and so reduce PPO activity, so preventing browning. ROS accumulation is a hallmark of plant stress responses, including those induced by in vitro culture conditions, which can exacerbate tissue browning and phenolic oxidation. Antioxidants are hypothesised to protect cellular integrity and increase biomass generation in callus cultures by neutralising ROS (MR Sahoo *et.al*., 2020) [11].

In callus cultures, phenolic compounds make up a significant class of secondary metabolites. They are crucial for plant defence and stress tolerance as well as PPO substrates. PPO activity and TPC are closely related because higher PPO activity usually corresponds to more phenolic oxidation and browning. Furthermore, antioxidants can enhance the growth and viability of callus cultures under stress conditions, contributing to higher biomass yields (Z Chen *et.al*., 2016) [3]. For species like *Solanum diphyllum* L., which is prized for its pharmacological and medicinal qualities, it is therefore essential to comprehend how phenolic metabolism, PPO activity, and antioxidant treatments interact to optimise callus culture procedures.

While browning control has been studied in many species, little is known about how it is biochemically regulated in medicinal plants such as *Solanum diphyllum* L. Investigating how antioxidants affect phenolic metabolism and stress-related pathways in in vitro culture can provide useful enhancements to tissue culture procedures. Furthermore, these discoveries may facilitate the development of pharmacologically active secondary metabolites, improved regeneration systems, and conservation strategies all of which are essential components of applied plant biotechnology (HN Murthy *et.al*., 2014) [9].

This study aims to evaluate the effects of ascorbic acid and citric acid (0-600 mg/L) on PPO activity, TPC, browning intensity, and biomass accumulation in callus cultures of *Solanum diphyllum* L. By integrating biochemical and

physiological analyses, the research seeks to elucidate mechanisms of browning inhibition and propose effective strategies to enhance callus viability and secondary metabolite yield in vitro (X Wen *et.al*., 2025) [15].

To date, no comprehensive study has reported the optimization of antioxidant-assisted in vitro callus culture in *Solanum diphyllum* L. This study is the first to investigate the effects of ascorbic and citric acid on oxidative browning, phenolic content, and biomass accumulation in this species, providing a novel protocol for enhancing callus viability and biochemical productivity. This approach may serve as a transferable model for tissue culture of medicinal plants.

Materials and Methods

Tissue culture and preservation

Seeds of *Solanum diphyllum* L. were surface sterilized sequentially using 0.1% Bavistin and 1% Basilicide for 10 minutes, followed by immersion in 0.1% mercuric chloride ($HgCl_2$) for 5 minutes and 70% ethanol for 30 seconds. Post-sterilization, seeds were thoroughly rinsed with sterile distilled water and inoculated onto MS basal medium devoid of PGRs. Upon germination, plantlets were established, and healthy leaves were excised and used as explants. Leaf explants were cultured on MS medium supplemented with 2, 4-dichlorophenoxyacetic acid (2, 4-D), 3% (w/v) sucrose, a two-fold concentration of standard MS vitamins, and selected antioxidants tailored to the experimental requirements. The pH of the medium was adjusted to 5.7 by using NaOH and HCl if needed, prior to autoclaving. Each leaf explant was inoculated individually into separate test tubes, resulting in a total of 320 cultured tubes. Cultures were incubated under standard controlled conditions, and callus formation was observed within 15-20 days of inoculation (Kumar *et.al*., 2022 & A Iftikhar *et.al*., 2015) [8, 7].

Experiment Design for Ascorbic Acid and Citric Acid

Two experiments were designed using a completely randomized design (CRD), consisting of 8 treatments, each replicated ten times (i.e., ten test tubes containing one callus each). The treatments involved the addition of ascorbic acid (ranging from 0 to 600 mg/L) or citric acid (also ranging from 0 to 600 mg/L) to the callus induction media. The cultures were maintained for a duration of 30 days under the previously described physical conditions, specifically at a temperature of 20˚ to 22 ˚C and a pH of 5.7 (G Amente et.al., 2021) [2]. These concentration ranges were selected based on preliminary screening experiments in laboratory and supported by reports in other *Solanum* species and medicinal plants, where antioxidant levels between 50-600 mg/L have been effective for reducing browning and improving callus viability (Murthy *et al*., 2014; Kumar *et al*., 2022) [9, 8]. This ensured that our design captured both low and potentially inhibitory high concentrations for comparative evaluation.

PPO extraction and activity assay

Thirty days after subculturing the callus, samples of one gram of fresh callus (comprising five mixed calli from each test tube) were extracted using 1 ml of extraction buffer, which consisted of phosphate buffer (Na_2HPO_4 and NaH_2PO_4) at 100 mM, pH 7.2, 0.1% (w/v) SDS, and 3 mM ascorbic acid, following the method described by Tang and Newton (2004) [14]. The samples were then subjected to sonication at 4 ˚C for 15 minutes. After sonication, the mixture was centrifuged at 15, 000×g for 20 minutes at 4 ˚C, and the supernatants were collected for the PPO enzyme activity assay. For the assay, 300 µl of the callus extracts were mixed with 300 µl of phosphate buffer (pH 7.4) and 400 µl of an ethanolic solution of 2, 6-dimethoxyphenol (0.5 mM). The absorbance of the mixture was measured at 468 nm using a UV spectrophotometer.

Assay and extraction of total phenolic

Thirty days after callus subculture, one gram of fresh calli, or five mixed calli from each test tube, were homogenized in 20 ml of 80% aqueous methanol. They were then sonicated for twenty minutes at 25 degrees Celsius and centrifuged for twenty minutes at 4 degrees Celsius. A vacuum evaporator was used to dry the supernatant, which was then dissolved in one milliliter of methanol. The TPC was ascertained using the Folin-Ciocalteu method (Ainsworth and Gillespie 2007) [1]. The solution was prepared by thoroughly mixing 0.1 ml of the extract with 0.8 ml of distilled water and 0.1 ml of Folin-Ciocalteu reagent. After shaking the mixture for three minutes, 3 ml of 2% sodium carbonate was added. The mixture was then allowed to stand at room temperature for two hours. Absorbance was measured at 760 nm using a UV-VIS spectrophotometer.

Growth (biomass) measurement

After 20 days in culture, the calli were weighed under sterile conditions prior to subculture. After 30 days, the calli were weighed again and the difference of fresh weight and dry weight of calli was considered as growth rates of calli.

Browning Assessment

To assess the extent of browning, calli were categorized into five groups based on the intensity of browning observed after 30 days of culture. The classification ranged from grade 1 (minimal browning) to grade 5 (severe browning).

Statistical assessment

Statistical analysis was performed using one-way ANOVA followed by an appropriate multiple comparison test in GraphPad Prism (version 10.5.0). A p-value of less than 0.05 was considered statistically significant.

Results

Effect of ascorbic acid on callus biomass, browning, PPO activity and phenolic content

Ascorbic acid significantly influenced the biochemical and physiological traits of *Solanum diphyllum* L. callus cultures (Table 1). A dose-dependent reduction in PPO activity was observed, with the lowest activity at 500 mg/L (0.012 ± 0.001) U, indicating strong browning inhibition. TPC increased with increasing concentrations of ascorbic acid, peaking at 500 mg/L (95.6 ± 0.16) GAE/g, suggesting enhanced phenolic biosynthesis. The optimal biomass production was recorded at 200 mg/L, with a fresh weight of 3.90 ± 0.032 g and dry weight of 0.020 ± 0.02 g, indicating effective stimulation of tissue proliferation at this concentration. However, higher concentrations (>500 mg/L) showed reduced growth and increased browning, suggesting possible phytotoxic effects.

Table 1: Effects of ascorbic acid on *Solanum diphyllum* L.

Treatments (Ascorbic acid)	PPO activity (U)	Total phenolic content (GAE/g)	Biomass (g)		Browning intensity
			fresh weight	dry weight	
0 mg/L	0.165±0.02	59.39±0.01	3.89±0.34	0.021±0.02	4.8±0.1
50 mg/L	0.092±0.004	73.88±0.02	3.61±0.42	0.014±0.09	4.2±0.12
100 mg/L	0.079±0.04	82.92±0.03	2.99±0.05	0.011±0.01	3.5±0.32
200 mg/L	0.041±0.32	84.1±0.01	3.90±0.032	0.02±0.02	2.8±0.21
300 mg/L	0.032±0.06	90.3±0.089	3.83±0.02	0.017±0.001	2.2±0.11
400 mg/L	0.019±0.021	92.7±0.09	3.69±0.01	0.019±0.032	1.3±0.01
500 mg/L	0.012±0.001	95.6±0.16	2.2±0.019	0.009±0.04	1.1±0.01
600 mg/L	0.038±0.006	89.31±0.06	3.10±0.2	0.012±0.021	3.7±0.04

U: activity unit, GAE: gallic acid equivalent, g: gram

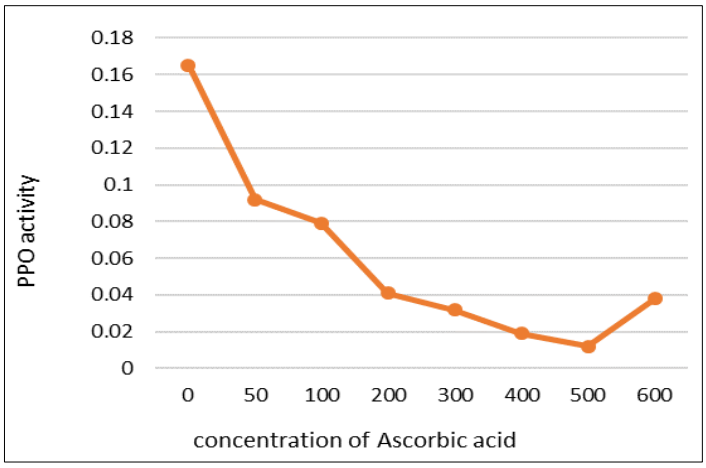


Fig 1: Effect of ascorbic acid on PPO activity in callus culture

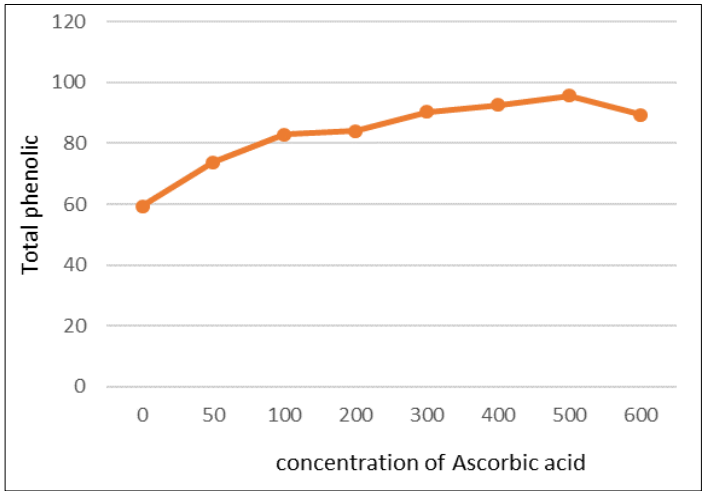


Fig 2: Effect of Ascorbic Acid on phenolic content in Callus Culture

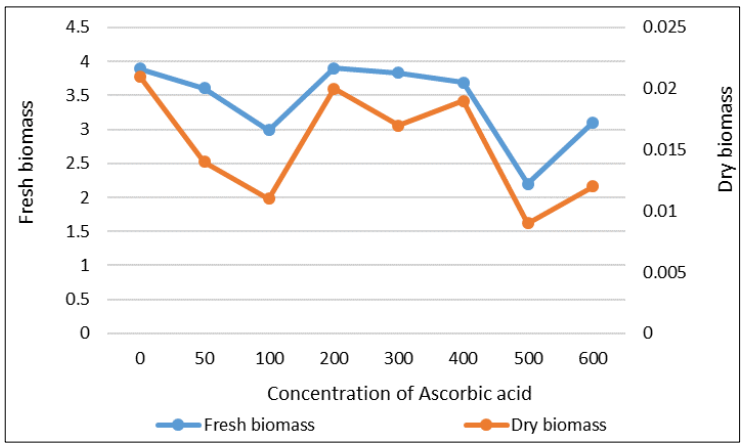


Fig 3: Effect of ascorbic acid on biomass of callus culture

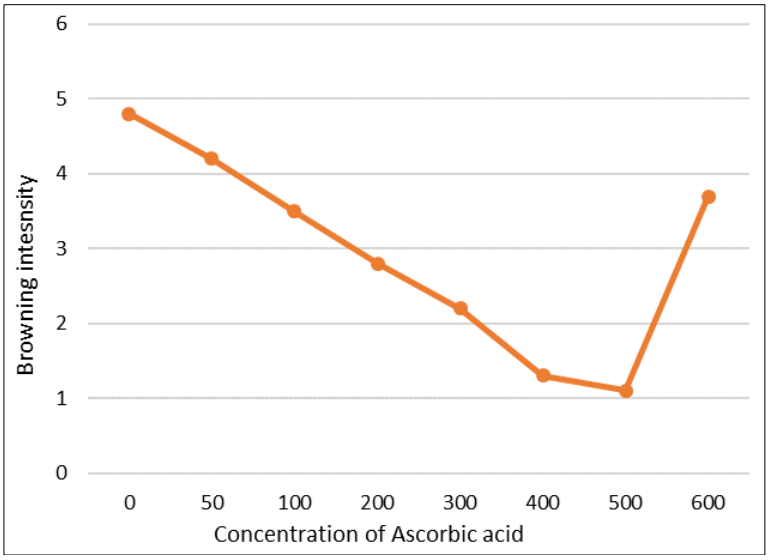


Fig 4: Effect of Ascorbic acid on Browning of tissue culture

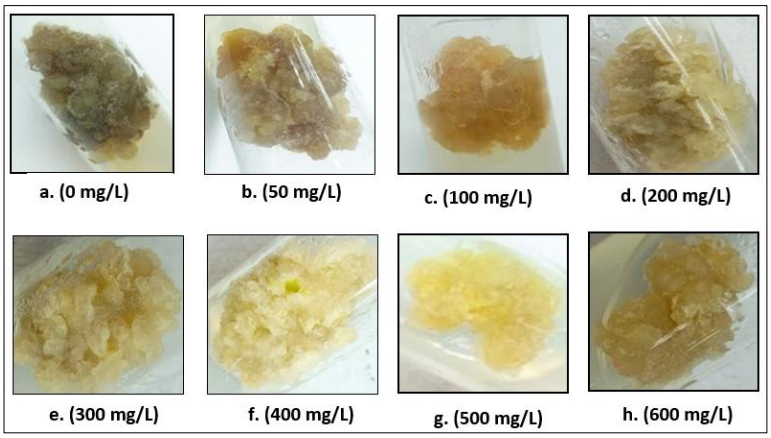


Fig 5: (a-h) Effect of increasing concentrations of ascorbic acid on callus morphology of *Solanum diphyllum* L.

Effect of citric acid on callus biomass, browning, PPO activity and phenolic content

Citric acid treatments also produced significant effects (Table 2). PPO activity decreased consistently across treatments, with the lowest value at 600 mg/L (0.011 ± 0.9) U, highlighting its strong enzyme-inhibitory effect. TPC was substantially higher in all citric acid-treated groups compared to the control, with the highest at 600 mg/L (94.3±0.02) GAE/g. Unlike ascorbic acid, citric acid treatments resulted in moderate biomass changes, with the best response at 300 mg/L (4.2±0.03) g. A clear inverse relationship between citric acid concentration and browning intensity was noted, with the lowest browning score of 1.0 ± 0.12 at 600 mg/L, indicating excellent control of oxidative discoloration. Notably, PPO activity suppression at 600 mg/L citric acid exceeded reductions reported in *Solanum lycopersicum* (Murthy *et al*., 2014) [9], indicating a potentially higher antioxidant responsiveness in *Solanum diphyllum* L. callus.

Overall, both antioxidants effectively mitigated PPO activity and tissue browning, though their effects on biomass and TPC differed. Ascorbic acid was more effective in enhancing growth and phenolic content, whereas citric acid provided stronger browning suppression with less impact on biomass accumulation.

Table 2: Effects of citric acid on *Solanum diphyllum* L.

Treatments (Citric acid)	PPO activity (U)	Total phenolic content (GAE/g)	Biomass (g)		Browning intensity
			fresh weight	dry weight	
0 mg/L	0.153±0.02	57.7±0.07	4.2±0.04	0.019±0.05	5.0±0.1
50 mg/L	0.089±0.03	73.1±0.1	3.9±0.2	0.016±0.03	4.3±0.02
100 mg/L	0.074±0.05	78.45±0.7	3.95±0.4	0.012±0.07	3.5±0.1
200 mg/L	0.059±0.08	84.3±0.09	4.0±0.02	0.025±0.01	2.8±0.02
300 mg/L	0.039±0.08	83.6±0.09	4.2±0.03	0.021±0.03	2.0±0.01
400 mg/L	0.038±0.02	91.8±0.01	3.3±0.03	0.014±0.02	1.5±0.2
500 mg/L	0.025±0.02	94.2±0.03	3.0±0.71	0.017±0.01	1.3±0.4
600 mg/L	0.011±0.9	94.3±0.02	3.1±0.05	0.022±0.012	1.0±0.12

U: activity unit, GAE: gallic acid equivalent, g: gram

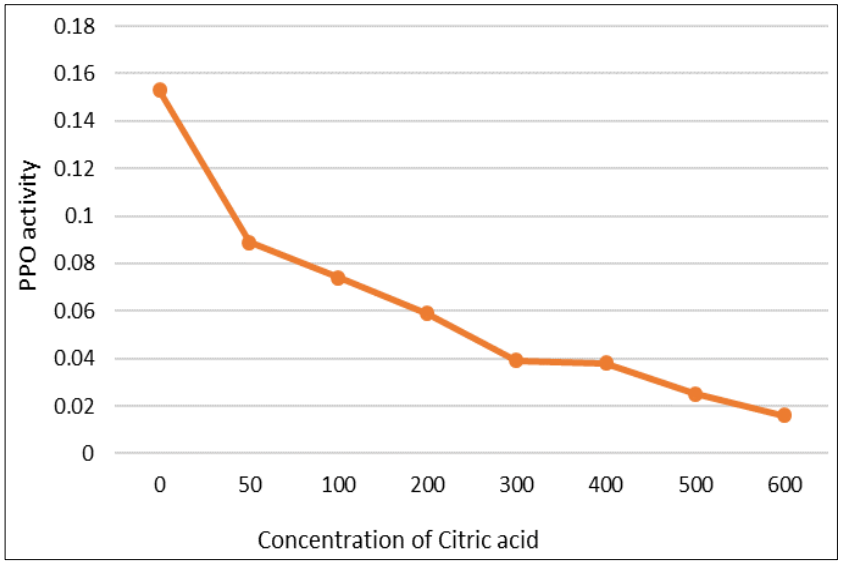


Fig 6: Effect of Citric Acid on PPO Activity in Callus Culture

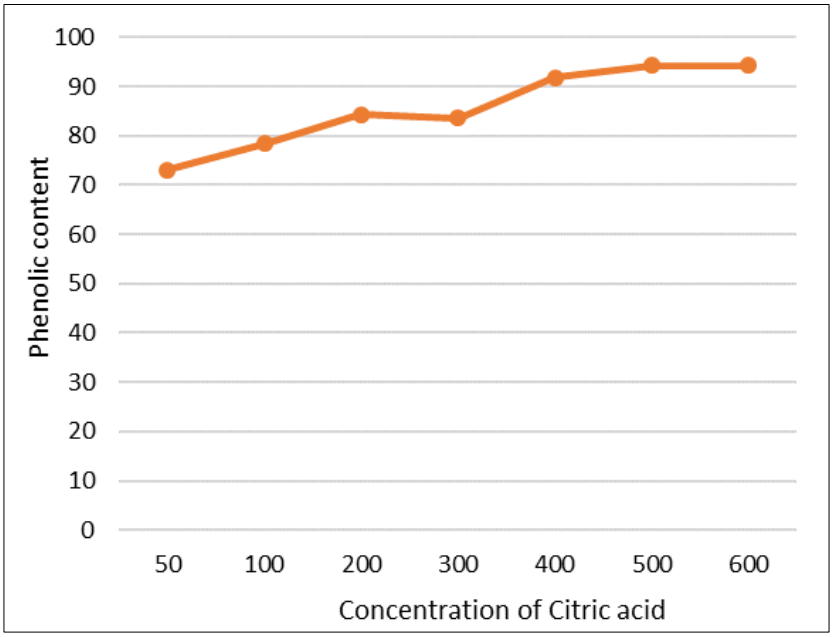


Fig 7: Effect of Citric Acid on phenolic content in Callus Culture

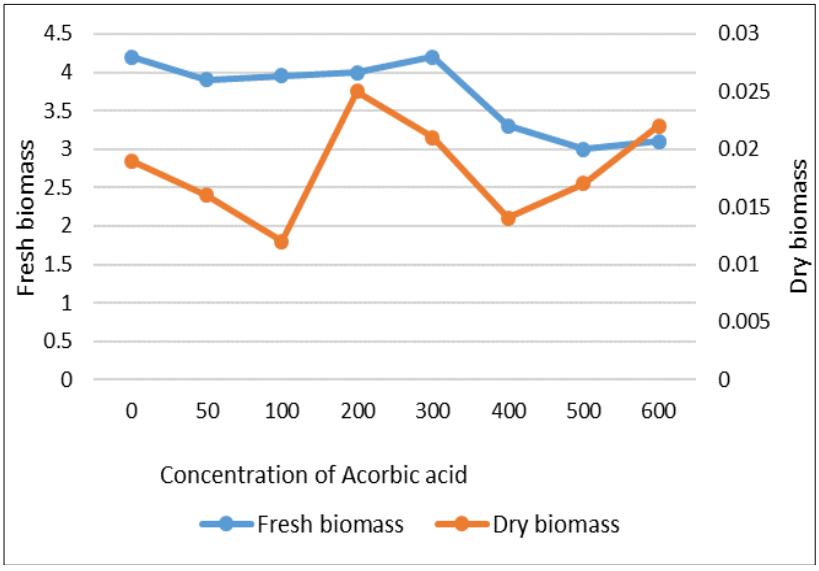


Fig 8: Effect of Citric acid on biomass of callus culture

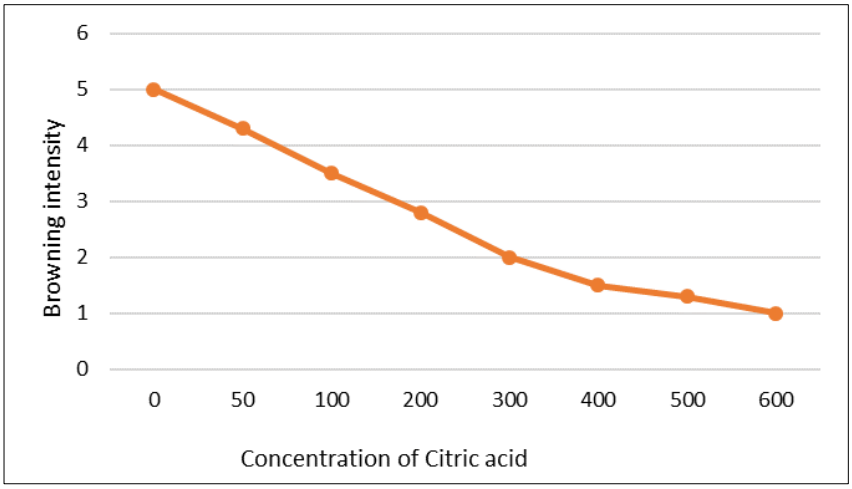


Fig 9: Effect of Citric Acid on browning in Callus Culture

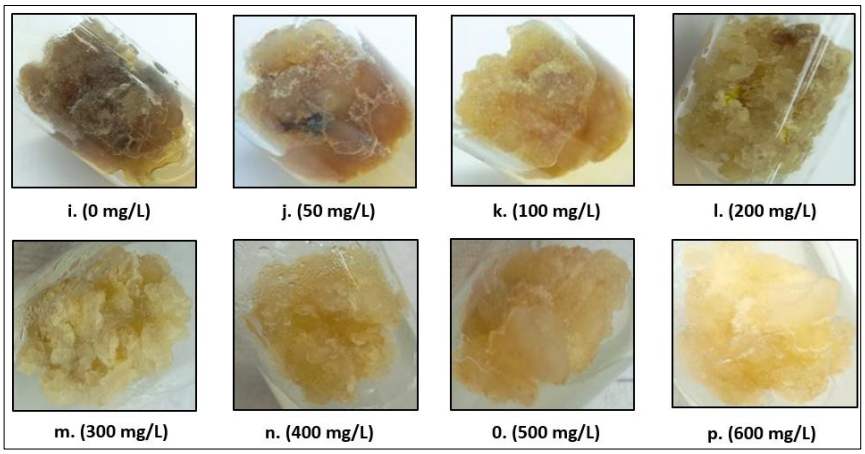


Fig 10: (i-p) Effect of increasing concentrations of citric acid on callus morphology of *Solanum diphyllum* L.

Discussion

The marked reduction in PPO activity following ascorbic acid treatment highlights its role as an effective antioxidant in preventing enzymatic browning in *Solanum diphyllum* L. callus cultures. Maximum inhibition was observed at 400-500 mg/L, indicating that ascorbic acid effectively limits oxidative reactions responsible for tissue discoloration. The concurrent increase in TPC suggests that ascorbic acid may not only prevent phenolic degradation but also stimulate their biosynthesis by maintaining redox homeostasis. The optimal biomass accumulation at 200 mg/L further supports a beneficial balance between metabolic activity and antioxidant protection at this concentration. However, concentrations exceeding 500 mg/L led to visible browning and reduced growth, likely due to phytotoxic effects such as altered pH, disrupted metabolic pathways, or oxidative imbalance.

Citric acid also proved effective in reducing PPO activity and browning intensity across all tested concentrations, with maximum inhibition observed at 600 mg/L. Its chelating properties likely contributed to PPO inactivation through the sequestration of metal cofactors essential for enzymatic activity. Interestingly, the enhancement of TPC was relatively consistent across citric acid treatments, indicating that even lower concentrations were sufficient to promote phenolic stability and accumulation. While biomass increase was modest, slight improvements at 300 mg/L suggest citric acid may support metabolic balance under oxidative stress, though not as strongly as ascorbic acid in promoting growth.

Similar browning-control effects of antioxidants have been reported in other Solanum species such as *S. lycopersicum* and *S. villosum*, though optimal concentrations in those studies often fell within the lower range (50-300 mg/L) (Kumar *et al*., 2022; Iftikhar *et al*., 2015) [8, 7]. In contrast, *Solanum diphyllum* L. in our study exhibited peak PPO inhibition at comparatively higher levels (500-600 mg/L), suggesting species-specific differences in antioxidant responsiveness. Furthermore, while previous reports in Solanaceae often note browning suppression without significant enhancement in secondary metabolites, our results demonstrate a dual benefit suppression of oxidative browning coupled with notable phenolic accumulation, particularly under ascorbic acid treatment. This combined effect underlines the novelty and broader applicability of our optimized protocol.

The inverse correlation between antioxidant concentration and browning intensity confirms the effectiveness of both acids in mitigating oxidative discoloration. However, their differential impact on biomass and phenolic content suggests that specific concentrations must be optimized based on target outcomes. These findings offer a reproducible protocol for reducing oxidative browning and enhancing callus growth in *Solanum diphyllum* L., which can be applied for large-scale micropropagation, conservation of rare genotypes, and enhanced recovery of pharmacologically important secondary metabolites under controlled conditions.

Conclusion

This optimized antioxidant-assisted callus culture protocol offers a valuable platform for future studies focused on secondary metabolite biosynthesis, while also supporting the conservation and biotechnological utilization of *Solanum diphyllum* L. The findings have practical relevance for applications such as micropropagation, enhanced in vitro metabolite production etc. By comprehensively assessing the effects of ascorbic and citric acids on browning inhibition, biomass accumulation, and enzyme activity, this study contributes to the refinement of tissue culture methodologies aimed at improving plant tissue viability and scalability.

The study concludes that by decreasing PPO activity and increasing phenolic content, ascorbic acid and citric acid both significantly reduce oxidative browning in *Solanum diphyllum* L. callus cultures. With best results at 200 mg/L and 500 mg/L, respectively, ascorbic acid was especially useful in promoting biomass accumulation and phenolic synthesis. Citric acid, on the other hand, showed greater PPO inhibition, especially at 600 mg/L, with little browning, despite having less of an impact on biomass. Higher levels of both antioxidants, however, had a detrimental effect on growth, suggesting possible phytotoxicity. All things considered, antioxidant supplementation, particularly at optimal concentrations, enhances callus viability and biochemical quality, providing a viable method to improve *Solanum diphyllum* L. in vitro culture results. This is the first optimized antioxidant-assisted callus culture protocol for *Solanum diphyllum L.*, potentially serving as a model for other medicinal plants prone to oxidative browning.

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