# Metabolic stability of *in vitro* plants derived from cryopreserved shoot tips of *Passiflora suberosa* – chromatographic analysis and assessment of antioxidant activity

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**Key words:**
- cryoprotection
- oxidative stress
- passion fruit
- secondary metabolites
- V-Cryo-plate

**Abstract**

*Passiflora suberosa*, commonly known as corky passionflower or corky stem passion fruit, is a wild passion fruit species with agronomic, ornamental, and medicinal potentials. In a previous study, we developed an efficient protocol for long-term conservation of *P. suberosa* through the cryopreservation of shoot tips, using the V-Cryo-plate technique, and identified osmoprotection and cryoprotectant exposure as the critical stages influencing post-freezing recovery. The present work aimed to evaluate the effect of antioxidants on recovery rates and the metabolic stability of plants derived from cryopreserved shoot tips. The addition of different concentrations of ascorbic acid or glutathione during the osmoprotection and cryoprotection stages did not improve the recovery rates. High-Performance Liquid Chromatography (HPLC) analysis of *in vitro* plants derived from cryopreserved materials indicated that the biosynthetic capacity of the recovered plants was unaffected by the cryopreservation process. Furthermore, no significant changes were observed in the antioxidant potential of the recovered plants, as assessed by their ability to scavenge DPPH radicals and chelate metal ions. These results confirm that cryopreservation is an effective long-term conservation strategy for *P. suberosa* and provide new insights into the cellular responses of tropical plants to the stress induced by the procedures involved in V-Cryo-plate protocols.

**Palavras-chave:**
- crioproteção
- estresse oxidativo
- maracujá
- metabólitos secundários
- V-Crioplaca

**Resumo**

*Passiflora suberosa*, conhecida popularmente como maracujazinho-cortiça, é uma espécie silvestre de maracujá com potencial agronômico, ornamental e medicinal. Em um trabalho anterior, foi desenvolvido um protocolo eficiente para a conservação em longo prazo de *P. suberosa* por meio da criopreservação de ápices caulinares, utilizando a técnica V-Crioplaca, sendo a osmoproteção e a exposição aos crioprotetores identificadas como etapas críticas. O objetivo do presente trabalho foi avaliar o efeito de antioxidantes nas taxas de recuperação pós-congelamento e na estabilidade metabólica das plantas recuperadas. A adição de diferentes concentrações de ácido ascórbico ou glutationa durante a osmoproteção e a exposição a crioprotetores não teve efeito positivo nas taxas de recuperação. A estabilidade metabólica das plantas derivadas de ápices criopreservados foi analisada por Cromatografia Líquida de Alta Eficiência (CLAE), indicando que sua

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capacidade biossintética não foi afetada pela criopreservação. Além disso, não foram observadas alterações na capacidade de captura do radical DPPH e da atividade quelante de íons metálicos. Esses resultados confirmam a eficiência da criopreservação como uma estratégia de conservação para *P. suberosa* e contribuem para a maior compreensão das respostas celulares de plantas tropicais ao estresse induzido pelos tratamentos que integram protocolos de V-Crioplaca.

## Introduction

Cryopreservation has been adopted as a complementary approach to the traditional methods of seed banks and field collections of *Passiflora* species (Pacheco *et al*. 2016; Simão *et al*. 2018; Araújo *et al*. 2019; Vianna *et al*. 2019; Generoso *et al*. 2019; Faria *et al*. 2020; Silva *et al*. 2022; Ferreira *et al*. 2024). This is particularly relevant considering the increasing genetic erosion within the genus, which has been exacerbated by the extensive land use for agricultural and industrial purposes (Faleiro *et al*. 2011).

*Passiflora suberosa* L. (Passifloraceae family, genus *Passiflora*, subgenus *Decaloba*) is an herbaceous, tendrilous climbing vine native to South and Central America, commonly known as corky passionflower or corky stem passion fruit. In Brazil, it mainly occurs in phytogeographic domains impacted by anthropic actions like the Amazon and Atlantic forests and Cerrado (Bernacci et al. 2015). *Passiflora suberosa* has great ornamental and agronomic potential, due to its exotic flowers, small purple fruits, and resistance to pathogens that affect the yellow passion fruit cultures (Gardner 1989; Otoni *et al*. 1996; Junqueira *et al*. 2005; Bernardes *et al*. 2020). It has also been used as a medicinal plant for the treatment of hypertension, diabetes, and inflammatory skin diseases, as well as a sedative (Dhawan *et al*. 2004; Bandara *et al*. 2018; Sudasinghe & Peiris 2018).

Several biotechnological studies have focused on establishing tissue culture systems for *P. suberosa* (Monteiro *et al*. 2000a; Garcia *et al*. 2011a; Rosa *et al*. 2016), which have laid the foundation for medium- and long-term *in vitro* conservation methods, including slow-growth storage and cryopreservation (Garcia *et al*. 2011b; Vianna *et al*. 2019). Garcia *et al*. (2011b) initially reported an encapsulation-vitrification protocol for *P. suberosa* shoot tips, which resulted in a regrowth rate of 28%. In a subsequent study using the same explants, the V-Cryo-plate technique reached a 60% recovery rate and a significant reduction in the plant recovery period. Furthermore, the levels of lipid peroxidation and antioxidant enzymes activities were higher after the osmoprotection and cryoprotection steps, when compared to the other stages of the protocol (Vianna *et al*. 2019).

Cellular damage induced by suboptimal cryopreservation conditions are collectively known as cryoinjury and arises from oxidative stress caused by the overproduction of reactive oxygen species (ROS), resulting in low post-freezing recovery rates and morphogenic and metabolic alterations (Wang *et al*. 2021). Therefore, the development of effective cryopreservation protocols for medicinal plants should also include the assessment of their post-freezing biosynthetic and bioactive capacities (Sharma *et al*. 2021; Wang *et al*. 2021; Markowski *et al*. 2024; Śliwińska *et al*. 2024). In the present work, we examined the effects of ascorbic acid and glutathione applied at the osmoprotection and cryoprotection stages of the V-Cryo-plate protocol for *P. suberosa* shoot tips. Additionally, we evaluated the metabolic stability of cryopreserved materials through qualitative and quantitative analyses of phytoconstituents and assessment of their antioxidant potential.

## Material and Methods

### *Plant material and culture conditions*

*In vitro*-grown plants of *P. suberosa* (Garcia *et al*. 2011a) maintained at the Plant Biotechnology Center of UERJ were used as sources of explants. Plants were monthly subcultured to solidified (0.7% w/v agar; Merck®) MSM basal medium (Monteiro *et al*. 2000b), containing half-strength of MSM salts and MS vitamins (Murashige & Skoog 1962), 1.5% (w/v) sucrose (Merck®), and devoid of growth regulators (1/2 MSM) (Garcia *et al*. 2011a). The pH of all media was adjusted for 5.8 before autoclaving for 15 min at 121 °C. All inorganic chemicals used were P.A. grade.

Cultures were maintained in a growth chamber at 25 °C ± 2 °C under a 16-h light photoperiod, with a total irradiance of 42 μmol $m^{-2}s^{-1}$ provided by white Light Emitting Diodes (LED).

### *Effect of antioxidants on shoot tips cryopreservation*

Shoot tips were cryopreserved according to Vianna *et al*. (2019), using the V-Cryo-plate technique, with some modifications. To evaluate the effects of antioxidants on post-freezing recovery rates, different concentrations of ascorbic

acid (0.15 mM, 0.30 mM) or glutathione (0.08 mM, 0.16 mM) (Sigma-Aldrich®, Brazil) were independently added to the loading solution or to PVS3. Recovery frequencies were recorded 60 and 90 days after rewarming.

### *Extracts preparation*

Leaves excised from *in vitro*-grown plants maintained on 1/2 MSM medium, as well as those from plants derived from cryopreserved shoot tips with or without glutathione, were prepared separately in 40% ethanol (1:50 plant:solvent, w/v), under reflux for 1 h (Birk *et al*. 2005). The extracts were then filtered, dried in hot water bath (90 °C) and stored at -20 °C. Samples were subjected to ultrasound treatment using methanol (Tedia®, Brazil) as solvent for further phytochemical characterization and antioxidant potential assays.

#### *HPLC Analysis*

Qualitative chromatographic analyses were carried out by High-Performance Liquid Chromatography coupled to a UV detector (HPLC-UV) in an Agilent© 1260 Infinity, equipped with LC-10AD pump, DGU- 14A degasser, CTO-10AS oven, SCL-10A controller and SPD-M10A DAD-UV detector. The analyses were performed on a Thermo-Scientific© Hypersil Gold RP18 column (250 mm × 4.6 mm i.d.× 5Å particle size), at a flow rate of 1.0 mL/min and oven temperature at 23 °C. All samples were solubilized in methanol at a final concentration of 1.0 mg/mL and the injected volume was 10 µL. Solvent system consisted of ultrapure aqueous acetic acid solution, pH 3.0 (solvent A) and acetonitrile (solvent B) (Tedia®, Brazil), with the following gradient elution: 95% A and 5% B (0–3 min); 5 to 100% B (3–40 min); 100% B (40–45 min); 95% A and 5% B (45–47 min).

### *Determination of total phenolic content*

Total phenolic content was determined using the Folin-Ciocalteau assay, with slight modifications (Holland *et al*. 2011), employing gallic acid (Sigma-Aldrich®, Brazil) as standard (mg/L). Briefly, 90 µL of extracts (0.25–2.5 mg/mL) resuspended in 0.5 mg/mL methanol were incubated with 10% (v/v) Folin-Ciocalteau solution (180 mL). After 5 min, 100 mM $NaCO_3$ solution (730 mL) were added to the solution, which was further incubated for 2 h in the dark. Quantification was performed spectrophotometrically (Shimadzu UV-B382), at 765 nm. Total phenolic content was further compared to the antioxidant potential to determine the correlation between phenolic content and antioxidant activity (Rudnicki *et al*. 2007).

### *Determination of antioxidant capacity*

#### *DPPH assay*

The antioxidant capacity was evaluated by the ability of the extracts to scavenge the DPPH radical (2,2-diphenyl-1-picrylhydrazyl) (Sigma-Aldrich®, Brazil), according to Sánchez-Moreno *et al*. (1998). Briefly, 25 µL of extracts diluted in 100% methanol (0.25–5 mg/mL) were added to a 60 µM DPPH MeOH solution (975 µL). The samples were incubated for 1 h in the dark, at room temperature, and the decrease in absorbance was spectrophotometrically quantified at 515 nm (Shimadzu UV-B382).

#### *Iron chelating assay*

The ability of extracts to chelate ferrous ion ($Fe^{2+}$) was also determined, according to Santos-Tierno *et al*. (2021). Extracts (250 µL, 0.5–5 mg/mL) were incubated with 0.1 mM $FeSO_4$ (Sigma-Aldrich®, Brazil) (250 µL) for 5 min, before the addition of 0.25 mM ferrozine solution (250 µL). The samples were incubated for 10 min, in the dark, and their absorbances were spectrophotometrically measured at 562 nm (UV-Vis BioMate 3 S, Thermo Scientific).

The extract concentration required for scavenging 50% of DPPH and for chelating 50% of the $Fe^{2+}$($EC_{50}$) was calculated by non-linear regression from graphs of % DPPH scavenging capacity or % chelation ability versus sample concentration (mg/mL).

#### *TLC-DPPH*

The qualitative evaluation of the antioxidant capacity was carried out by TLC-DPPH (Simão *et al*. 2016). For TLC analysis, 20 µL of each extract (5 mg/mL) were directly applied on TLC aluminum plates (Si gel 60 $UV_{254nm}$, Marchery-Nagel, 20 × 20 cm plates). Flavonoid analysis was carried out using ethyl acetate:formic acid:acetic acid:water (100:11:11:26, v/v) as mobile phase (Wagner & Bladt 2001), whereas phenolic acids and saponins were analyzed using chloroform:ethyl acetate:acetone:formic acid (40:30:20:10 v/v) and chloroform:acetic acid:methanol:water (60:32:12:18, v/v) as the mobile phases, respectively (Simão *et al*. 2016; Jesionek *et al*. 2015). The solvents used for the TLC analyses were analytical grade. Methanol was purchased from Tedia®, Brazil, whereas ethyl acetate and chloroform were purchased from Labsynth®, Brazil, and formic acid, acetone and glacial acetic acid were purchased from Sigma-Aldrich®, Brazil. For evaluation of robustness the acetonitrile used was the Merck® brand.

To detect antioxidant activity, all plates were sprayed with 0.02 % DPPH methanolic solution, and maintained in the dark for 30 min. The presence of antioxidant compounds was evidenced by yellow

spots against a purple background (Masoko & Eloff 2007).

### *Statistical analysis*

Cryopreservation experiments were repeated at least twice, using twelve explants per treatment. The determination of total phenolics and the antioxidant assays were carried out in triplicates, in two independent experiments. Statistical evaluation of experimental data was performed by analysis of variance (ANOVA) and Tukey-Kramer comparison post-test (0.05% significance level), using GraphPad Instat (GraphPad Software Inc., San Diego, CA), or MStat-C statistical package. The correlation of the antioxidant potential and total phenolic content was carried out using Pearson Correlation Coefficient at 0.05% significance level using GraphPad Instat.

## Results

### *Effect of antioxidants on shoot tips cryopreservation*

The addition of ascorbic acid significantly reduced regrowth rates, particularly when applied during the cryoprotection stage (Fig. 1). Additionally, whole plant regeneration was achieved 90 days after rewarming (Fig. 2). In contrast, glutathione supplementation accelerated regrowth, regardless of the stage of the cryopreservation protocol or the concentration used, with initial plant regeneration observed 30 days after rewarming and full development within 60 days (Fig. 1). Hence, only plants obtained from this treatment were further analyzed regarding metabolic stability.

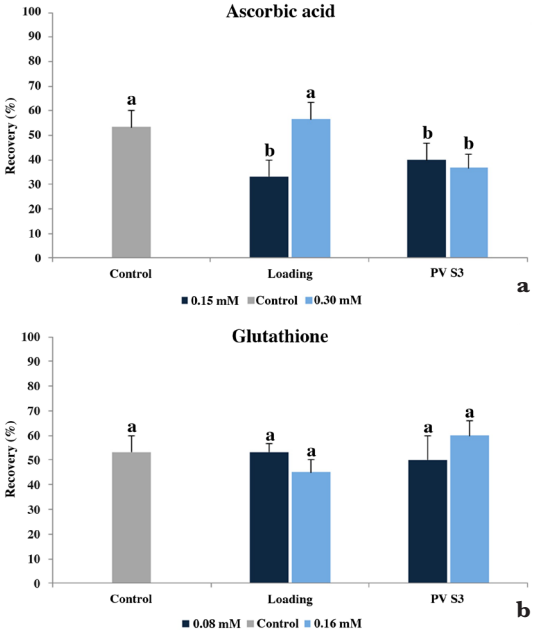


**Figure 1** – a-b. Effect of ascorbic acid and glutathione treatment during osmoproctection and cryoprotection stages of *Passiflora suberosa* shoot tips cryopreservation using the V-Cryo-plate protocol – a. recovery (%) after ascorbic acid treatment; b. recovery (%) after glutathione treatment.

### *Evaluation of metabolic stability after cryopreservation*

#### *HPLC analysis*

HPLC analysis of all samples revealed similar chromatographic patterns of distinct compounds, with retention times (Rt) ranging from 13 to 16 min. Leaves from non-cryopreserved plants showed the highest number of peaks, including two exclusive compounds (peaks 7 and 10, with = 14.8 and 39.5 min, respectively). All detected compounds showed absorption spectra compatible with phenolic acids or flavonoids (λmax~ 330 nm and 360 nm) (Fig. 3).

Compounds with the same retention time were compared based on their relative peak areas. Although there was a decrease in the intensity of all peaks from the cryopreserved-derived plant extracts when compared to non-cryopreserved samples, compound 5 (Rt ~ 13.71 min) showed a comparatively higher signal, suggesting its predominance in all extracts (Fig. 3).

### *Evaluation of the antioxidant potential and correlation with phenolic content*

All extracts showed high antioxidant potential as assayed by the reduction of the DPPH radical, with $EC_{50}$ ranging from 2.58 to 2.72 mg/mL. Extracts also showed high antioxidant potential as metallic ions chelators, with $EC_{50}$ values between 0.92 and 0.99 mg/mL (Tab. 1).

The TLC-DPPH screening technique revealed yellow spots indicating DPPH scavenging activity in extracts from both cryopreserved and non-cryopreserved samples, regardless of the mobile phase used. Saponin analysis depicted three intense yellow spots, while phenolic acid and flavonoid analyses showed that all detected compounds exhibited antioxidant activity (data not shown).

No significant differences in the total phenolic content were detected in plants derived from cryopreserved shoot tips in comparison to control samples (Tab. 1). Furthermore, there was a positive correlation between phenolic contents and antioxidant potential of all materials (Fig. 4).

## Discussion

The V-Cryo-plate technique has been widely adopted for plant cryopreservation due to the high post-freezing recovery rates (Niino *et al*. 2013; Yamamoto *et al*. 2011; Cordeiro *et al*. 2015; Rafique *et al*. 2015; Simão *et al*. 2018). The increased efficiency is mainly associated with the reduced amount

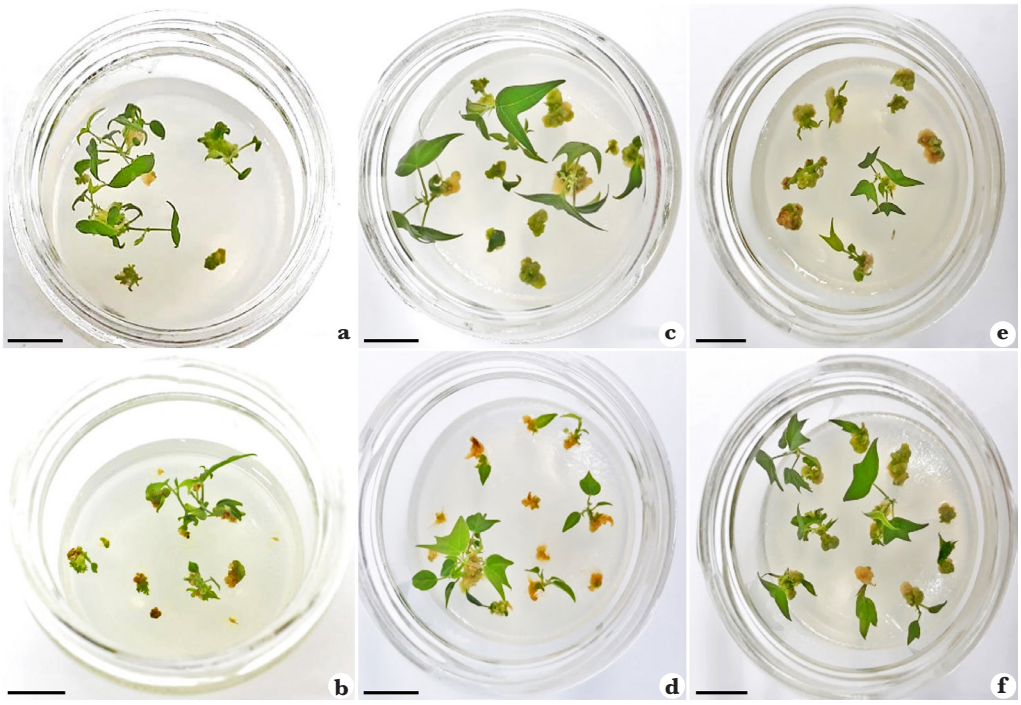


**Figure 2** – a-f. Shoot development from cryopreserved shoot tips of *Passiflora suberosa* after treatment with ascorbic acid or glutathione – a-b. Ascorbic acid (0.30 mM) – a. during loading; b. during PVS3 exposure; c-d. Glutathione (0.08 mM) – c. during loading; d. during PVS3 exposure; e-f. Glutathione (0.16 mM) – during loading; f. during PVS3 exposure. Scale bar = 1 cm.

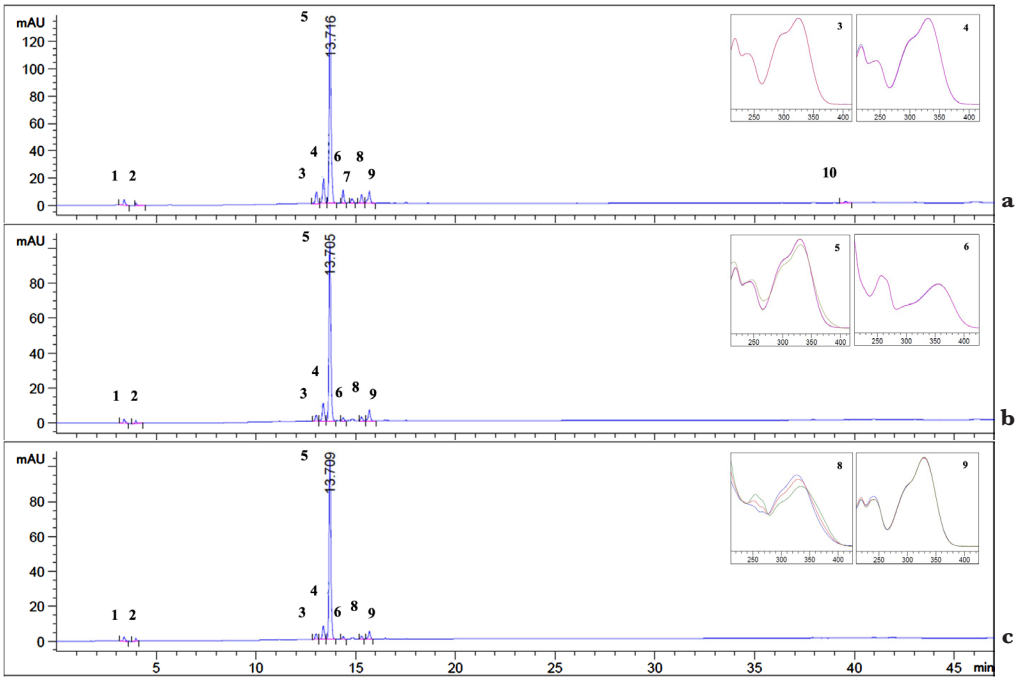


**Figure 3** – a-c. Chromatographic profile by HPLC-DAD-UV ($UV_{340\ nm}$) analysis of leaf extracts from non-cryopreserved and cryopreserved plants of *Passiflora suberosa* – a. leaves of *in vitro* plants derived from non-cryopreserved shoot tips; b. leaves of *in vitro* plants derived from cryopreserved shoot tips, without glutathione treatment; c. leaves of *in vitro* plants derived from cryopreserved shoot tips treated with glutathione. Details show the UV spectra of some peaks.

**Table 1** – Total phenolics and antioxidant activity of leaf extracts of non-cryopreserved and cryopreserved plants of *Passiflora suberosa*.

| Samples | DPPH assay ($EC_{50}$ - mg/mL) | Iron chelating assay ($EC_{50}$ - mg/mL) | Phenolic content (mg GAE/g) |
|---|---|---|---|
| Non-cryopreserved plants | 2.58 ± 0.0[a] | 0.92 ± 0.06[a] | 64.9 ± 4.3[a] |
| Cryopreserved plants (-glutathione) | 2.68 ± 0.047[a] | 0.98 ± 0.03 [a] | 63.0 ± 2.3[a] |
| Cryopreserved plants (+ glutathione) | 2.72 ± 0.044[a] | 0.99 ± 0.06 [a] | 58.9 ± 0.9[a] |

Data represent mean ± standard deviation. The same letters in a column indicate that means are not statistically different according to Tukey-Kramer test ($p < 0.05$).

of cryoprotectant solutions, and the temperature exchange rates at the aluminum base, which lead to lower toxicity and reduce the chance of physical damage caused by the formation of ice crystals (Niino *et al*. 2013; Yamamoto *et al*. 2015; Białoskórska *et al*. 2024). However, plant cryopreservation generally involves adaptations and adjustments in the protocols for each species aiming at reducing cryoinjury and, thus, enhancing regrowth.

The exogenous application of ascorbic acid and glutathione at specific steps of the cryopreservation protocol has been considered a key mechanism to complement the cellular antioxidant system and optimize recovery for several species (Uchendu *et al*. 2010; Zhang *et al*. 2015; Chen *et al*. 2016; Bi *et al*. 2018). In the present work, however, the addition of antioxidant compounds did not contribute to increasing recovery rates from cryopreserved *P. suberosa* shoot tips. Although the use of glutathione has not affected plant development rates after rewarming, the addition of ascorbic acid induced a significant decrease in plant recovery. Similar results have been reported for shoot tips cryopreservation of *Nephelium ramboutan-ake* (Chua & Normah 2011), *Mentha* × *piperita* L. (González-Benito *et al*. 2016), and orchid protocorms (Khor *et al*. 2020). On the contrary, Zhang *et al*. (2015) observed a positive effect in the cryopreservation of embryonic calluses of *Agapanthus praecox*, after treatment with ascorbic acid or glutathione during PVS2 exposure.

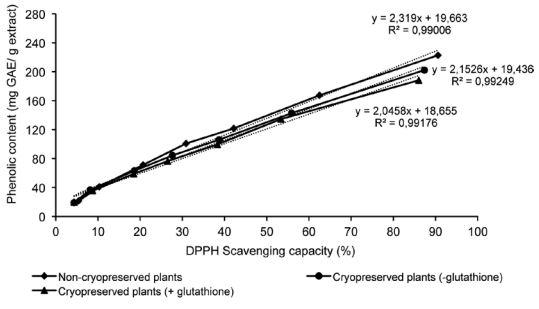


**Figure 4** – Correlation between total phenolic content and antioxidant activity of leaf extracts of non-cryopreserved and cryopreserved plants of *Passiflora suberosa*.

The evaluation of biosynthetic capacity after cryopreservation is essential for the long-term storage of plants with medicinal potential, as it ensures the preservation of metabolic traits and confirms that the biosynthesis of bioactive compounds remains unaffected. In this work, HPLC analysis revealed that although cryopreservation did not qualitatively affect the phytochemical composition of *P. suberosa* plants derived from cryopreserved shoot tips, two exclusive substances (peaks 7 and 10) were detected in leaves of non-cryopreserved plants, with absorption spectra compatible with flavonoids. On the contrary, Bruňáková & Cellárová (2017) observed a significant increase in hypericin and phloroglucinol levels in plants derived from cryopreserved shoot tips of *Hypericum perforatum* and *H. tetrapterum*, respectively, while Śliwińska *et al*. (2024) found an increase in chlorogenic acid content in hairy roots of *Polyscias filicifolia* after dehydration.

The preservation of the antioxidant potential in plants derived from *P. suberosa* cryopreserved shoot tips was another aspect evaluated in this work. Leaves from *in vitro*-grown plants and those derived from cryopreserved shoot tips, with or without glutathione, exhibited high antioxidant potential, as evidenced by both DPPH radical reduction and metal ion chelation. These findings, along with the TLC-DPPH assay results and the correlation between phenolic content and antioxidant capacity, suggest that the antioxidant activity of the extracts is primarily associated with phenolic compounds, particularly flavonoids and phenolic acids, as previously reported for other *Passiflora* species (Lugato *et al*. 2014).

Phenolic compounds are widely recognized for their antioxidant properties, primarily due to their reducing capacity, which enables them to effectively scavenge free radicals. Additionally, they can chelate metals, particularly iron and copper, thereby inhibiting free radical formation (Vuolo

*et al*. 2019). However, their antioxidant efficiency varies depending on the number and arrangement of hydroxyl groups in their structure (Sroka & Cisowski 2003).

Interestingly, our TLC-DPPH analysis also suggests that the antioxidant capacity of *P. suberosa* leaf extracts may be influenced by the presence of saponins. Similar findings have been reported in various species (Francis *et al*. 2002; Ashraf *et al*. 2013; Chen *et al*. 2014), including *Passiflora* biotechnological materials, such as adventitious root cultures of *P. pohlii* (Simão *et al*. 2016).

Our results further support the effectiveness of cryopreservation as a long-term conservation strategy for *P. suberosa* and contribute to the growing body of knowledge on metabolic stability in cryopreserved tropical plants.

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## Data availability statement

In accordance with Open Science communication practices, the authors inform that there is no data sharing of this manuscript.

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