

Unlocking the Potential of Phloroglucinol and Tocopherol in Improving the Photosynthetic Efficiency, Membrane Stability, and Antioxidant Enzyme Activities of Cryopreserved In Vitro Raised Plantlets of *Citrus maxima* Merr. And *Citrus jambhiri* Lush

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Abstract

This study examined the effects of tocopherol (Vitamin E) and phloroglucinol (1,3,5-trihydroxybenzene) on cryopreserved plantlets of *Citrus jambhiri* Lush and *Citrus Maxima* Merr., two important *Citrus* species used in *citrus* breeding and variety development. After undergoing cryopreservation using conventional desiccation methods, the seeds of these two species were propagated in Murashige and Skoog (MS) medium with various concentrations of tocopherol and phloroglucinol. Plantlets raised from both cryopreserved and non-cryopreserved seeds were multiplied using clonal propagation on MS medium supplemented with the specified two chemicals. The clones were subsequently assessed by a range of procedures, including the quantification of chlorophyll levels, measurement of electrolyte leakage, and evaluation of specific antioxidant enzyme activities, including superoxide dismutase, catalase, and peroxidase. Cryopreserved and non-cryopreserved *C. jambhiri* and *C. maxima* clones subjected to 2.0 μM and 15.0 μM tocopherol showed improved membrane stability compared to their control counterparts. Increased photosynthetic efficiency was attained by supplementing 0.3 μM and 4.0 μM phloroglucinol to *C. jambhiri* and *C. maxima*, respectively. Both cryopreserved and non-cryopreserved *C. jambhiri* clones had their maximum antioxidant enzyme activity when cultured in a medium with 2.0 μM tocopherol and 0.3 μM phloroglucinol. In *C. maxima*, cryopreserved and non-cryopreserved clones had higher antioxidant enzyme activity in medium with 15.0 μM tocopherol and 4.0 μM phloroglucinol. In conclusion, the use of vitamin E and phloroglucinol in cryopreservation can greatly improve the important developmental traits of cryopreserved *citrus* plantlets.

Key Message

This study explores tocopherol and phloroglucinol effects on cryopreserved *Citrus jambhiri* and *Citrus maxima* *in vitro* plantlets. Optimal concentrations of which enhances membrane stability, photosynthesis, antioxidant activity, and plantlet regeneration potential.

Introduction

The United Nation Human Right Commission (UNHRC) recognises that all persons born on this planet have a moral right to food. This need has been partially satisfied as a result of technological developments like gene technology, nanotechnology, cultured meat, food irradiation, etc. (Siegrist and Hartmann 2020). However, restricted or limited accessibility to genetic resources can impede the advancement of scientific research and hinders the development of new products and technologies. Priority has therefore been given to conservational efforts that facilitates the use of technologies and to sustainable approaches. Article 9 of the Nagoya Protocol of the Convention on Biological Diversity (CBD) recognized the importance and benefits of *ex situ* conservation techniques such as cryopreservation over *in situ* conservational approaches (CBD 2019). Cryopreservation, a non-lethal storage of biological samples at extremely low temperature remains the only practical method available today for the long-term preservation of germplasm of vegetatively propagated species and those with recalcitrant seeds (Benelli 2021; Roque-Borda et al. 2021). The advancements in plant cryopreservation studies however,

does not match the success of cryo-retrieved plantlets. Of the many hindrances, the production of oxidative stress mediated by reactive oxygen species (ROS) during any of the steps involve in cryopreservation can impair the regenerative potential of cryo-retrieved plantlets (Ren et al. 2021). Reactive oxygen species (ROS) formed as a result of cryopreservation, can affect various aspects of plant physiology, including chlorophyll integrity, membrane stability, and antioxidant enzyme activity (Chen et al. 2015; Liu et al. 2021; Ren et al. 2021; Sachdev et al. 2021; Sahu et al. 2022). Therefore, the success of any cryopreservation protocols greatly depends on minimizing or suppressing the stress on cells or tissue exposed right from explant extraction stage to pre-culture treatments, desiccation, exposure to liquid nitrogen, thawing and specifically during reestablishment in the culture medium.

Addition of antioxidants in the regeneration medium can minimise oxidative stress, control the formation of ROS, increase the activity of antioxidant enzymes, prevent lipid peroxidation, and prevent DNA degeneration. (Liu et al. 2021). Antioxidants added to the regeneration medium typically resulted in excellent cell viability, demonstrating that oxidative stress plays a role in the success of cryopreservation (Len et al. 2019). Phloroglucinol and tocopherol are two such compounds that has been used successfully to improve numerous developmental aspects of cryo-retrieved plantlets *in vitro*. Phloroglucinol (1,3,5-trihydroxybenzene) is a phenolic compound that has a significant importance to plant tissue culture, since it has been considered as an alternative supplement to plant growth regulators (Teixeira da Silva et al. 2013; Aremu et al. 2015). In cryopreservation, phloroglucinol is utilized as a cryoprotectant because it has a cryoprotective action against oxidative stress and associated metabolism brought on by free radicals, which increases the lifetime of cryopreserved explants (Londe et al. 2017). Although the exact mechanism by which phloroglucinol promotes growth is unclear, one study has found that the substance has a significant impact on the lignin biosynthetic pathway by enhancing the expression of key enzymes, including cinnamyl alcohol dehydrogenase (CAD), cinnamoyl-CoA reductase (CCR), and Caffeic acid O-methyltransferase, which increases lignin production (Farmer, 1985). On the other hand, Tocopherol, often known as vitamin E, is employed as an antioxidant to protect plant cells from oxidative damage during the process of plant tissue culture and cryopreservation of plant genetic resources (Falk and Munné-Bosch 2010). Tocopherol has been demonstrated to increase the viability of cryopreserved plant cells and tissues, as well as the growth and development of plant cells in culture (Uchendu et al. 2010; Mène-Saffrané 2017; Vidya Muthulakshmi et al. 2023). The ability of this lipid-soluble compound to regulate plant growth and development lies in its antioxidant properties (Traber and Atkinson 2007). Tocopherol can scavenge free radicals and shield plant cells from oxidative damage thereby increasing the activities of antioxidant enzymes such as nitrate reductase and Glutathione reductase (Munné-Bosch and Alegre 2002).

Citrus maxima Merr., the world's largest *Citrus* fruit primarily farmed in Southeast Asia, is known for its high nutritional value, antibacterial properties, and versatile culinary uses (Oyedepo 2012; Shivananda et al. 2013; Karo et al. 2020; Tocmo et al. 2020). It's also a valuable genetic resource for breeding initiatives, with genetic diversity and adaptable traits (Krueger and Navarro 2007; Talon and Gmitter 2008; Ding et al. 2022). On the other hand, *Citrus jambhiri* Lush, or rough lemon, native to India and Asia, is prized for its disease resistance, high acidity for culinary purposes, and essential oil production (Chaudhari et al. 2014;

Pandove et al. 2016; Pathak et al. 2021). As rootstock, it enhances the resilience of grafted *citrus* plants and supports their growth in challenging conditions (Hamzé et al. 1986; Bowman and Joubert 2020; Narukulla et al. 2023). Therefore, preserving their genetic diversity is crucial for the future development of pest and disease-resistant *citrus* cultivars in the face of climate change and environmental challenges.

Change in environmental conditions due to climatic shift is a significant threat to *citrus* species and citriculture worldwide (Balfagón et al. 2022). The temperature increase is changing the environmental conditions in the areas where citrus varieties are currently being developed, along with other unfavourable climate phenomena. This will probably increase the negative effects of unfavourable environmental factors on citrus physiology and production, with possible extinction risks (Shafqat et al. 2021; Balfagón et al. 2022). Furthermore, climate change poses an increased risk of intensification of plant diseases, putting at risk the world's food supply and natural plant biodiversity (Singh et al. 2023). Further, long-term conventional seeds storage for both *C. maxima* and *C. jambhiri* is not possible owing the recalcitrant nature of their seeds (Rohini et al. 2016; Khopkar et al. 2017). Therefore, the aim of this research investigation was to perform an antioxidant or elicitors-assisted cryopreservation of seeds of the two species with the sole purpose to improve the performances of cryo-retrieved *in vitro* raised and clonally propagated plantlets of *C. jambhiri* and *C. maxima*.

Materials and Methods

Mature fruits of *C. jambhiri* and *C. maxima* were collected from different locations situated in the Northeastern Indian states of Meghalaya (Fig. S1, S2; Table S1). The seeds were extracted from the collected mature fruits and were washed thoroughly under running tap water for 30 to 45 min followed by adding 5.0 mL of Labolene (HiMedia, Mumbai, India) and 500 mL of water in a 1L beaker. The beaker was then shaken vigorously for 5 to 10 min to remove any surface contaminants followed by placing the seeds under running tap water for up to 1 h. Only seeds that sank to the bottom of the beaker were chosen, those that floats on the surface of the water were considered unviable and were all discarded. In order to prevent any moisture content fluctuation until they were used, these chosen seeds were sun-dried for up to 1 wk before being kept in airtight containers (minor modification of Sangma et al. 2020).

Determination of seed moisture content

The moisture content of seeds from mature fruits of *C. jambhiri* and *C. maxima* were analysed following protocols laid down by the International Seed Testing Association (ISTA, 1999). The formula employed for the calculation of seed moisture content is given below:

$$\text{Moisture content (\%)} = \frac{W1 - W2}{W1 - W3} \times 100$$

Where,

‘W1’ is the weight of seeds and moisture bottle

'W2' is the weight of dry seeds and moisture bottle and

'W3' is the weight of empty moisture bottle.

The resulting moisture contents from the above formula were considered as the original seed moisture content.

Dehydration

The dehydration of seeds was conducted using the procedure outlined by Kaya et al. (2017) for *C. jimbhiri* and Wen et al. (2010) for *C. maxima*, respectively, with significant modifications. The seeds of *C. jimbhiri* and *C. maxima* were initially sterilized by treating them with a solution of 0.1% mercuric chloride (HgCl_2) (HiMedia). Subsequently, the seeds are rinsed multiple times with double-distilled water (DDW), often ranging from 5 to 10 repetitions. The seeds were subsequently subjected to a 4% sodium hypochlorite (NaOCl) solution (HiMedia), followed by treatment with 70% ethanol ($\text{CH}_3\text{CH}_2\text{OH}$) (HiMedia), and then rinsed 5 to 10 times with distilled deionized water (DDW). The seeds were dehydrated inside a laminar air flow cabinet (Thermo Fisher Scientific, Waltham, MA) for a duration of 0 to 18 h, with a 3 h interval for *C. jimbhiri* and a duration of 0 to 12 h, with a 2 h interval for *C. maxima*. During each of these designated dehydration periods, the moisture content of roughly 4 g of seeds was assessed using the procedure established by ISTA (1999) as previously mentioned in the Materials and Methods section. One group of desiccated seeds was introduced to the regrowth medium (MS), while another group of seeds was subjected to freezing at a temperature of -196°C using liquid nitrogen. These frozen seeds were then stored for a duration of 1 h, after which they were thawed at a temperature of $42 \pm 2^\circ\text{C}$ for a period of 2 min. The seeds from both sets were introduced into a regrowth medium (MS) (HiMedia), which contained different amounts of tocopherol (ranging from 1.0 to 5.0 μM) and phloroglucinol (ranging from 0.1 to 0.5 μM) for *C. jimbhiri*, and tocopherol (ranging from 5.0 to 25.0 μM) and phloroglucinol (ranging from 1.0 to 5.0 μM) for *C. maxima*. The seeds were then kept in darkness for a period of 7 d before being transferred to a controlled environment in the culture room. The germination followed by regeneration of the treated seeds were consistently evaluated. The appearance of the radicle is considered a sign of seed germination. The sprouted seeds were thereafter evaluated periodically for indications of regrowth. The appearance of the initial pair of leaves, known as seed leaves, due to the extension of the plumule and the division of the cotyledons, is regarded as a sign of regeneration. The study utilized two characteristics, namely germination and regeneration, which were quantified as percentages. The aforementioned tests were replicated three times, with 18 replicates per treatment.

Clonal propagation

A single healthy cryopreserved and non-cryopreserved mother plantlet (representing each treatment) was selected for subsequent clonal multiplication in MS medium supplemented with above mentioned same treatments. Clones were obtained using shoots, apical meristems, and axillary buds in each treatment. In all the above experiments, the pH of the medium was adjusted to 5.8 using 0.1N hydrochloric acid (HCl) (HiMedia) or sodium hydroxide (NaOH) (HiMedia) before autoclaving at 104 kPa and 121°C for 20 min.

After inoculation, the cultures were maintained inside a culture room with a controlled environmental condition set at 75 to 80% relative humidity, $25 \pm 2^\circ\text{C}$ temperature, a 14 h photoperiod, and a light irradiance of $50\mu\text{Mol m}^{-2}\text{s}^{-1}$ which was provided by cool fluorescence tube lighting (Phillips, Mumbai, India). Clones of both species, which had been treated and were cryopreserved or not, underwent each of the subsequent below mentioned experiments.

Estimation of Chlorophyll content

The chlorophyll content was determined using the Lichtenthaler (1987) approach with minor modifications. Acetone (CH_3COCH_3 , 100%) (HiMedia) was used to extract chlorophylls from each leaf material. The extracts were centrifuged at 3000g for 5 min before analyzing with a UV-Vis spectrophotometer (Agilent Technology, Cary 60 UV-Vis, Los Angeles, CA) at 663.2, 646.8, and 470 nm. The chlorophyll a, b, total chlorophyll, and carotenoid contents were estimated by the following formulas:

$$Chla = (12.25A_{663.2} - 2.79A_{646.8}) \times VS \times DF/SM (g)$$

$$Chlb = (21.21A_{646.8} - 5.1A_{663.2}) \times VS \times DF/SM (g)$$

$$TotalChl = Chla + Chlb$$

$$Carotenoids = \frac{[(1000A_{470} - 1.8Chla - 85.02Chlb)]}{198} \times VS \times DF/SM (g)$$

Where,

'*Chl a*' is Chlorophyll a, '*Chl b*' is Chlorophyll b, '*Total Chl*' is the total chlorophyll '*VS*' is the volume of the supernatant (mL), '*DF*' is the dilution factor, '*SM*' is the sample mass '*A_{663.2}*' is the absorbance reading at 663.2 nm, '*A_{646.8}*' is the absorbance reading at 646.8 nm, '*A₄₇₀*' is the absorbance reading at 470 nm.

Assessment of Electrolyte Leakage

As detailed by Lutts et al. (1996), electrolyte leakage (LE) was employed to analyse membrane stability by quantifying the amount of solutes leaching out of the leaves of the clones of *C. jambhiri* and *C. maxima*. Young leaves were cleaned three times with double-distilled water (DDW) to remove any surface contaminants before being chopped into small leaf discs and incubated for 22 h on a rotary shaker in sealed vials containing 10 mL of DDW. The solution's initial electrical conductivity (CE1) was measured immediately after incubation followed by autoclaving the sealed vials with the leaf discs at 120°C for 20 min, the final electrical conductivity (CE2) was measured after the fluid had cooled to ambient room temperature. The electrolyte leakage (LE) was then calculated using the following formula:

$$LE = \frac{CE1}{CE2} \times 100$$

The value obtained from the above formula was expressed in percentage.

Determination of antioxidant enzyme activities

Preparation of extract

Fresh clones leaf tissue weighing 0.5 g was homogenised in 5.0 mL of a solution containing 50 mM potassium phosphate buffer (K_3PO_4 , pH = 7) (HiMedia), 2 mM ethylenediaminetetraacetic acid ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_8$) (HiMedia), and 1% (volumetric weight) polyvinylpyrrolidone ($\text{C}_6\text{H}_9\text{NO}$) (HiMedia), which acted as the extraction buffer, in order to prepare the sample for measuring the activity of antioxidant enzymes. After that, the homogenous mixture was centrifuged using a SIGMA 3-16PK chilled centrifuge (SIGMA Laborzentrifugen GmbH, Osterode am Harz, Germany) for 15 min at 15,000 rpm and 4°C. According to Ozden et al. (2009), the supernatant from the centrifugation was kept as an extract at -84°C until the necessary studies could be carried out.

Peroxidase (POD) enzyme activity

Using a spectrophotometer (Agilent Technology, Cary 60 UV-Vis) at a wavelength of 470 nm, the increase in light absorption that came from the oxidation of guaiacol ($\text{C}_7\text{H}_8\text{O}_2$) in the presence of hydrogen peroxide (H_2O_2) was used to measure the activity of this enzyme, in accordance with the protocol outlined by Hemeda and Klein (1990). 50.0 μL of the enzymatic extract, 90.0 μL of 0.3% H_2O_2 (HiMedia), 90.0 μL of 1% $\text{C}_7\text{H}_8\text{O}_2$ (HiMedia), and 780.0 μL of 50 mM K_3PO_4 , (pH7.0) were combined to create the reaction mixture. Next, the quenching coefficient of 26.6 mM cm^{-1} was used to determine the enzyme activity, which was then reported as $\mu\text{M H}_2\text{O}_2$ degraded $\text{min}^{-1} \text{ mg}^{-1}$ protein.

Superoxide Dismutase (SOD) enzyme activity

Using the method outlined by Beauchamp and Fridovich (1971), the activity of SOD was ascertained by measuring the decrease in light absorption of the nitro blue tetrazolium chloride ($\text{C}_{40}\text{H}_{30}\text{Cl}_2\text{N}_{10}\text{O}_6$) (HiMedia) complex in the presence of enzyme activity. Using this approach, 70.0 μL of the extracted enzyme extract, 50.0 mM K_3PO_4 , (pH 7.8), 13.0 mM L-methionine ($\text{C}_5\text{H}_{11}\text{NO}_2\text{S}$) (HiMedia), 75.0 μM $\text{C}_{40}\text{H}_{30}\text{Cl}_2\text{N}_{10}\text{O}_6$, 0.1 μM $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_8$, and 4.0 μM riboflavin ($\text{C}_{17}\text{H}_{20}\text{N}_4\text{O}_6$) (HiMedia) were combined to create a 3.0 mL reaction mixture. Following a 15 min exposure to a light chamber with approximately $300 \text{ mol m}^{-2} \text{ s}^{-1}$ of fluorescent lamps (Phillips, India), the reaction mixture was evaluated for absorbance at 560 nm using an Agilent Technology Cary 60 UV-Vis spectrophotometer. The uptake rate was determined to be 50% of the uptake read for the control. The enzyme activity was reported as $\text{mg protein min}^{-1}$.

Catalase (CAT) enzyme activity

The activity of CAT was assessed utilising the Cakmak and Marschner (1992) approach. Using this approach, 50.0 μL of the recovered enzyme extract was combined with 1.0 mL of catalase assay, which contained 10.0 mM H_2O_2 and 50.0 mM K_3PO_4 (pH 7.0). The absorbance was then determined for 1 min at 240 nm using an Agilent Technologies Cary 60 UV-Vis spectrophotometer. An extinction value of 39.4 mM cm^{-1} was used to calculate the enzyme activity ($\mu\text{mol min}^{-1} \text{ g FW}$).

Statistical and Graphical Analysis

All data obtained in this study were subjected to one-way ANOVA. Tukey's test was performed to test the level of significance of the means of all collected data at the significance level ($p \leq 0.05$) using Origin Pro statistical software (PC version Origin Pro 2021 7.0, Northampton, MA). Principal component analysis and network plot analysis were performed using PAST statistical software (PC version PAST4.13 2022) (Hammer 2001).

Results and Discussion

Effect of dehydration time, moisture content, and cryopreservation on germination and regeneration percentage

Seeds of *C. jambhiri* are relatively smaller than those of *C. maxima*, seeds of *C. jambhiri* are obovoid, sometimes flattened, and can be white or green in colour. In contrast, the seeds of *C. maxima* are larger and have a different shape, with a thicker endocarp. The size of seeds in *citrus* fruits can be influenced by various factors, including the specific variety of the citrus fruit. In the case of *C. jambhiri* and *C. maxima*, the difference in seed size may be attributed to genetic factors and the ploidy level of the fruits. Research has shown that the ploidy level of the parent fruits can affect the size and ploidy level of the seeds in citrus fruits (Yahata et al. 2021). Additionally, the genetic characteristics of each citrus variety can lead to differences in seed size. For example, a study on the fruit quality of a new pummelo cultivar, *C. maxima*, reported differences in the fruit characteristics, including seed size and content, among different cultivars (Pan et al. 2023). The results of this study reveal that the initial moisture contents of *C. jambhiri* and *C. maxima* seeds were 38.55% and 41.23%, respectively. When these seeds were inoculated in the MS medium supplemented with 2.0 μM and 15.0 μM of tocopherol, respectively, without undergoing dehydration or cryopreservation, the germination and regeneration rates were found to be 97.45% and 100% for *C. jambhiri*, and 87.41% and 98.25% for *C. maxima*, respectively (Fig. 1A&B). Nevertheless, the rates of germination and regeneration of the seeds in their respective media were considerably reduced when the seeds were cryopreserved without undergoing the process of dehydration. The cryopreservation of seeds demonstrated a higher efficacy in the production of shoots and roots in both species, surpassing their respective germination potentials. It is worth noting that cryopreserved *C. jambhiri* seeds exhibited a more favourable response to regeneration in comparison to regeneration of *C. maxima*. Nevertheless, the seeds of both species exhibited the capacity for cryopreservation by dehydration, as evidenced by successful germination and regeneration. The dehydration process of *C. jambhiri* seeds for a duration of 9 h, resulting in a moisture content of 33.12%, and *C. maxima* seeds for a duration of 6 h, resulting in a moisture content of 36.41%, yielded optimal germination and regeneration percentages. Specifically, *C. jambhiri* exhibited approximately 65.23% germination and 77.78% regeneration, while *C. maxima* exhibited germination and regeneration percentages of approximately 42.55% and 57.77% respectively. However, in both scenarios, an increase in dehydration exacerbates the reduction in moisture content, leading to diminished rates of germination and regeneration (Fig. 1).

The observable responses of cryopreserved plantlets subjected to varying concentrations of tocopherol, with lower concentrations for *C. jambhiri* and higher concentrations for *C. maxima*, suggest the unique physiological requirements of each plant species and the specific composition of the growing medium. Several authors have highlighted the importance of balancing multiple parameters, including as plant growth regulators, nutritional concentration, rooting, and shooting medium, in order to achieve optimal outcomes in *in vitro* propagation across different plant species. For instance, Hossain et al. (1994) underlined its importance in *Aegle marmelos*, Pant et al. (2015) in *Chrysanthemum morifolium*, and Emoghene et al. (2018) in *Phoenix dactylifera*. This observation might also be attributed to the differences in seed sizes exhibited by these two *Citrus* species. According to Akinyosoye et al. (2014), it has been observed that larger seeds possess a greater nutritional reserve compared to smaller seeds. Upon germination, a significant portion of these reserves is utilized to facilitate the initial phase of plant growth immediately following germination. This phenomenon frequently results in the production of more robust seedlings. After the exhaustion of this nutrient supply, the developing plantlets must depend on the macro and micronutrients present in the surrounding natural environment or *in vitro* culture to ensure consistent growth. Hence, it may be inferred that the growth of seedlings in *C. maxima* is dependent on a greater concentration of tocopherol in larger seeds, but smaller seeds of *C. jambhiri* can sustain seedling growth with a comparatively lower concentration of tocopherol, owing to their diminished nutritional reserves.

The results obtained from our experimental study further suggest that the moisture content of seeds plays a vital role in the successful germination and regeneration of cryopreserved seeds in *Citrus* species. Previous research has demonstrated a correlation between elevated moisture levels and reduced rates of germination and regeneration in the context of cryopreservation (Engelmann 2000; Malik et al. 2020). The increased moisture content found in frozen seeds might result in the production of larger ice crystals, which can have severe negative effects on cellular structures, hence increasing the likelihood of cellular damage. As stated by Wesley-Smith et al. (2014), the process of cellular or tissue dehydration has the capacity to decrease the probability of ice crystal formation and alleviate damage to vital cellular constituents. Moreover, previous studies have provided evidence that the process of dehydrating citrus seeds prior to their immersion in liquid nitrogen is a noteworthy method for preserving plant germplasm (Aldemir et al. 2015). In addition, the cryopreservation of polyembryonic seeds, a characteristic feature of *Citrus* species, can be achieved through the utilization of the dehydration process. The research conducted by Kaya et al. (2017) shown that the implementation of dehydration methods resulted in a germination rate of 93.30% in *C. jambhiri* and the process encompassed reducing the moisture content of the seeds to 21.10% within a time frame of 12 h. In accordance with the findings of Kaya et al. (2017), our investigation on the identical species exhibited variability with regard to germination rates and the duration required to attain the optimal moisture level. Wen et al. (2010) reported that the optimal moisture content for cryopreservation in *Citrus* spp. is dependent upon the particular species and methodologies employed. Furthermore, it is important to acknowledge that the genetic variability within a given species can potentially influence an individual's response to cryopreservation (Khan et al. 2012). It is crucial to understand that there might be variations in physiological parameters, even among individuals within the

same species. Damasco and Refuerzo (2016) have provided evidence of a degree of variation in the responses of several species of *Calamansi* (*Citrofortunella microcarpa*) to seed desiccation and cryopreservation.

Effect of antioxidant treatments on the electrolyte leakage in the treated clonally propagated plantlets

Leaf membrane stability was evaluated by quantifying electrolyte leakage in both cryopreserved and non-cryopreserved clones. Irrespective of the plant species under investigation, it was consistently observed that the amount of electrolyte leakage decreased in both the non-cryopreserved and cryopreserved samples when supplemented with either tocopherol or phloroglucinol. Specifically, clones of *C. jambhiri* and *C. maxima* exhibited the lowest level of electrolyte leakage from the cellular membrane when subjected to 2.0 μM and 15.0 μM of tocopherol, respectively (Table 1). Enhanced membrane stability was observed until a specific threshold is attained, after which the membrane stability declines.

The electrolyte leakage test is a technique used to evaluate the cold tolerance and tissue damage in plants. It measures the amount of electrolytes lost by plant cells when they experience injury or stress. This test has been documented by Tinus (2002), Demidchik et al. (2014), Hatsugai and Katagiri (2018), and Kovalesski and Grossman (2021). Electrolyte leakage serves as an indicator of membrane stability. Plant cells exhibit electrolyte leakage when they experience damage or death, indicating a compromised cell membrane. Hence, there exists an inverse relationship between electrolyte leakage and membrane stability, suggesting that elevated levels of electrolyte leakage are linked to reduced membrane stability (Venkateswarlu and Ramesh, 1993). Multiple research studies have provided support for our results that tocopherol contributes to the stabilization of membrane integrity in both species. Tocopherols have the ability to stabilize membranes and remove various reactive oxygen species (ROS) and lipid oxidation products. This helps to extend the lifespan of seeds and avoid lipid peroxidation during germination (Sattler et al. 2004; Munoz and Munne-Bosh 2019). The relationship between tocopherols and the regulation of jasmonic acid levels in plants supports their involvement in intracellular signalling (Munne-Bosh 2007). Tocopherols and tocotrienols, which are constituents of vitamin E, are lipid-soluble antioxidants that regulate lipid peroxidation. The capacity of vitamin E to preserve the integrity of cellular membranes is widely acknowledged (Wang and Quinn 2000).

Effect of different antioxidant treatments on photosynthetic efficiency in the treated clonally grown plantlets

The photosynthetic efficiency of plantlets derived from clonal propagation of the two *Citrus* species was evaluated by measuring the levels of chlorophyll A, chlorophyll B, total chlorophyll, and carotenoids following exposure to different amounts of antioxidants. Incorporating either tocopherol or phloroglucinol into the MS medium resulted in elevated levels of chlorophyll content and carotenoids in both *C. jambhiri* and *C. maxima* species, as compared to the control group lacking these antioxidants. Additionally, the assessment of photosynthetic pigments in *C. jambhiri* shown that enhancing the efficiency of photosynthesis in clonally cultivated plantlets can be achieved by subjecting them to a growth medium

containing either 2.0 μM tocopherol or 0.3 μM phloroglucinol. Nevertheless, clonally grown *C. maxima* plantlets showed enhanced photosynthetic efficiency when exposed to basal conditions supplemented with either 15.0 μM tocopherol or 4.0 μM phloroglucinol, regardless of whether they were cryopreserved or not (Fig. 2 & S2). Although the photosynthetic efficiency of clonally propagated plantlets decreased when higher concentrations of antioxidants were used, it is important to note that plantlets grown in MS medium with any concentration of antioxidants showed better photosynthetic efficiency compared to the control (Fig. 2).

The photosynthetic efficiency of green plants and algae is the percentage of light energy that is converted into chemical energy. Increases in photosynthetic efficiency are directly correlated with increases in the amounts of photosynthetic pigments (e.g., chlorophyll a, chlorophyll b, carotenoids, etc.) present in a species' leaves. Tocopherols, a lipid-soluble antioxidants are produced by all plant species and especially abundant in seeds and photosynthetic tissues (Maeda et al. 2006; Ali et al. 2019; Naqve et al. 2021; Shah et al. 2021; Niu et al. 2022; Mesa and Munne-Bosh 2023). Under conditions of intense light and heat stress, tocopherols, especially α -tocopherol, are caused to accumulate at high levels. The role of tocopherol in the photosystem II (PSII) reaction centre is to scavenge the singlet oxygen that is produced by the chlorophyll triplet states. Tocopherol improves the development, biochemistry, and yield of water-deficiency wheat cultivated in the field (Ali et al. 2019). This provides more evidence that tocopherol is involved in better growth, which in turn leads to higher photosynthesis and an improved antioxidant defence mechanism. Tocopherol likely improves photosynthetic efficiency by protecting chloroplasts from damage, scavenging singlet oxygen in PS II, and preventing singlet oxygen from entering the photosystem.

In various horticultural and grain crops, phloroglucinol, a growth stimulant, has been found to enhance somatic embryogenesis and shoot development. Recent research on the microalga *Thalassiosira pseudonana* has shown that phloroglucinol can boost growth, production of fucoxanthin as well as photosynthetic efficiency (Liu et al. 2022). Phloroglucinol treatment enhanced the amounts of foliar carotenoid and chlorophyll in a research on *Coccoloba uvifera* plantlets, which are important markers of photosynthetic activity (Manokari et al. 2021). Phloroglucinol may have different impacts on photosynthetic pigment concentrations depending on the species and ambient factor, therefore further study is required to completely grasp its role.

Effect of different antioxidant treatments on antioxidant enzyme activities of clonally propagated plantlets

Superoxide dismutase, peroxidase, and catalase enzymes exhibited elevated levels of activity in clonally produced plantlets of *C. jambhiri* when tocopherol (1.0 to 5.0 μM) and phloroglucinol (0.1 to 0.5 μM) were added to the MS medium, as compared to the control. The maximum levels of antioxidant activity were seen at concentrations of 2.0 μM tocopherol and 0.3 μM phloroglucinol in the medium, as shown in Fig. 3. The enzyme activity in clonally grown plantlets of *C. maxima* exhibit a similar pattern to those of *C. jambhiri*, with the sole exception being that higher levels of antioxidants (15.0 μM tocopherol and 4.0

μM phloroglucinol) were necessary for *C. maxima* (Fig. 4). The antioxidant enzyme activity was higher in clonally formed plantlets that were not cryopreserved compared to their cryopreserved counterparts in both species. The addition of any level of the two antioxidants to the media increases the activity of antioxidant enzymes in the plantlets of both species. Nevertheless, when the concentrations are increased, the antioxidants exhibit a reciprocal correlation with the enzyme activity.

Adding antioxidants during cryopreservation has been shown to reduce oxidative stress and enhance the function of antioxidant enzymes (Liu et al. 2021;). Enzymatic antioxidants, such as SOD, PRX, and CAT, as well as non-enzymatic antioxidants like salicylic acid and ascorbic acid, are essential components of an efficient antioxidant defence system. Hence, the decline in antioxidant enzyme activity seen in controlled cryopreserved plantlets could be attributed to the damage incurred during the cryopreservation procedure, potentially affecting the efficiency of both enzymatic and non-enzymatic antioxidant defence mechanisms.

Tocopherol is a cryoprotective agent (CPA) used in the cryopreservation of plant tissues. The compound tocopherol has shown promising effects in reducing oxidative damage and promoting regeneration in experiments conducted on the shoot tips of blackberries (Ren et al. 2021). Furthermore, tocopherol, together with other antioxidants, has been employed to enhance the genetic stability of cryopreserved mint shoot tips (González-Benito et al. 2016). The involvement of Vitamin E in low-temperature adaptation and phloem loading has been identified as highly significant in *Arabidopsis* (Maeda et al. 2006). Phloroglucinol is a contemporary cryopreservation research compound that has received significant traction. It has been utilised to assist in the cryopreservation of several plant species, such as *Astronium urundeuva* (Paula et al. 2022), *Melocactus zehntneri*, *Cereus gounellei* (Vendrame et al. 2020), *Catsetum atratum* (Prenzier Suzuki et al. 2018), and others. The efficacy of these two chemicals in cryopreservation may stem from their ability to operate as cryoprotectants, shielding cells from harm induced by ice crystallisation during the freezing and thawing processes. Phloroglucinol acts as a hydrogen bond donor, whereas tocopherol functions to stabilise and safeguard membranes, hence reducing the amount of free water capable of forming ice.

The Figs. 5A and 5B demonstrate the principal component analysis, which provides a comprehensive view of the correlation between different antioxidant enzymes activities and treatments with different dosages of tocopherol and phloroglucinol in clonally propagated plantlets of *C. jambhiri*. The bi-plot analysis revealed that the combined influence of PC1 and PC2 explained 99.54% and 99.80% of the total variation in the tocopherol and phloroglucinol data sets, respectively (Fig. 6A and B). PC1 explains the majority of variance in both antioxidants, with 98.375% for tocopherol and 99.198% for phloroglucinol. PC2 explains a lower fraction, with 1.165% for tocopherol and 0.60% for phloroglucinol. The superoxide dismutase (SOD) enzyme activities in cryopreserved and non-cryopreserved plantlets of *C. jambhiri* has a substantial impact on the first principal component (PC1) when tocopherol is used as an antioxidant in the growing medium. This is evidenced by the magnitude of the lines in the bi-plot graph. In addition, the biplot graph clearly showed distinct clusters in the upper and lower right quadrants. These clusters corresponded to different concentrations of tocopherol (2.0 μM , 3.0 μM , 4.0 μM , and 5.0 μM) and

phloroglucinol (0.3 μ M, 0.4 μ M, and 0.5 μ M). This implies that the observations within each cluster exhibit inherent similarities or associations with one other. Both graphs also unequivocally illustrate that there is no correlation between the activities of antioxidant enzymes. The findings provide valuable insights into the relationship between these variables and highlight potential interdependencies that can guide further study.

Further, the PCA bi-plot graphs (Fig. 5C and 5D) provide a correlation between the antioxidant enzyme activities in cryopreserved and non-cryopreserved plantlets of *C. maxima* with respect to varying treatment concentrations of tocopherol and phloroglucinol. The bi-plot analysis revealed that PC1 and PC2 accounted for 98.44% and 1.16%, respectively, of the variation in tocopherol, while PC1 and PC2 explained 97.38% and 1.55% of the variation in phloroglucinol. These components made a noteworthy contribution to the observed variation in the dataset. Furthermore, Catalase (CAT) enzyme activities in cryopreserved plantlets derived from a single clone significantly affects PC1 when phloroglucinol is used as an antioxidant in the growing media. This can be observed by examining the line lengths in the bi-plot graph. In addition, the bi-plot graph clearly showed distinct clusters (2.0 μ M, 3.0 μ M, and 4.0 μ M phloroglucinol) in the upper and lower right quadrants, indicating that these data have shared similarities or connections. Moreover,, superoxide dismutase (SOD) enzyme activities in both cryopreserved and non-cryopreserved plantlets of *C. maxima* significantly affects the first principal component (PC1) when tocopherol is used as an antioxidant in the growing medium. This is also evidenced by the magnitude of the lines in the bi-plot graph. In addition, the bi-plot graph clearly showed distinct clusters (10.0 μ M, 15.0 μ M, 20.0 μ M, and 20.0 μ M tocopherol) in the upper and lower right quadrants, indicating that these observations had shared similarities or correlations with each other.

PCA, or Principal Component Analysis, is a statistical method employed in plant science research to identify correlations among variables. Multiple studies have utilised it to investigate various phenomena, such as characteristics associated with peanut flavour, cows' linear type attributes, the capacity of water resources, and the metabolic profile of different plant components (Morris and Antonious 2013; Chen et al. 2020; Liu et al. 2022; Zhang et al. 2023). The PCA method has been employed in plant tissue culture to examine various phenomena such as secondary metabolites in suspension cells, salt tolerance of different accession lines, basal media utilised in tissue culture, growth dynamics of seedlings cultivated in tissue culture, and the effects of growth regulators on plantlets (Singh et al. 2001; Badr et al. 2015; Kanwal et al. 2021; Liu et al. 2021).

The Fruchterman-Reingold algorithm was used to construct network analysis plots in this study. These plots were based on the Euclidean similarity index and were used to visualise the relationship between different treatments and their effects on antioxidant enzyme activities. The analysis revealed that in both *C. jambhiri* and *C. maxima*, supplementing the growth medium with different concentrations of tocopherol and phloroglucinol resulted in increased activities of SOD, POX, and CAT compared to the control. This can be observed from the size of the nodes in Figs. 6A and B, which represent each treatment. Moreover, based on the magnitude of the lines connecting each therapy in this plot analysis, a connection between treatments can be inferred. Based on the findings, it can be inferred that for *C.*

jambhiri, the optimal concentrations of phloroglucinol and tocopherol for improving antioxidant enzyme characteristics in clonally propagated plantlets are 0.3 μM and 2.0 μM , respectively. On the other hand, in *C. maxima* clonally propagated plantlets, antioxidant enzyme activities can be enhanced by using 15.0 μM of tocopherol and 4.0 μM of phloroglucinol, respectively (Fig. 6A-B). Prior research has utilised this form of data visualisation to illustrate the correlation between various plant growth regulators (PGRs) and the shooting and rooting responses in the plant tissue culture experimental of *Dendrobium* sp. (Subrahmanyeswari et al. 2022).

Conclusion

Currently, there are established and reliable cryopreservation methods for preserving the seeds of *C. jambhiri* and *C. maxima*. However, there is limited knowledge regarding the biochemical and physiological characteristics of these cryopreserved plantlets. To summarise, this study indicates that adding tocopherol and phloroglucinol to the basal medium can enhance the success of cryopreservation using the dehydration method. Additionally, these supplements can also improve the physiological and biochemical characteristics of cryopreserved clones of the two *Citrus* species. Hence, this procedure can be utilised in the future to optimise the likelihood of survival of cryopreserved plantlets of other citrus species.

Declarations

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Data availability (data transparency)

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Competing Interests

The authors declare that there are no known financial or non-financial competing interests.

Authors Contribution

M Wanlambok Sanglyne: Collection and identification of samples, experimentation, compilation of data, statistics, data visualization and initial draft

Lavinia Alexis Kurbah: Experimentation (Cryopreservation), data visualization and drafting

Dr. Meera C. Das: Supervision, final editing, concepts and experimental designs

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Table

Table I: Electrolyte leakages from the leaves of clonally propagated, cryopreserved and non-cryopreserved *Citrus jambhiri* Lush and *Citrus maxima* Merr.

<i>Citrus</i> spp.	Explant	Treatments	Conc. (μ M)	Electrolyte leakage (%)	
				-LN	+LN
<i>Citrus jambhiri</i>	Seeds	Tocopherol	Control	77.52 $\pm$ 0.06 ^A	87.78 $\pm$ 0.06 ^A
			1	70.03 $\pm$ 0.01 ^F	80.29 $\pm$ 0.02 ^F
			2	59.94 $\pm$ 0.15 ^H	70.20 $\pm$ 0.16 ^H
			3	68.71 $\pm$ 0.46 ^G	78.97 $\pm$ 0.46 ^G
			4	69.53 $\pm$ 0.17 ^{F,G}	79.79 $\pm$ 0.17 ^{F,G}
		Phloroglucinol	5	71.28 $\pm$ 0.04 ^E	81.54 $\pm$ 0.04 ^E
			0.1	74.44 $\pm$ 0.09 ^C	84.70 $\pm$ 0.10 ^C
			0.2	73.65 $\pm$ 0.01 ^C	83.91 $\pm$ 0.01 ^C
			0.3	69.53 $\pm$ 0.23 ^{F,G}	79.79 $\pm$ 0.23 ^{F,G}
			0.4	72.46 $\pm$ 0.11 ^D	82.73 $\pm$ 0.11 ^D
			0.5	75.79 $\pm$ 0.22 ^B	86.06 $\pm$ 0.23 ^B
<i>Citrus maxima</i>	Seeds	Tocopherol	Control	70.03 $\pm$ 0.02 ^A	82.38 $\pm$ 0.02 ^A
			5	65.6 $\pm$ 0.15 ^D	77.95 $\pm$ 0.15 ^D
			10	64.36 $\pm$ 0.06 ^E	76.71 $\pm$ 0.06 ^E
			15	55.31 $\pm$ 0.45 ^G	67.67 $\pm$ 0.45 ^G
			20	64.45 $\pm$ 0.10 ^E	76.80 $\pm$ 0.10 ^E
			25	66.57 $\pm$ 0.16 ^C	78.93 $\pm$ 0.16 ^C
		Phloroglucinol	1	69.45 $\pm$ 0.12 ^A	81.81 $\pm$ 0.12 ^A
			2	68.51 $\pm$ 0.04 ^B	80.87 $\pm$ 0.08 ^B
			3	68.07 $\pm$ 0.06 ^B	80.42 $\pm$ 0.03 ^B
			4	59.72 $\pm$ 0.07 ^F	72.08 $\pm$ 0.07 ^F
			5	69.40 $\pm$ 0.09 ^A	81.75 $\pm$ 0.21 ^A

(Means that do not share the same letters are significantly different as shown by Tukey's test of significance at $p \leq 0.05$; Tukey's tests was performed separately between antioxidant treatments in both the species)

Figures

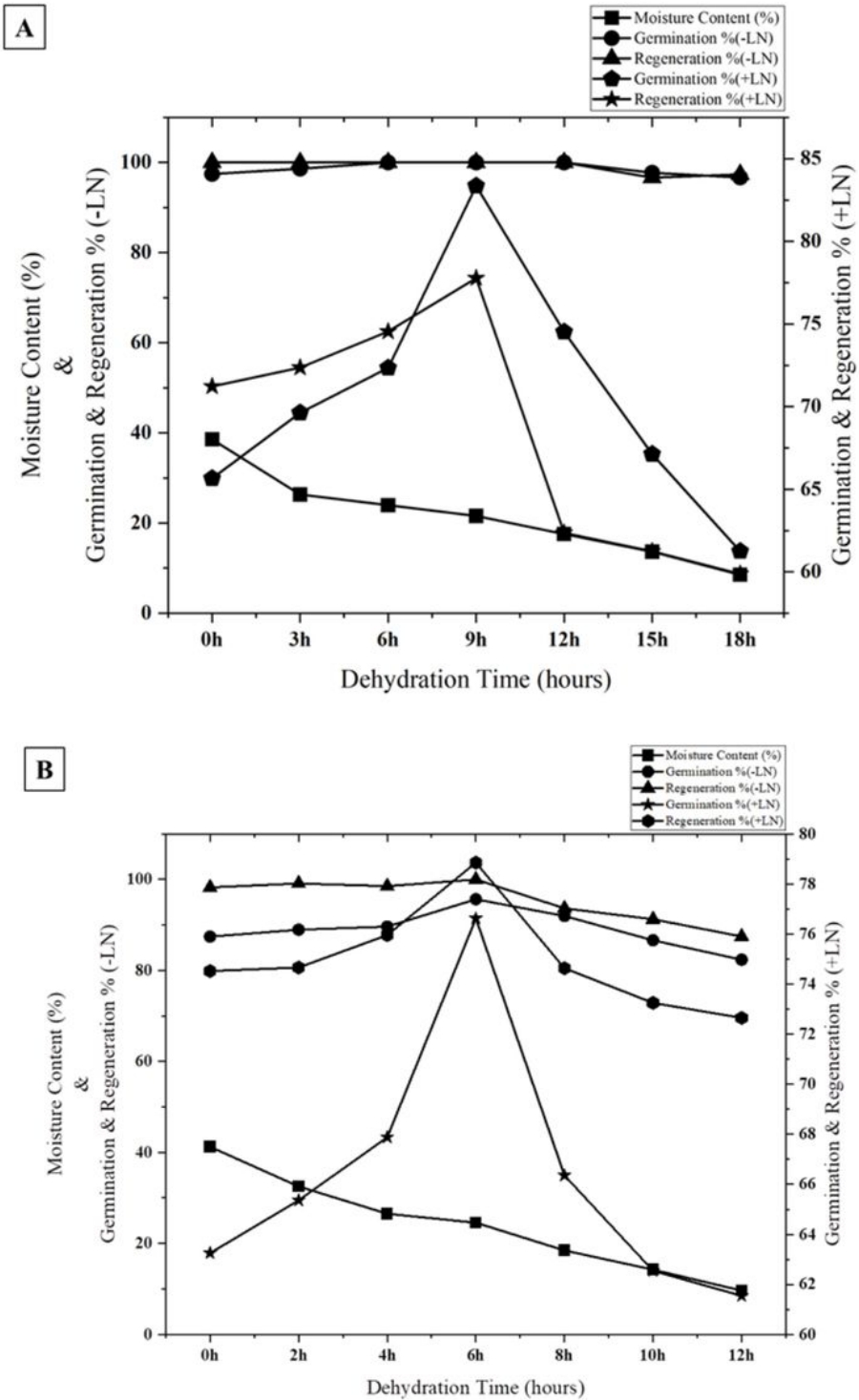


Figure 1

A: Moisture content, *in vitro* germination and regeneration percentage of seeds of *Citrus jambhiri* Lush with (+LN) and without (-LN) liquid nitrogen treatments after dehydration for several hours (0 to 18h); B: Moisture content, *in vitro* germination and regeneration percentage of germinated seeds of *Citrus maxima* Merr. plantlets that has been treated with (+LN) and without (-LN) liquid nitrogen after dehydration for several hours (0 to 12h) {Regeneration medium was supplemented with 2µM (*Citrus jambhiri* Lush) and 15µM (*C. maxima* Merr.) of tocopherol}

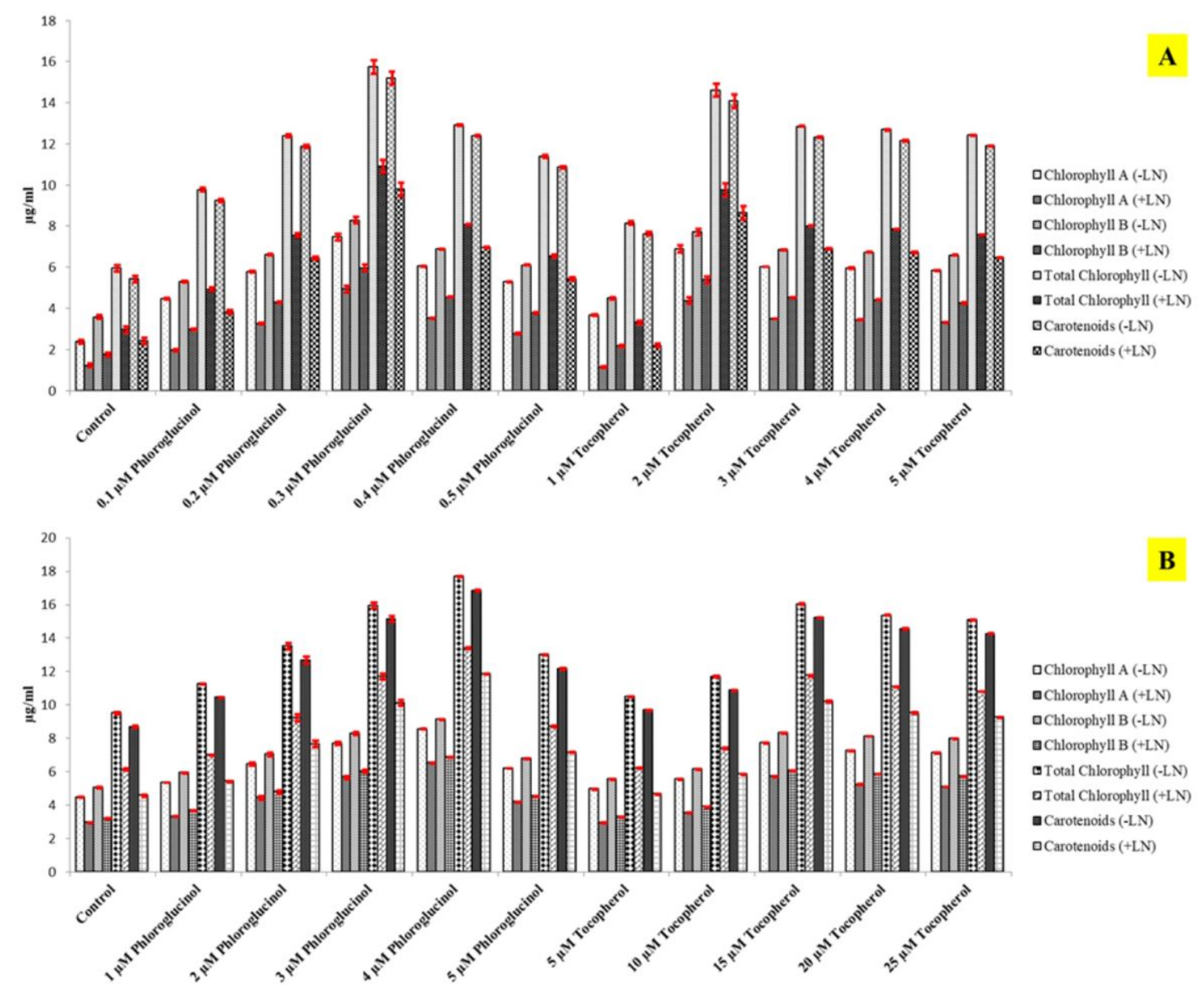


Figure 2

A: Chlorophyll A, Chlorophyll B, Total Chlorophyll and Carotenoids contents from the leaves of clonally propagated and *in vitro* grown plantlets of *Citrus jambhiri* Lush cryopreserved (+LN) and non-cryopreserved (-LN) seed. B: Chlorophyll A, Chlorophyll B, Total Chlorophyll and Carotenoids contents from the leaves of clonally propagated and *in vitro* grown plantlets of *Citrus maxima* Merr. raised from cryopreserved (+LN) and non-cryopreserved (-LN)

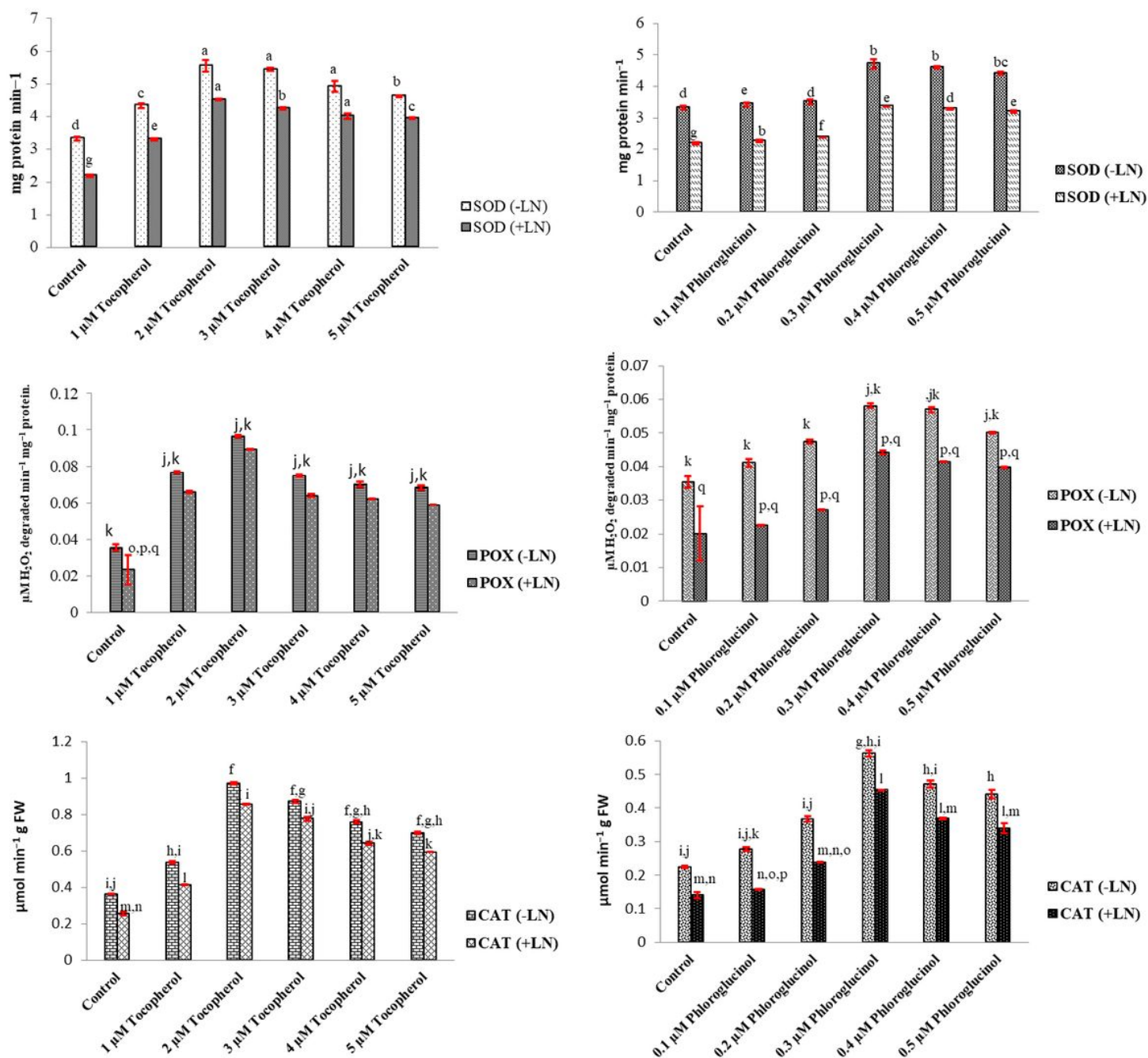


Figure 3

Superoxide Dismutase, Peroxidase and Catalase enzymes activities from the leaves of clonally propagated, *in vitro* grown and cryopreserved and non-cryopreserved plantlets of *Citrus jambhiri* Lush

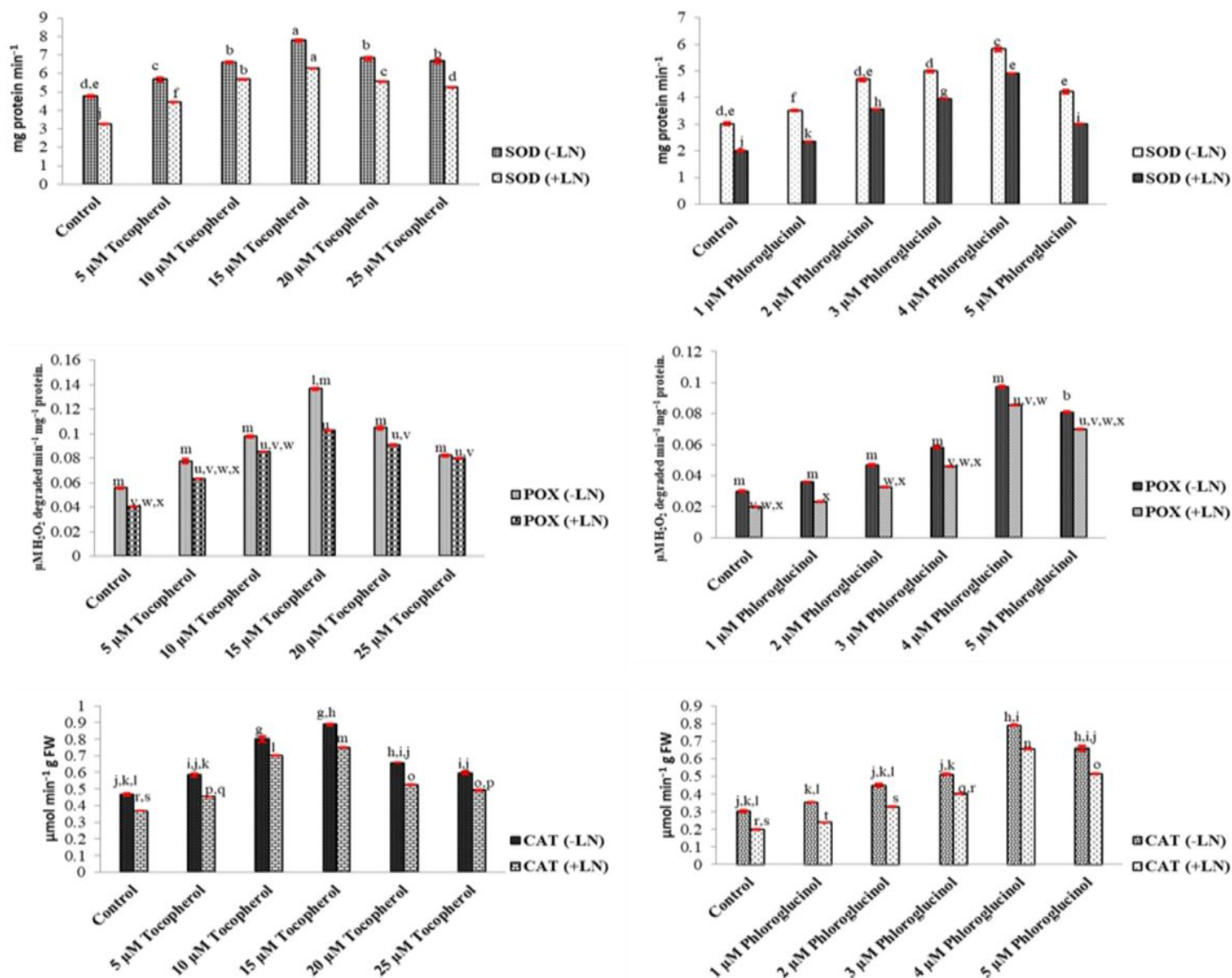


Figure 4

Superoxide Dismutase, Peroxidase and Catalase enzymes activities from the leaves of clonally propagated, *in vitro* grown and cryopreserved and non-cryopreserved plantlets of *Citrus maxima* Merr.

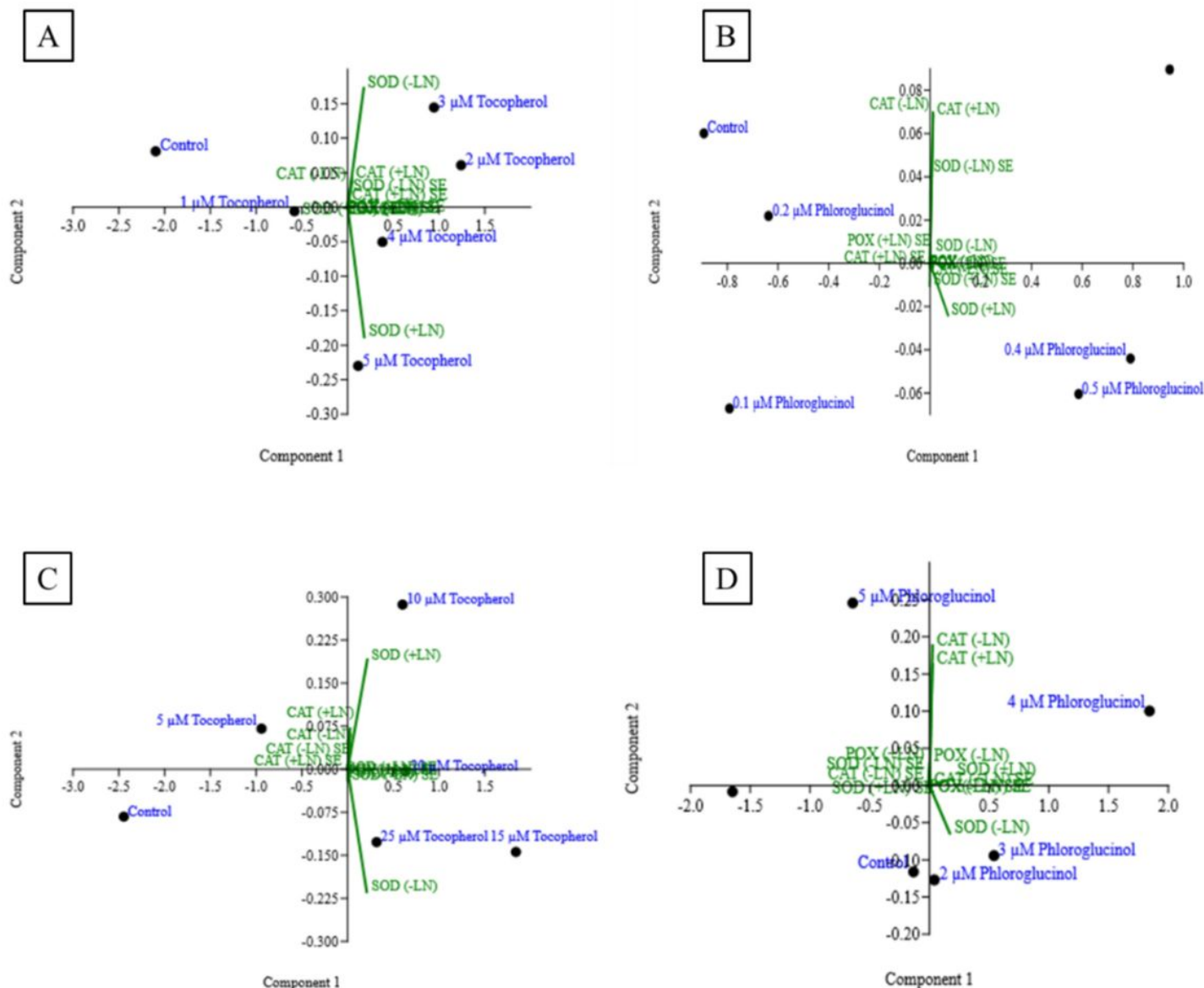


Figure 5

A: Principal component analysis bi-plot graphs based on antioxidant enzyme activities of cryopreserved and non-cryopreserved *in vitro*-grown plantlets of *Citrus jambhiri* Lush treated with different concentrations of tocopherol; B: Principal component analysis bi-plot graphs based on antioxidant enzyme activities of cryopreserved and non-cryopreserved *in vitro*-grown plantlets of *Citrus jambhiri* Lush that has been treated with different concentrations of phloroglucinol; C: Principal component analysis bi-plot graphs based on antioxidant enzyme activities of cryopreserved and non-cryopreserved *in vitro*-grown plantlets of *Citrus maxima* Merr. treated with different concentrations of tocopherol; D: Principal component analysis bi-plot graphs based on antioxidant enzyme activities of cryopreserved and non-cryopreserved *in vitro*-grown plantlets of *Citrus maxima* Merr. treated with different concentrations of phloroglucinol

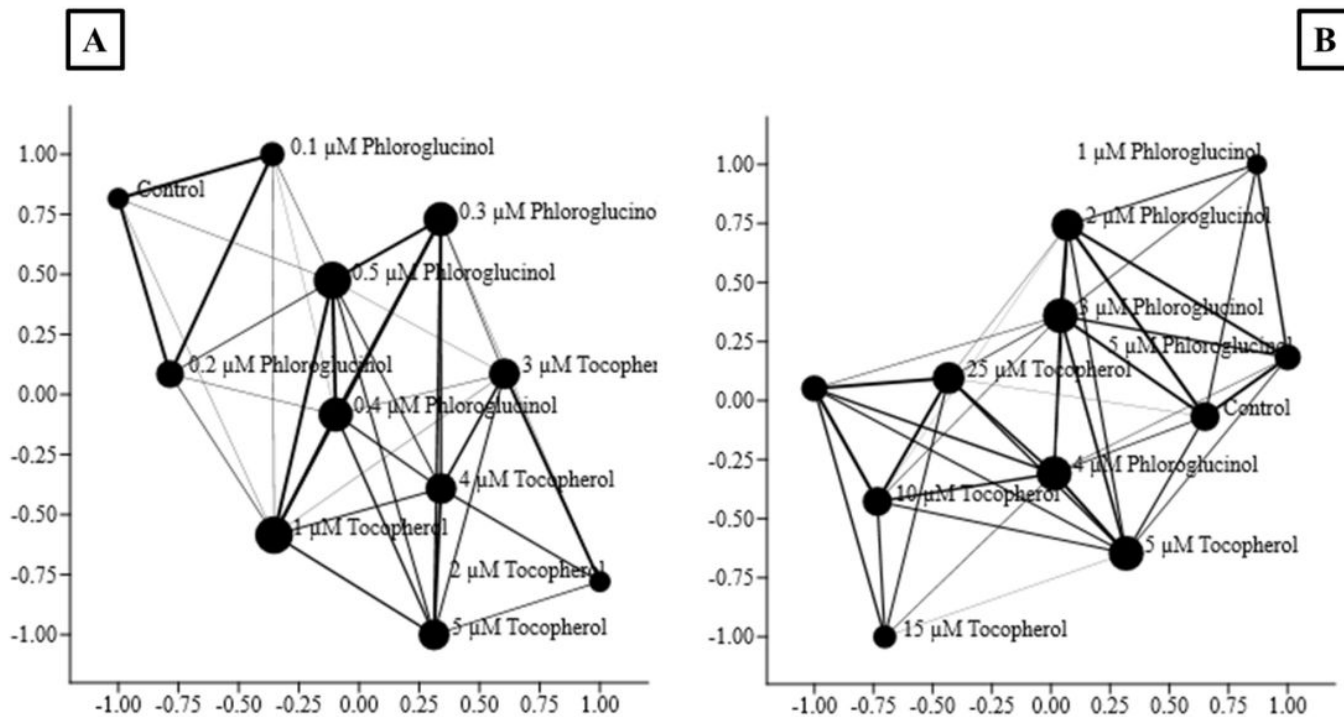


Figure 6

Network Plot analysis (based on Euclidean similarity index and Fruchterman-Reingold algorithm) explaining the association of elicitors (μ M) in connection to their individual influence on antioxidant enzymes activities (SOD, POX and CAT) of cryopreserved and non-cryopreserved clonally propagated plantlets of A: *Citrus jambhiri* Lush and B: *Citrus maxima* Merr.

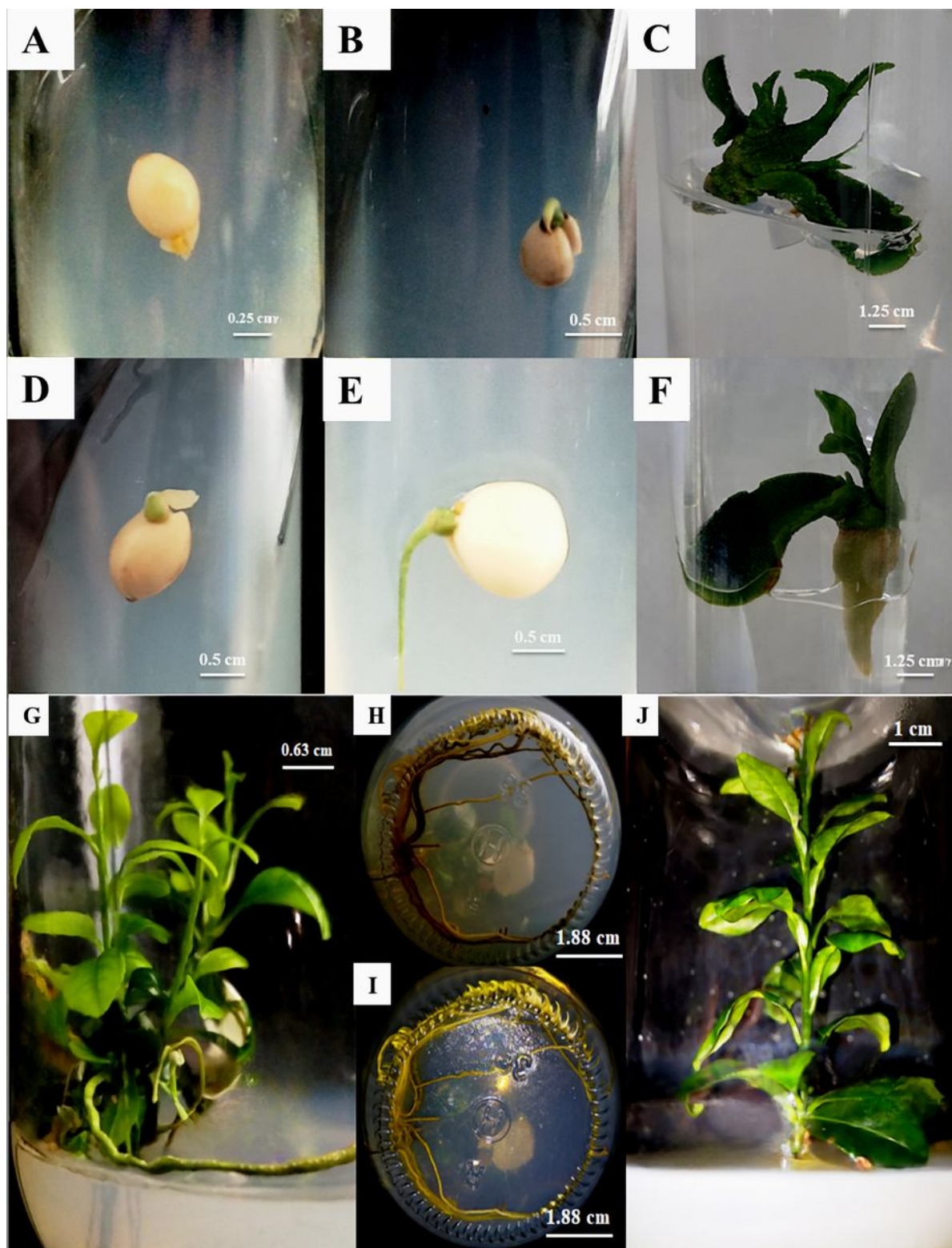


Figure 7

A: Dehydrated and cryopreserved seeds of *Citrus jambhiri* Lush inoculated in MS medium supplemented with antioxidants, B: 15 d old germinating dehydrated and cryopreserved seeds of *Citrus jambhiri* Lush inoculated in MS medium supplemented with antioxidants, C: 30 d old plantlets of *Citrus jambhiri* Lush raised from dehydrated and cryopreserved seeds; D: Dehydrated and cryopreserved seed of *Citrus maxima* Merr. inoculated in MS medium supplemented with antioxidants, E: 15 d old germinating,

dehydrated and cryopreserved seeds of *Citrus maxima* Merr. inoculated in MS media supplemented with antioxidants. F: 30 d old plantlets of *Citrus maxima* Merr. raised from dehydrated and cryopreserved seed. G: 60 days old plantlets of *C. jambhiri* raised from dehydrated and cryopreserved seeds exhibiting multiple shooting; H: Roots of 60 d old plantlets of *Citrus jambhiri* Lush raised from dehydrated and cryopreserved seeds in MS media supplemented with antioxidants; I: Roots of 60 d old plantlets of *Citrus maxima* Merr. raised from dehydrated and cryopreserved seeds in MS media supplemented with antioxidants; J: 60 d old plantlets of *Citrus maxima* Merr. raised from dehydrated and cryopreserved seeds.

Supplementary Files

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