

Autotetraploid synthetic seeds of *Ficus carica* L. exhibit a superior cold acclimation system than their origin diploids

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Abstract

The current study investigates cold conservation of Synseeds of two fig cytotypes using sodium alginate 3% and nodal segments as explants. The biometrical and some important physiochemical parameters were evaluated to determine the cold acclimation responses of both cytotypes' Synseeds. Results demonstrated that in the 9th month of conservation, 37.5% of autotetraploid Synseeds had survived, whereas all diploid Synseeds had died. The survival rate of autotetraploid Synseeds (87.5%) in the 6th month of conservation was significantly higher than that of their origin diploids (54.16%). Autotetraploid Synseeds produced significantly lower amounts of stress indexes such as H_2O_2 and MDA (53.7% and 31.7%, respectively) during the cold conservation period, particularly in the 6th month of conservation, than their origin diploids; which were accompanied by a lower ion leakage (49.75%) and a higher water content (8.34%) compared to their origin diploids. Furthermore, autotetraploid Synseeds exhibited superior capability of accumulating cell osmolytes in the 6th month of conservation than their origin diploids, including higher contents up to 34.62% TSS, 73.78% proline, and 43.96% glycine betaine. The activity of two key antioxidant enzymes of CAT and APX was also significantly higher in autotetraploid Synseeds up to 48.14% and 30.29% in the 6th month of conservation, respectively, than in their origin diploids. According to the findings, it could be concluded that the autotetraploid cytotype has significantly more tolerance to cold stress due to its superior cold acclimation strategies, mainly including the osmolytes accumulation and cell detoxification dependent on antioxidant enzymes, than their origin diploid cytotype.

Key Message

The cold conservation capability of tetraploid and diploid synthetic seeds of the fig tree was evaluated, and tetraploids exhibited higher survival ability through elevated cold acclimation strategies.

1. Introduction

The common fig (*Ficus carica* L.) is a fruit tree belonging to the Moraceae family (Kislev et al. 2006; Flaishman et al. 2008). Due to its well-adaptation to harsh climates, it is considered a promising fruit tree for sustainable production in arid and semi-arid areas worldwide. Over the past two decades, the Middle East and Mediterranean regions, which are the major fig cultivation areas, have confronted some limiting factors, such as increased average temperature, severe depletion of underground waters, and decreased annual precipitation, which have seriously threatened many agricultural activities. The common fig has a great potential for expanding cultivation and maintaining sustainable production in these areas due to its great tolerance to a wide range of abiotic stresses (drought, heat, saline, and alkaline soils), as well as its high nutritional value and high economic importance (Crisosto et al. 2010; Badgujar et al. 2014; Doaa et al. 2015; Ibrahim et al. 2017).

Polyploidy (whole genome multiplication) is considered a significant phenomenon with a crucial role in plant speciation and adaptation during evolutionary periods (van de Peer et al. 2021). Plant breeders

have thus considered synthetic polyploidy induction as a pivotal breeding method to reinforce plants' tolerance to a wide range of abiotic stresses, such as drought, salinity, cold, and heat (Sattler et al. 2016). Our previous research successfully developed an *in vitro* polyploidy induction method in the fig tree (Abdolinejad et al. 2021), which resulted in the production of autotetraploid plants that exhibited a significantly greater drought tolerance than their origin diploids by augmenting both enzymatic and non-enzymatic defensive strategies (Abdolinejad and Shekafandeh 2022).

The synthetic seed (artificial seed or Synseed) technology was defined for the first time by Murashige (Murashige 1977) as “an encapsulated single somatic embryo”. Since then and working on various explants, the concept was extended to “artificially encapsulated various *in vitro* derived explants, including, somatic embryos, shoot tips, single nodes (microshoots), cellular aggregates or any other micropropagules that have the capability of regrowing and converting to a plant under either *in vitro* or *ex vitro* conditions” by other scientists (Rihan et al. 2017). Nowadays, Synseed technology and cryopreservation techniques are extensively employed for the propagation and conservation of many plant species and transport of elite, pathogen-free, and genetically modified plant germplasms between national and international laboratories (Faisal and Alatar 2019; Rai et al. 2021). In this regard, sodium alginate, a polysaccharide derived from various species of seaweed and bacteria, is the most used agent for plant material encapsulation. The characteristic of forming a semi-permeable gelling coat around the propagules, and the capability of preserving water and solutes, keeping a balance between O₂ and CO₂ gasses, controlling biochemical changes, and delaying damage to tissues (Martínez-Cano et al. 2022) have made sodium alginate the best choice for the encapsulation-conservation of *in vitro*-derived explants.

Storing the encapsulated propagules at low temperatures for medium to long periods could adversely affect explant tissues and reduce their regrowth potential by interfering with the normal functions of plant cells at biometrical, physiological, and cellular levels. This is associated with symptoms such as tissue browning, necrosis, and chlorosis, and increased levels of phenolic compounds and anthocyanins (Bhandari and Nayyar 2014). Cold stress has a range of harmful consequences on plant cells, initiating with membrane rigidification as the first influence of cold-stress responses and continuing with disrupting the stability of protein complexes, decreasing the activity of enzymes, particularly ROS scavenging enzymes, and subsequently affecting gene expression and protein synthesis, by hindering the RNA processing events (Ding et al. 2019). Plants have developed some defensive mechanisms to mitigate cold stress injuries, such as cold acclimation, which is considered the main strategy plants employ to deal with cold stress conditions (Thomashow 1999), including a series of physiological mechanisms, including enhancing the enzymatic and non-enzymatic defensive activities and accumulating osmoprotectants and protective proteins (Kaplan et al. 2007).

In this study, the cold-conservation capability of diploid and induced autotetraploid Synseeds of *F. carica* cv. Sabz was investigated over 12 months. Furthermore, the parameters related to regrowth ability alongside those physiological and biochemical mechanisms relating to the cold acclimation process

were also assessed every three months intervals to determine the differences between diploid and autotetraploid in their cold acclimation responses.

2. Material and methods

2.1. Plant material and explant preparation

The *in vitro* growing plants from two cytotypes of *F. carica* cv. Sabz, including diploid ($2n = 2x = 26$) and induced-autotetraploid ($2n = 4x = 52$) plants obtained from our previous study by Abdolinejad et al. (2021), were used in this study. Initially, four hundred stem segment explants of both cytotypes were cultured on MS medium (Murashige and Skoog 1962) supplemented with 87.64 mM sucrose, 19.32 mM agar, 8.84 μM 6-Benzylaminopurine (BAP), and 1.07 μM 1-Naphthaleneacetic acid (NAA) and environmental conditions of 16/8 h light/darkness (light intensity of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$) and $25 \pm 1^\circ\text{C}$ for four weeks, to obtaining enough uniform explants for the encapsulation cold conservation experiment. Stem segment explants consisting of shoot tips and single nodes (microshoots), approximately 5 mm in length and 1.5-2 mm thickness, were determined to be appropriate for encapsulation. The defoliation process and cutting of microshoots were conducted in Petri dishes under a laminar airflow cabinet, then excised explants were instantly transferred to a liquid MS medium to avoid dehydration under airflow.

2.2. Encapsulation and synthetic seed production

The microshoots were then submerged in a 250 ml beaker containing 100 ml of 3% (w/v) sodium alginate dissolved in MS medium but without calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) and agar, and supplemented with 3% sucrose, 2.21 μM BAP, and 0.54 μM NAA. The medium containing microshoots was drawn into the tip using a 1000 μl micropipette equipped with a tip adapter of 5 mm (in diameter) and then microshoots were slowly dispensed one by one into a 250 ml beaker containing 100 ml of 1% (w/v) CaCl_2 to generate spherical synthetic seeds (Synseeds). The beaker was shaken gently for 30 min and followed by rinsing the synthetic seeds with 100 ml of sterile distilled water for 5 min (Fig. 1).

2.3. Cold conservation and regrowth medium conditions

All generated Synseeds were divided into four sections, each containing 600 Synseeds, placed into a 500 ml jar and kept in a refrigerator at 4°C , in darkness. The experiment lasted 12 months, and every 3-month 150 Synseeds of both cytotypes were taken away, of which 100 Synseeds (five grams) for physiochemical assessments and the other 48 for the regrowth capability experiments were used. The regrowth medium consisted of MS medium supplemented with 87.64 mM sucrose, 19.32 mM agar, 4.42 μM BAP, and 0.55 μM NAA and the cultures were placed in the growth room under a 16/8 h light/darkness photoperiod (light intensity of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$) and $25 \pm 1^\circ\text{C}$ conditions.

2.4. Synseed regrowth and biometrical parameters

Parameters related to Synseed recovery and growth, including browning index, bud break percentage, average growth rate (AGR), and plant height, were evaluated as follows:

The browning index was visually estimated based on scoring of browning intensity from 1 (lowest browning index) to 5 (highest browning index).

The bud break percentage was calculated until 10 days after culturing the Synseeds on the regrowth medium using the following formula:

$$\text{Budbreak (\%)} = \left(\frac{\text{Number of germinated Synseeds}}{\text{Total number of incubated Synseeds}} \right) \times 100$$

The average growth rate parameter was calculated four weeks after culturing the Synseeds on the regrowth medium using the following formula:

$$\text{AGR} = \left(\frac{H2 - H1}{T} \right)$$

where H1 = First measurement, H2 = Second measurement, and T = Time interval between starting bud break to the second measurement (5 days).

Plant height was measured by a ruler.

2.5. Physiological measurements

The physiological measurements were conducted by removing the nodal explants of both cytotypes from sodium alginate coatings.

2.5.1. Relative water content (RWC) and ion leakage (IL)

To calculate the Synseeds RWC, the initial weight, dry weight, and turgid weight of samples were estimated, and then the RWC was determined using the following formula:

$$\text{RWC (\%)} = \left(\frac{\text{Freshweight} - \text{Dryweight}}{\text{Turgidweight} - \text{Dryweight}} \right) \times 100$$

The ion leakage rate of Synseeds was determined by recording the initial electrical conductivity (EC1) and the second electrical conductivity (EC2) and finally calculating the electrolyte leakage using the following formula:

$$\text{Ionleakage (\%)} = \left(\frac{\text{EC1}}{\text{EC2}} \right) \times 100$$

2.6. Biochemical

In every sampling time, including the 3rd, 6th, and 9th months of the cold conservation period, five grams of stem explants were collected following the extraction from the Synseeds and immediately powdered in liquid nitrogen and kept at – 80°C for further experiments.

2.6.1. Total carotenoid content

According to Roy et al. (2021) method, total carotenoid content was extracted by homogenizing 50 mg of stored powder with an extraction solution consisting of ethanol, acetone, and distilled water (2.25: 2.25: 0.5) and placed in a cold environment (4°C) for 24 h. The mixtures were then centrifuged at 10000 g for 10 min at 4°C and then subjected to 470 nm light absorbance.

2.6.2. Hydrogen peroxide (H₂O₂) content

According to Sarker and Oba (2018), 100 mg of stored powder was mixed with 500 µl of 0.1% TCA and left to stand for 5 min, then the samples were centrifuged at 13000 g for 10 min at 4°C. The reaction mixture consisting of 250 µl of supernatant, 250 µl of 100 mM potassium phosphate buffer (pH = 7), and 500 µl of 1 M potassium iodide was kept in the dark for 60 min and light absorbance was recorded at 390 nm.

2.6.3. Malondialdehyde (MDA) content

To estimate the MDA content in explants, 50 mg of stored powder was homogenized in 1 ml of ethanol: distilled water (80% v/v) solution, then centrifuged at 10000 g for 15 min. 500 µl of the supernatant was mixed with 500 µl of thiobarbituric acid (TBA) solution consisting of 20.0% (w/v) trichloroacetic acid (TCA) and 0.01% butylated hydroxytoluene. The mixture was then heated at 95°C for 30 min, cooled on ice, and centrifuged at 7000 g for 10 min. The light absorbance of the samples was recorded at 414 nm, 532 nm, and 600 nm (Hodges et al. 1999). The MDA content was calculated using the following formula:

$$\text{MDA} = 6.45(\text{Abs } 532 - \text{Abs } 600) - 0.56 \text{ Abs } 414$$

2.6.4. Total soluble sugars (TSS)

The method of Patade et al. (2012) was used for the determination of the samples TSS. The procedure included mixing 50 mg of stored powder with 5 ml of an ethanol: distilled water solution (4:1 v: v) and keeping it at room temperature for 30 min, followed by centrifugation at 10000 g for 15 min at 4°C. 1 ml of the supernatant was then mixed with 3 ml Anthrone reagent (150 mg anthrone + 100 ml H₂SO₄ 72%) and placed in a water bath at 100°C for 15 min before being cooled on ice. The light absorbance was read at 620 nm and glucose was used as the standard sample.

2.6.5. Total phenolic content (TPC)

According to Harzallah et al. (2016), the total phenolic content of samples was quantified using the Folin-Ciocalteu method. 100 mg of stored powder was combined with 2 ml of a methanol: distilled water: HCl extraction solution (8:1.9:0.1 v/v/v) and incubated at room temperature for 2 h. The mixture was then centrifuged at 7000 g for 15 min, and the supernatant was moved to a new vial. 100 µl of this extract was mixed with 750 µl of a 10-fold diluted Folin-Ciocalteu reagent, allowed to rest for 5 min at room temperature, and then 750 µl of 0.7 M sodium bicarbonate solution was added and incubated for 90 min

at room temperature and finally, light absorbance was read at 725 nm using a microplate spectrophotometer.

2.6.6. Proline content

The free proline content of samples was determined by homogenizing 50 mg of stored powder with 2 ml of 3% sulfosalicylic acid and centrifuging at 7000 g for 5 min. Then, 1 ml of the supernatant was reacted with 1 ml of acid ninhydrin and 1 ml of glacial acetic acid and kept in a water bath at 100 ° C for 60 min before being immediately cooled on ice. Thereafter, 2 ml of toluene was added to the mixture and vortexed for 20 seconds and left to stand for 15 min at room temperature. The light absorbance of the supernatant was spectrophotometrically recorded at 520 nm (Bates et al. 1973).

2.6.7. Glycine betaine content

To quantify the glycine-betaine content of samples, 50 mg of stored powder was homogenized with 2 ml of distilled water and shaken for 16 h at ambient temperature. Then, 2 ml of sulfuric acid (1:1, v/v) was added to the sample and left undisturbed for 1h before filtration through 45 µm filters. The filtered sample was then kept on ice for 60 min. 500 µl of the extract was mixed with 200 µl of iodine-potassium iodide (KI-I₂) reagent and kept at 4°C for an additional 16 h. The mixture was centrifuged at 10000 g for 5 min at 0°C and then lyophilized. The resulting per-iodide crystal was dissolved in 1 ml of 1,2-dichloroethane and the light absorbance was measured by a spectrophotometer at 365 nm (Patade et al. 2012).

2.7. Antioxidant enzymes measurements

To determine the activity of antioxidant enzymes, initially, an explant extract was provided according to Abdolinejad and Shekafandeh (2022) and used for further experiments.

2.7.1. Superoxide dismutase (SOD) activity

SOD activity was assessed via its capacity to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Manquían-Cerda et al. 2016). The reaction mixture included 300 µl of enzyme extract, 400 µl of potassium phosphate buffer (100 mM, pH 7.0), 10 µl EDTA (10 mM), 50 µl methionine (260 mM), 80 µl NBT (4.2 mM), and 170 µl riboflavin (130 µM). Illumination was provided by cool-white fluorescent tubes at a light intensity of 60 µmol m⁻² s⁻¹ for 15 min and light absorbance was taken at 560 nm using a spectrophotometer.

2.7.2. Catalase (CAT) activity

The CAT activity of the samples was assessed by the method of Chance and Maehly (1955). Accordingly, 15 µl of enzyme extract, 2885 µl potassium phosphate buffer (50 mM, pH 7.0), and 100 µl hydrogen peroxide (10 mM) were mixed, and the light absorbance was spectrophotometrically recorded at 240 nm. The CAT activity was determined by recording the decrease in optical absorbance occurring due to the decomposition rate of H₂O₂ over one min.

2.7.3. Ascorbate peroxidase (APX) activity

The APX activity of the samples was assessed using the method of Nakano and Asada (1981). Accordingly, 20 µl of enzymatic extract, 1950 µl potassium phosphate buffer (50 mM, pH 7.0) supplemented with 100 µM EDTA and 100 µM sodium ascorbate, and 30 µl hydrogen peroxide (2.5 mM) were mixed, and the light absorbance was spectrophotometrically read at 290 nm.

2.7.4. Glutathione peroxidase (GPX) activity

The GPX activity was determined by assessing the guaiacol oxidation in the presence of hydrogen peroxide during 2 min and 20 seconds intervals (Ozden et al. 2009). A reaction mixture containing 50 µl of guaiacol (20 mM), 2900 µl of potassium phosphate buffer (10 mM, pH 7.0), and 50 µl of enzyme extract, was initiated by adding 20 µl of H₂O₂ (40 mM), and the light absorbance was spectrophotometrically recorded at 470 nm.

2.8. Experimental design and data analysis

The current study was conducted in a factorial experiment with two factors, including cytotype (diploid and autotetraploid) × time (0, 3, 6, and 9 months) in a completely randomized design with 6 replications and 8 explants per replicate. All collected data were subjected to analysis of variance using the GLM procedure of SAS software version 9.4 (SAS institute, USA), and the means were compared by Duncan's multiple range test ($p \leq 0.05$). Where the data contained zero, it was subjected to a logarithmic transformation.

3. Results

The results of ANOVA revealed that the interactions between cytotype and cold conservation time were statistically significant for all investigated parameters (Supplementary files, S1, S2, and S3). Autotetraploid Synseeds exhibited superior resilience to cold conservation conditions than their origin diploids, as they could survive for 9 months while their origin diploids only survived until the 6th month of conservation (Fig. 1). Thus, the comparative results of the two cytotypes have mainly focused on this conservation time. It is worth noting that the regrowth and biometrical responses of two cytotypes were evaluated only for the 3rd, 6th, and 9th months of cold conservation but not for the freshly prepared explant in the control (0 month). Furthermore, as neither autotetraploid nor diploid Synseeds had not survived in the 12th month of the conservation (Fig. 1), none of the biometrical and physiochemical parameters were measured.

3.1. Biometrical responses

Results demonstrated that the browning index of both cytotypes Synseeds significantly increased over the conservation time. However, the browning index was significantly higher in diploid explants than in autotetraploids. The highest browning index (4.33/5) was found in diploid explants of the 9th month of conservation and the minimum browning index (1.33/5) was observed in autotetraploid explants of the 3rd month of conservation. Indeed, the browning index of explants was 1.87, 1.81, and 1.62-fold higher in

diploids compared with autotetraploids in the 3rd, 6th, and 9th months of conservation, respectively (Fig. 2, A).

The results indicated that the ability of both cytotypes' Synseeds to initiate bud growth declined significantly over the cold conservation time. Although there was no significant difference between the two cytotypes in bud break percentage in the 3rd month of the conservation, autotetraploid Synseeds exhibited a significantly higher bud break capability (87.5%) than their origin diploid Synseeds (54.16%) in the 6th month of the conservation. Furthermore, in the 9th month of conservation in which diploid Synseeds did not exhibit any bud break, autotetraploid Synseeds displayed a 37.5% bud break (Fig. 1 and Fig. 2, B).

Although the cold conservation conditions adversely affected the shoot length and AGR features of both cytotypes, autotetraploid Synseeds were significantly less affected; thus, the autotetraploid shoot length and AGR measurements were 1.85 and 2.64 folds greater in the 3rd month and 2.05 and 4.33 folds greater in the 6th month of conservation than their respective diploids (Fig. 1 and Fig. 2, C, D).

The rooting and acclimatization capabilities of both cytotypes were also dramatically affected by the cold conservation conditions. Although the results did not show a significant difference between the two cytotypes in the 3rd month of the conservation, the rooting and acclimatization capability of autotetraploid Synseeds were significantly higher than their origin diploids in the 6th and the 9th months of conservation. Indeed, while autotetraploid Synseeds retained their rooting and acclimatization capability at a high level of 81.93% until the 6th month of conservation, the diploid explants displayed a significant decrement of up to 30.53 and 41.67% in their rooting and acclimatization capability, respectively. In the 9th month of conservation, where the diploid Synseeds were not survived, the autotetraploid explants did not exhibit a significant decrease in their rooting and acclimatization capability than their status in the 6th month of conservation (Fig. 1 and Fig. 2, E, F).

3.2. Physiological responses

The RWC and ion leakage parameters of both cytotype Synseeds were significantly affected during cold conservation time. The results demonstrated that the RWC decrement rate in the autotetraploid Synseeds followed a gentle slope from 93.63% in the control explants to 76.43% in the Synseeds of the 9th month of conservation, whilst diploid Synseeds displayed a drastic decrement in their RWC from 95.15% in control explants to 46.68% in the Synseeds of the 9th month of conservation (Fig. 3, A). Significant differences between autotetraploid Synseeds and their origin diploids in RWC at all sampling times were found, so that, the RWC in autotetraploid Synseeds was 8.34% and 63.7% higher than their origin diploids in the 6th and 9th months of conservation, respectively (Fig. 3, A). The ion leakage alteration also followed the same trend but contrariwise to the RWC alterations during the cold conservation period. Compared to the origin diploid Synseeds, the ion leakage rate was significantly lower in autotetraploid Synseeds up to 29.31%, 49.75%, and 39.44% in the 3rd, the 6th, and the 9th months of the cold conservation, respectively (Fig. 3, B).

The H₂O₂ and MDA contents of both cytotype Synseeds were significantly increased by increasing the cold conservation duration. However, autotetraploid Synseeds accumulated significantly lower levels of H₂O₂ and MDA than their origin diploids in the 3rd, 6th, and 9th months of conservation. Thus, the H₂O₂ and MDA contents in autotetraploid Synseeds were 53.7% and 31.7% less than their origin diploids in the 6th month of conservation, respectively, as well as 32.87% and 36% lower in the 9th month of conservation (Fig. 3, C, D).

3.3. Osmoprotectants responses

The results demonstrated that autotetraploid Synseeds had significantly accumulated greater cell osmolytes, including TSS, proline, and glycine betaine contents, compared to their origin diploid Synseeds during the cold conservation period. In the 3rd and 6th months of cold conservation, autotetraploid Synseeds accumulated significantly higher TSS contents, up to 29.27% and 34.62%, than their origin diploids, respectively. In the 9th month of conservation where the diploid Synseeds were not survived, the TSS content in autotetraploid Synseeds was 49.38% higher than their origin diploids (Fig. 4, A). The autotetraploid Synseeds also dramatically accumulated a greater content of proline than their origin diploids, so that the proline content of autotetraploid Synseeds was 82.29% and 73.78% higher in the 3rd and 6th months of conservation, respectively, than their origin diploids (Fig. 4, B). The two cytotypes also displayed a significant difference in glycine betaine content during the cold conservation period. There was no significant difference between diploid and autotetraploid Synseeds in the 3rd month of conservation, but autotetraploid Synseeds had accumulated a significantly higher level of glycine betaine (43.96% higher) in the 6th month of conservation (Fig. 4, C).

A significant difference in the total carotenoid content was observed between autotetraploid Synseeds and their origin diploids during the cold conservation period. While there was no significant difference between the two cytotype Synseeds in the 3rd month of conservation, autotetraploid Synseeds had biosynthesized significantly higher contents of carotenoids (60.56% higher) compared to their origin diploid Synseeds in the 6th month of conservation (Fig. 4, D).

According to the results, diploid Synseeds produced a significantly greater TPC in all sampling times of the cold conservation period than their origin diploid. The TPC contents of autotetraploid Synseeds were up to 47.02%, 30.31%, and 41.11% higher than their origin diploid Synseeds in the 3rd, the 6th, and 9th months of conservation, respectively (Fig. 4, E).

3.4. Enzymatic activity responses

The results revealed that the antioxidant defensive system of both cytotypes, including the activities of SOD, CAT, APX, and GPX enzymes, was significantly increased influenced by the cold conservation conditions. Accordingly, SOD activity was significantly higher in diploid Synseeds than in autotetraploid Synseeds. Indeed, the SOD activity was 12.5% and 16.81% less in autotetraploid Synseeds than their origin diploid Synseeds in the 3rd and 6th months of conservation, respectively (Fig. 5, A). The CAT

activity was dramatically higher in autotetraploid Synseeds than their origin diploid Synseeds, up to 65.5% and 93.15% in the 3rd and 6th months of conservation, respectively, which indicated a statistically significant difference between them. However, the CAT activity of autotetraploid Synseeds in the 9th month of conservation significantly decreased, up to 38.18% than its activity in the 6th month of conservation (Fig. 5, B). The autotetraploid cytotype also exhibited superiority in APX activity during cold conservation conditions. The APX activity in autotetraploid Synseeds was 48.14% and 30.29% higher than their origin diploids in the 3rd and 6th month of conservation, respectively. Nevertheless, autotetraploid Synseeds displayed a 20.2% decrement in their APX activity in the 9th month of conservation than in the 6th month of conservation (Fig. 5, C). The GPX activity was significantly higher in the diploid Synseeds, up to 6% and 12.17%, respectively, under the optimal growing conditions (control) and the 3rd month of conservation compared to the autotetraploid Synseeds. However, a significant increase in GPX activity up to 3.95% was observed in the autotetraploid Synseeds than in their origin diploid Synseeds in the 6th month of cold conservation. Nonetheless, increasing the conservation time from the 6th month to the 9th month, led to a significant decrement of 24% in GPX activity in autotetraploid Synseeds (Fig. 5, D).

3.5. Principal component analysis

The principal component analysis, as displayed in Fig. 6 (score plot (A) and loading plot (B)), was performed based on the physiological and biochemical responses of Synseeds of two fig cytotypes during cold conservation conditions. The results revealed that PC1 and PC2 accounted for 59.4% and 33.7% of the variation, respectively. Accordingly, under the optimum growth conditions (control treatment) two cytotypes were linked to the RWC, bud break, and AGR features. The positive side of PC1, which was gathered by both cytotype Synseeds in the 3rd and 6th month of cold conservation, was marked by maximum biosynthesis of those physiological and biochemical responses relating to the enzymatic and non-enzymatic defensive system, i.e., TSS, carotenoids, proline, glycine betaine, SOD, CAT, APX, and GPX. While the positive side of PC2, which consisted of the physiological and biochemical responses of diploid Synseeds in the 6th and the 9th months of cold conservation and autotetraploid Synseeds in the 6th month of conservation, was linked to the high values of stress markers such as browning index, ion leakage, TPC, H_2O_2 , and MDA (Fig. 6).

4. Discussion

The current experiment lasted for 12 months, and neither autotetraploids nor diploids could survive for 12 months (Fig. 1). However, the autotetraploids displayed a significantly better cold acclimation performance than their origin diploids since they kept their regrowth potential for 9 months, while their diploid progenitors could only survive for 6 months. Mid- to long-term Synseeds conservation under low temperatures is associated with chilling injuries, especially in the case of subtropical crops such as *F. carica*. In our experiment, diploid Synseeds were remarkably much more affected by chilling injuries, as they exhibited a significantly higher browning index, lower bud break percentage, and lower growth rate

than autotetraploid Synseeds. Furthermore, a dramatic decrease in rooting and acclimatization capability also occurred. Although the vital requirements of explants, including sucrose, vitamin, minerals, and water, were provided for them by encapsulation during the conservation period, low temperature causes suspension of the vital physiological and biological processes related to cell metabolism, stability of membrane proteins, and membrane rigidification and resulting in interference with all various membrane functions (Siddiqui and Cavicchioli 2006; Seydi et al. 2020). Cold stress also disrupts the mitochondrial and chloroplastic electron transfer reactions, which leads to the overgeneration of reactive oxygen species (ROS). ROS damage cell membranes and lead to increased ion leakage (Ruelland et al. 2009).

Autotetraploid Synseeds remarkably produced lower amounts of stress markers such as H_2O_2 and MDA, than their origin diploids, indicating that they benefit from better acclimation mechanisms to preserve their cell membranes from oxidative damages, leading to lower ion leakage and keeping RWC in a low-risk area and preventing cell dehydration (Liu et al. 2011; Jan et al. 2018). Lower accumulated stress markers such as H_2O_2 and MDA under stressful conditions are considered a primary marker for identifying tolerate genotypes to different abiotic stresses (Jiroutova et al. 2021), whereas a high-level generation of H_2O_2 and MDA is associated with damage to cell compartments and disrupting biological systems (Yadav 2010). It has been proven a relationship between polyploidy induction and enhanced tolerance to cold stress (Jan et al. 2018; Lourkisti et al. 2020; Syngelaki et al. 2021), which is mainly related to the defense mechanisms and some augmented physiological responses, e.g., maintaining cell membrane stability, preventing extreme cell dehydration, decrement ion leakage, and accumulating lower levels of H_2O_2 and MDA. These physiological mechanisms are regulated by two major widespread defensive strategies, including the accumulation of cell osmolytes and increased biosynthesis of antioxidant enzymes (Ruelland et al. 2009). In our study, PCA indicated that cold acclimation mechanisms are highly related to osmolytes accumulation and the activity of antioxidant enzymes in both cytotypes (Fig. 6). Thus, we followed the accumulation and activity alterations of two main representatives, osmolytes and antioxidant enzymes.

Soluble sugars (mono and disaccharides) are the most common cell osmolytes, and their concentrations increase in various abiotic stresses, including cold stress (Tarkowski and Van den Ende 2015). As multifunctional molecules, they alleviate the adverse effects of cold stress by contributing to different mechanisms, including cell energy supply, stabilization of structure and conformations of various enzymes, proteins, and cell membranes, preventing cell dehydration and plasmolysis via filling intermembrane spaces, and ROS scavenging (Tarkowski and Van den Ende 2015; Aftab and Hakeem 2021). A synergistic correlation of elevated levels of cell osmolytes such as TSS, proline, and GB has improved stress tolerance in numerous studies (Bhandari and Nayyar 2014; Tan et al. 2015), and our results also confirmed this. Indeed, autotetraploid Synseeds exhibited elevated levels of osmoprotectants during the cold conservation period, especially in the critical period of the 6th month (Fig. 4), during which their diploid progenitors displayed a drastic decrement in bud break percentage and osmoprotectants accumulation. This indicates that elevated levels of osmolytes have significantly protected autotetraploid Synseed cells and improved their tolerance in confronting long-term exposure to cold stress. Like soluble

sugars, proline and GB are also involved in cold acclimation mechanisms such as maintaining cell turgor, stabilization, and protection of cell membranes and macromolecules, ROS scavenging, regulating the expression of osmotic stress-related genes, regulating fluid and ion homeostasis, and redox balance during stressful conditions (Khan et al. 2017; Dikilitas et al. 2020). Abundant evidence has shown that neopolyploids acquire improved tolerance to cold stress compared to their diploid progenitors (Li and Van de Peer 2020), in most cases by the mechanisms related to the increased expression of stress related genes and increased cell size (Doyle and Coate 2019). Tetraploidy induction in *Plumbago auriculata* led to an increment in cold stress tolerance via augmented ROS scavenging, lower production of MDA, and enhanced stability of cell membranes (Jiang et al. 2020). Polyploidization has also improved the cold acclimation capability in *Nicotiana benthamiana* (Deng et al. 2012) and grafted Clemantines on tetraploid rootstocks (Oustric et al. 2017) by reinforcing the mechanisms related to ROS scavenging, increasing photosynthesis efficiency, decreasing H₂O₂ and MDA accumulation, and decrement ion leakage percentage.

Regarding the suspension of vital physiological and biological activities in Synseeds under cold conservation conditions, the production of ROS increases exponentially in cell compartments such as mitochondria, chloroplasts, and plasma membranes (Awasthi et al. 2015). Since overaccumulation of ROS is lethal for plant cells, they must be eliminated instantly by plant antioxidative defense systems. Antioxidant enzymes have been proven to play a crucial role in plant detoxification under environmental stresses, including cold stress (Awasthi et al. 2015; Hasanuzzaman et al. 2020; de Araújo et al. 2021). In the present study, antioxidant enzymes played a determinant role in alleviating the cold conservation harmful effects in both cytotype Synseeds, as the activity of all assayed enzymes had significantly increased compared to the control growth conditions. However, autotetraploid Synseeds displayed a more efficient ROS scavenging system than their origin diploids, especially in the 9th month of conservation, where diploid Synseeds could not survive. PCA also indicated a close relationship between cold acclimation and antioxidant enzyme activities in both cytotypes (Fig. 6). According to the results (Fig. 5), SOD activity revealed that a balance between the generation and removal of ROS has been established in autotetraploid Synseeds in the 3rd to the 9th months of conservation, while a significant increasing trend was found in their origin diploids in the 3rd to the 6th months of conservation. On the other hand, the CAT and APX activities of autotetraploid Synseeds in the 3rd and 6th months of conservation were dramatically higher than their origin diploids, two strong antioxidant enzymes that can convert millions of molecules of H₂O₂ into H₂O and O₂ in a min, indicating their crucial role in cell detoxification under stressful conditions. Polyploidization in different plant species has significantly reinforced the antioxidant defense system, by enhancing the activity of SOD, CAT, APX, and GTX, in various abiotic stresses (Deng et al. 2012; Oustric et al. 2017; Tossi et al. 2022; Abdolinejad and Shekafandeh 2022).

5. Conclusion

In the current study, a method based on alginate encapsulation was established for cold conservation of *in vitro*-derived diploid and autotetraploid explants in *F. carica*. The results revealed that autotetraploid

Synseeds were significantly less affected by the adverse effects of the cold conservation period. Indeed, they survived until the 9th month of conservation, while their origin diploids only survived for 6 months. In addition, in the 3rd and 6th months of conservation, autotetraploid Synseeds displayed a remarkably higher bud break, AGR, rooting, and acclimatization than their origin diploids, which indicated they had employed more efficient cold acclimation strategies. From the physiological aspect, autotetraploids accumulated significantly fewer amounts of H_2O_2 and MDA than their origin diploids, which means less damage to the cell membrane and resulted in remarkably lower ion leakage and keeping higher RWC. Our findings also highlighted that the higher resilience of autotetraploid Synseeds to cold conservation conditions could be closely related to their reinforced cold acclimation strategies compared to their origin diploids. Autotetraploids significantly accumulated higher amounts of cell osmolytes such as soluble sugars, proline, and GB. Also, the activity of antioxidant enzymes CAT and APX, which are responsible for converting the cold stress-induced generated H_2O_2 into H_2O and O_2 during the cell detoxification process, was significantly higher in autotetraploids Synseed than in their origin diploids.

Declarations

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contribution

RA Performed practical experiments, data collection, data analysis, and writing original draft, Writing – review & editing. **AS** Conceptualization, validation, supervision, writing, review & editing, and funding acquisition. All authors have reviewed and agreed to the final draft of the manuscript.

Data availability: The datasets generated during the current study are available from the corresponding author on reasonable request.

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Figures

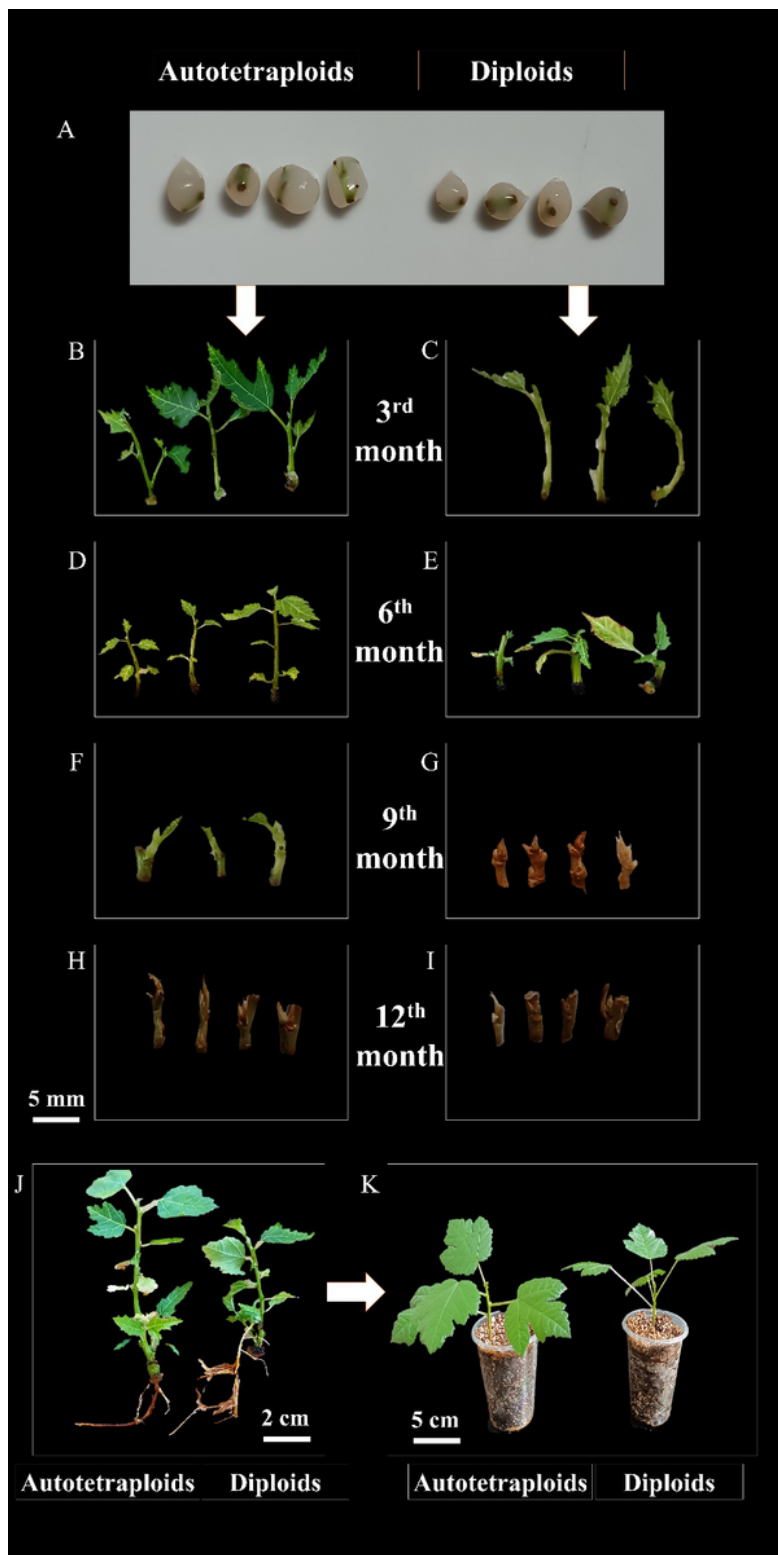


Figure 1

Synseed production of diploid (right sides) and autotetraploid (left sides) fig cytotypes using stem segments as explants (0.5 mm in length). A: generated Synseeds ready to cold conservation, B and C: bud break and regrowth capability in Synseeds of both cytotypes in the 3rd month of conservation and 7 days after culturing on the regrowth medium, D and E: bud break and regrowth capability in Synseeds of both cytotypes in the 6th month of conservation and 7 days after culturing on the regrowth medium, F

and G: bud break and regrowth capability in Synseeds of both cytotypes in the 9th month of conservation and 7 days after culturing on the regrowth medium, H and I: the situation of both Synseeds cytotypes in the 12th month of conservation and 7 days after culturing on the regrowth medium, J: rooting of successfully recovered explants related to the 3rd month of conservation of both cytotypes, K: greenhouse successful acclimatization of *in vitro* rooted plants of both cytotypes

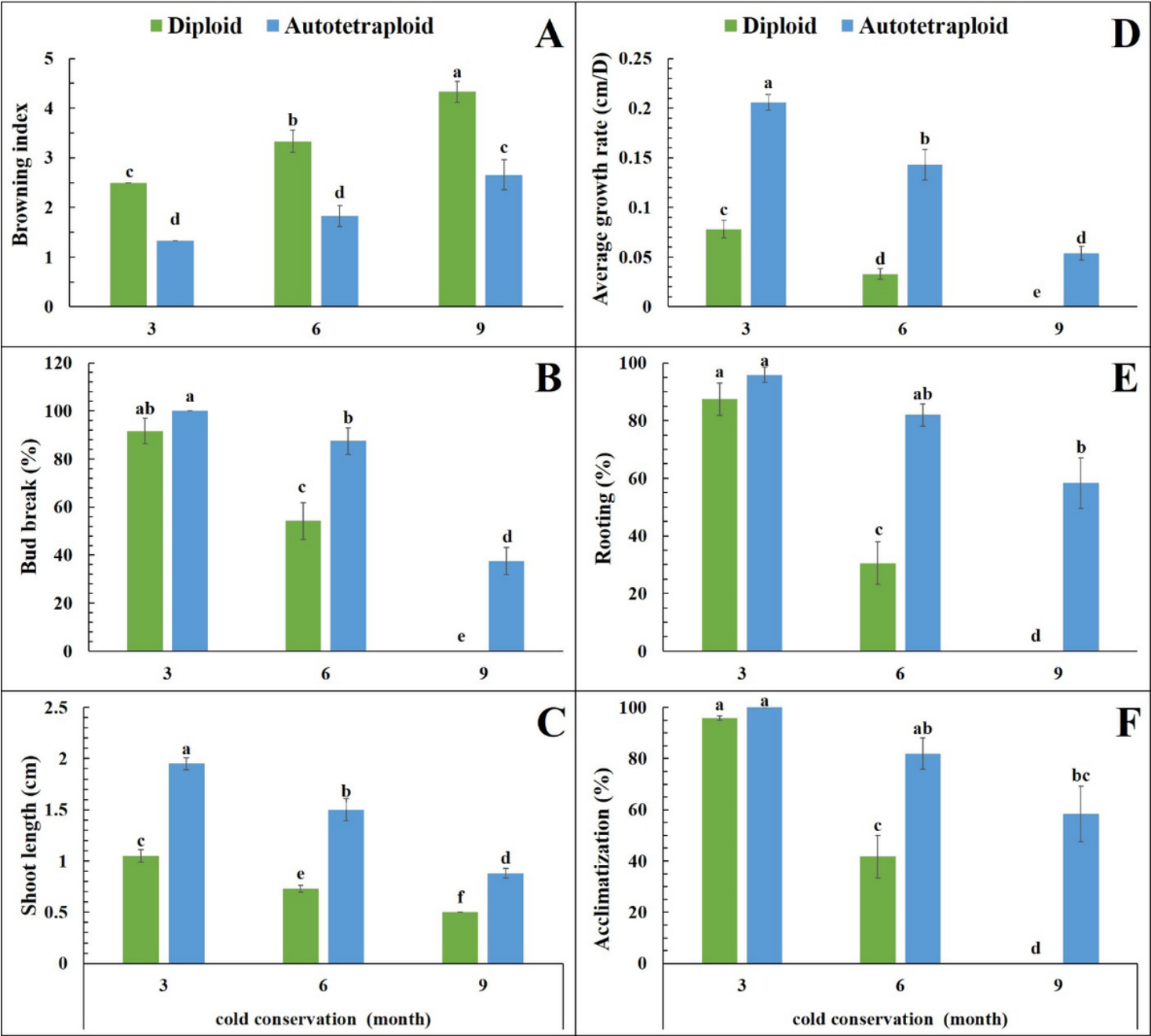


Figure 2

Biometrical responses of diploid and autotetraploid Synseeds of *Ficus carica* cv. Sabz during different times of the cold conservation period. **A:** Browning index of Synseeds after removing them from the refrigerator, **B:** Bud break percentage of Synseeds 7 days after culturing on the regrowth medium, **C and D:** Growth parameters of Synseeds at the end of 4th week culturing on the regrowth medium, **E and F:**

Rooting and acclimatization capabilities of plantlets obtained from Synseed regrowth. Data represent mean $\pm$ SE, and different letters on the columns show significant differences at $P \leq 0.05$ according to Duncan's multiple range tests

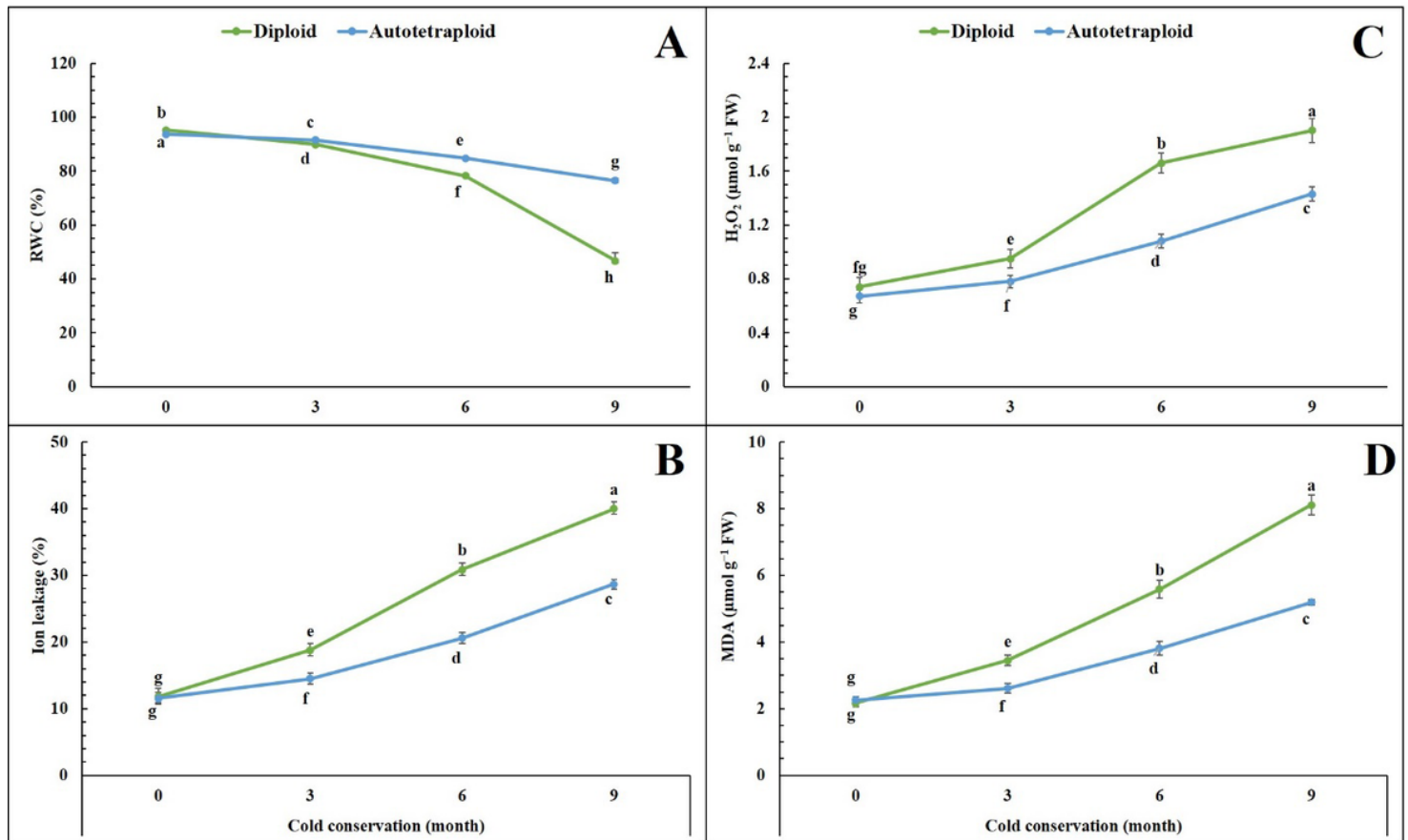


Figure 3

The physiological responses, including **A:** RWC%, **B:** ion leakage%, **C:** H₂O₂ content, and **D:** MDA content, of diploid and autotetraploid Synseeds of *Ficus carica* cv. Sabz during different times of cold conservation. Data represent mean $\pm$ SE, and different letters in each graph show significant differences at $P \leq 0.05$ according to Duncan's multiple range tests

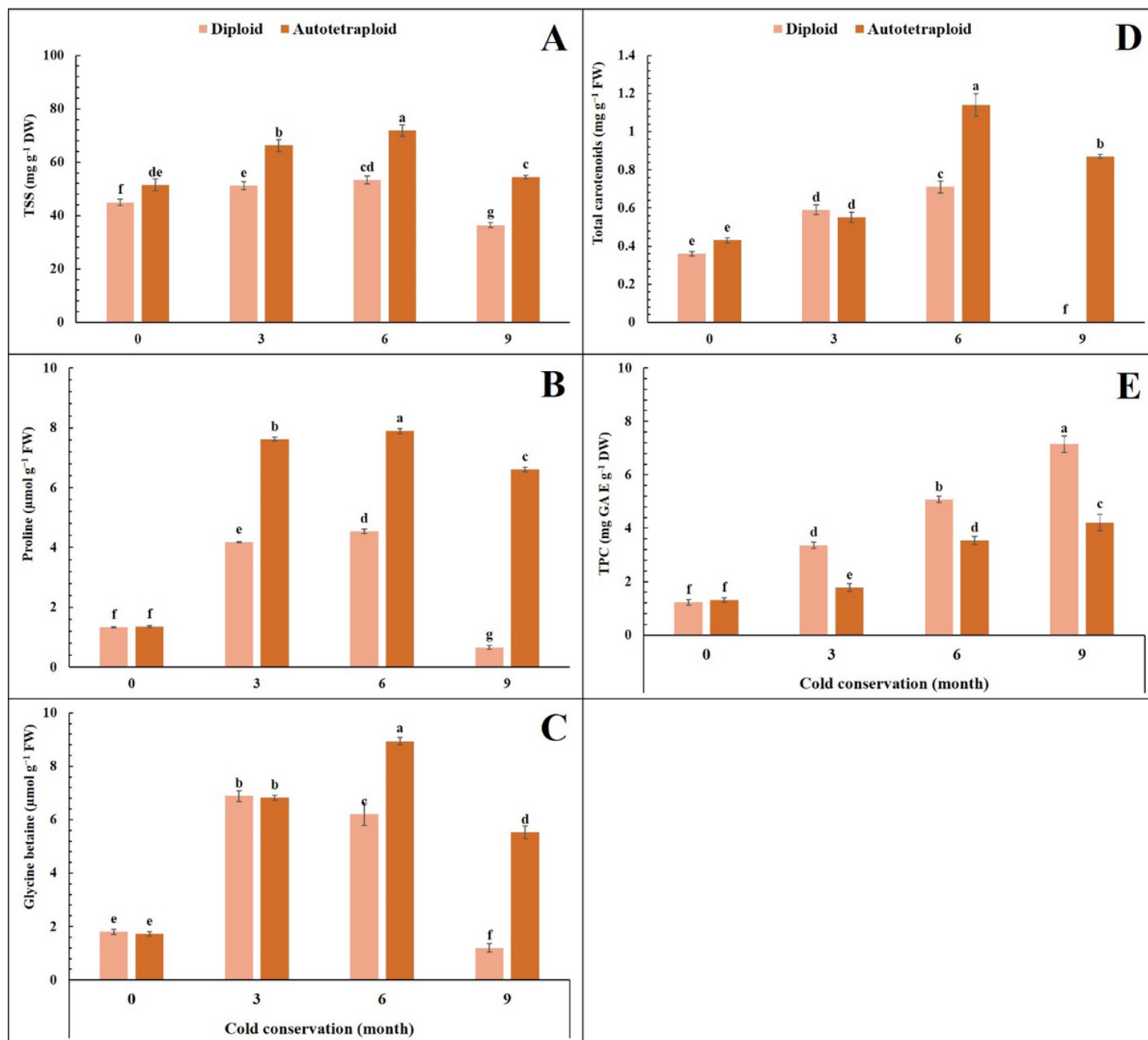


Figure 4

Biochemical responses of diploid and autotetraploid Synseeds of *Ficus carica* cv. Sabz, including the accumulation of **A**:total soluble sugars, **B**: proline, **C**: glycine betaine, **D**:total carotenoids, and **E**: total phenolic compounds during different times of cold conservation. Data represent mean $\pm$ SE, and different letters on the columns show significant differences at $P \leq 0.05$, according to Duncan's multiple range tests

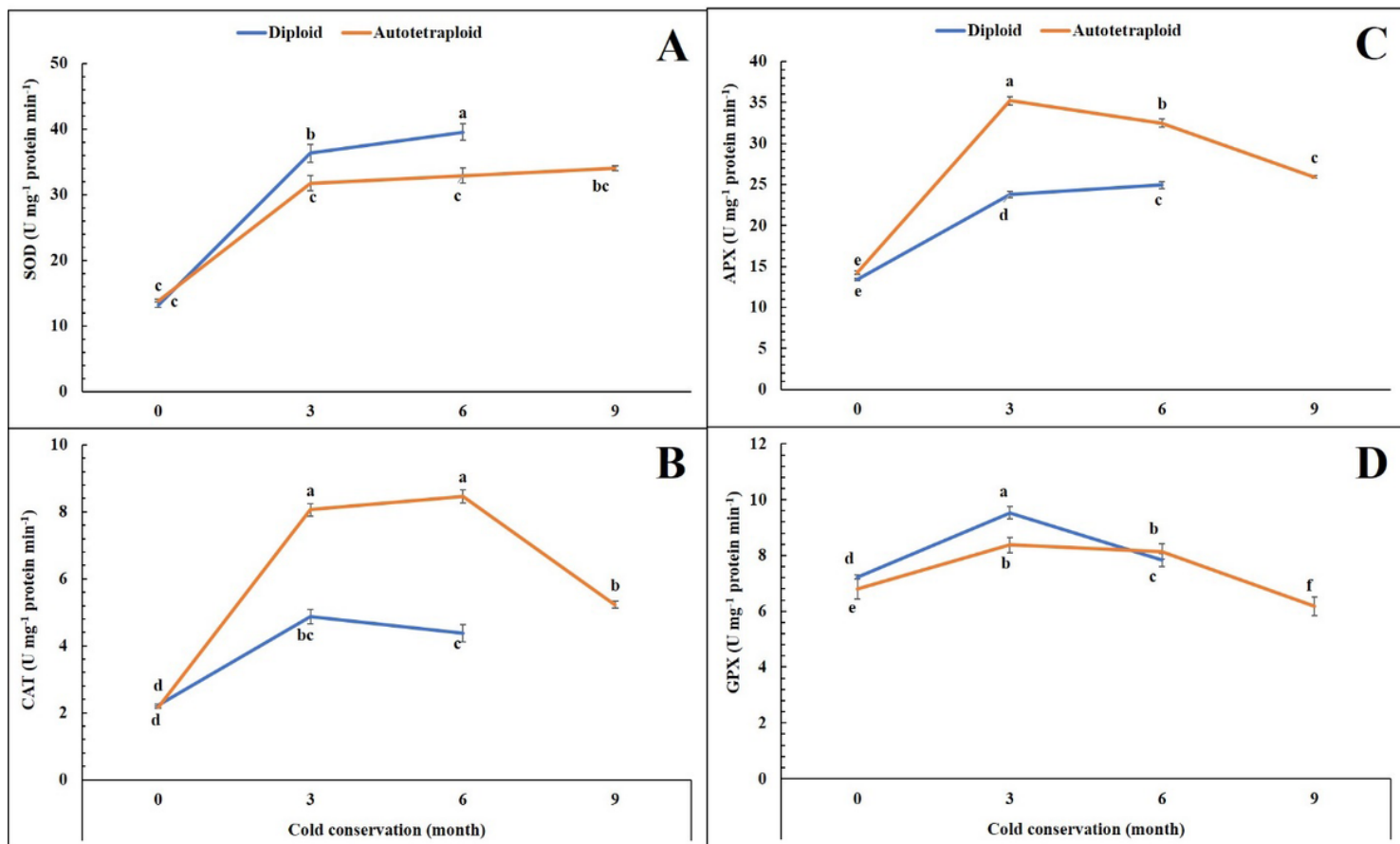


Figure 5

Antioxidant enzyme activities in diploid and autotetraploid Synseeds of *Ficus carica* cv. Sabz during the cold conservation period. **A:**Superoxide dismutase activity, **B:** Catalase activity, **C:** Ascorbate peroxidase activity, and **D:** Glutathione peroxidase activity. Data represent mean $\pm$ SE, and different letters in each graph show significant differences at $P \leq 0.05$ according to Duncan's multiple range tests

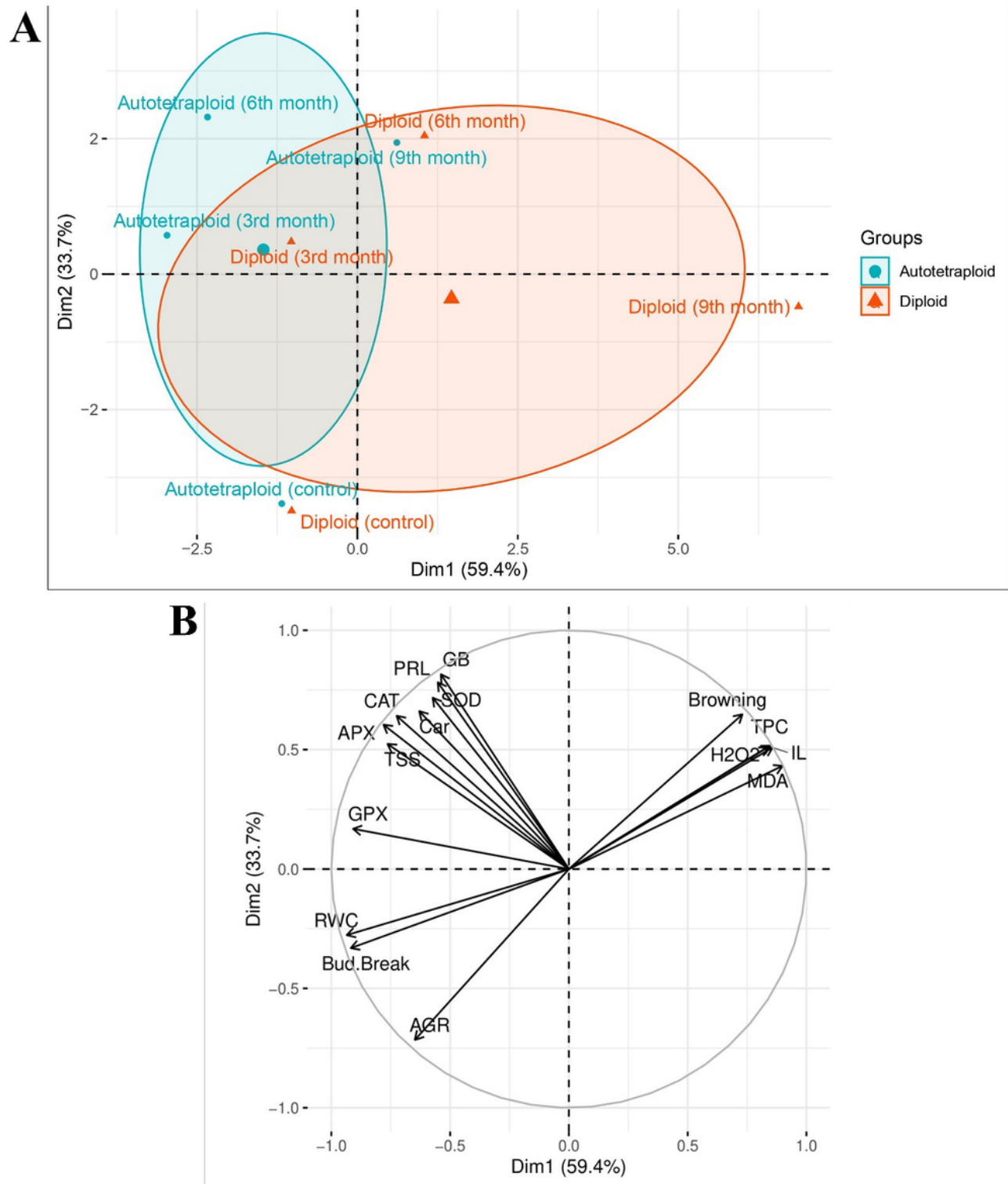


Figure 6

Principal component analysis (PC1 and PC2) of the score plot position of diploid and autotetraploid Synseeds (**A**) and loading plot of resulting physiological and biochemical responses (**B**) at the 0 (control), 3rd, 6th, and 9th months of cold conservation period. Abbreviations: RWC (relative water content, IL (ion leakage), AGR (average growth rate), MDA (malondialdehyde), TPC (total phenolic compounds), Car (total

carotenoids), TSS (total soluble sugars), PRL (proline), GB (glycine betaine), SOD (superoxide dismutase), CAT (catalase), APX (ascorbate peroxidase), GPX (glutathione peroxidase)

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