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Changes in soluble carbohydrates and related enzymes induced by low temperature during early developmental stages of quinoa (*Chenopodium quinoa*) seedlings

Mariana Rosa[1], Mirna Hilal[1], Juan A. González[2], Fernando E. Prado[1] *

[1] Cátedra de Fisiología Vegetal, Facultad de Ciencias Naturales e IML, Universidad Nacional de Tucumán, Miguel Lillo 205, 4000 Tucumán, Argentina

[2] Instituto de Ecología Vegetal, Area Botánica, Fundación Miguel Lillo, Miguel Lillo 251, 4000 Tucumán, Argentina

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Summary

Low temperature represents one of the principal limitations in species distribution and crop productivity. Responses to chilling include the accumulation of simple carbohydrates and changes in enzymes involved in their metabolism. Soluble carbohydrate levels and invertase, sucrose synthase (SS), sucrose-6-phosphate synthase (SPS) and α-amylase activities were analysed in cotyledons and embryonic axes of quinoa seedlings grown at 5 °C and 25 °C in the dark. Significant differences in enzyme activities and carbohydrate levels were observed. Sucrose content in cotyledons was found to be similar in both treatments, while in embryonic axes there were differences. Invertase activity was the most sensitive to temperature in both organs; however, SS and SPS activities appear to be less stress-sensitive. Results suggest that 1) metabolism in germinating perispermic seeds would be different from endospermic seeds, 2) sucrose futile cycles would be operating in cotyledons, but not in embryonic axes of quinoa seedlings under our experimental conditions, 3) low temperature might induce different regulatory mechanisms on invertase, SS and SPS enzymes in both cotyledons and embryonic axes of quinoa seedlings, and 4) low temperature rather than water uptake would be mainly responsible for the changes observed in carbohydrate and related enzyme activities.

Key words: Invertase – low temperature – soluble carbohydrates – sucrose-6-phosphate synthase – sucrose synthase

Abbreviations: SPS = sucrose-6-phosphate synthase. – SS = sucrose synthase. – UDP = uridine 5′ diphosphate. – UDPGlc = uridine 5′ diphosphate glucose

Introduction

Among the abiotic stresses, low temperature constitutes one of the major environmental limitations to crop productivity and wild species distribution. Plant metabolism displays a striking capacity for remodelling in response to environmental changes, which elicit complex responses at the cellular, developmental and physiological levels (MacKintosh 1998). Alterations in carbohydrate levels are claimed to be an effective stress tolerance mechanism (Morgan 1992). It has been

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* E-mail corresponding author: fepra@csnat.unt.edu.ar

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shown that an increased requirement of carbohydrate supply under stress conditions and hence, the expression of degradative and biosynthetic enzymes, can be stress-induced and additionally upregulated by a number of environmental stimuli that affect source-sink relations (Roitsch 1999). Plant adaptation to low (nonfreezing) temperatures often involves the accumulation of soluble sugars, the most common and abundant of which is sucrose (Guy et al. 1992).

Sucrose plays a central role in higher plants not only as a substrate to sustain the heterotrophic growth of sink tissues, but it is also an important signalling molecule that regulates both source and sink metabolism under stress conditions (Roitsch 1999). There are three enzyme systems directly involved in sucrose metabolism. The first one is invertase (EC 3.2.1.26), which is a hydrolytic enzyme that catalyses the irreversible sucrose cleavage reaction. The second is sucrose synthase (SS, EC 2.4.1.13), which catalyses the reversible reaction UDPGlc + fructose = sucrose + UDP. The third is sucrose-6-phosphate synthase (SPS, EC 2.4.1.14), which is a soluble enzyme located in the cytoplasm that catalyses the reaction UDPGlc + fructose-6-phosphate = sucrose-6-phosphate + UDP. In plant tissues invertase and SS play important roles in sink capacity. Thus, it was suggested that the invertase pathway might be generally directed toward growth and expansion, whereas the SS pathway might be characteristic of a metabolism associated with polysaccharides synthesis, for example, in cell wall formation (ap Rees 1988). Additionally it was also considered to make a major contribution to sucrose synthesis in potato tubers (Geingenberger and Stitt 1993). SPS is thought to play a major role in sucrose biosynthesis because the hydrolysis of SPS by an accompanying specific phosphatase renders the synthetic reaction irreversible in favour of sucrose accumulation (Huber and Huber 1996). Consequently, invertase, SS and SPS may actually be considered as central modulators of carbon partitioning due to the fact that they supply carbohydrates, as well as amplify signals that regulate source-sink relationships (Roitsch 1999).

Quinoa and other members of the Chenopodiaceae family have long been known for their extraordinary tolerance to cold, drought and salt (National Research Council 1989). Quinoa seeds have an amylaceous perisperm (Fig. 1) and a high nutritional value which makes it a very suitable food and gluten-free flour source (Caperuto et al. 2000). In perispermic seeds, perisperm is the unique carbohydrate source for cotyledons and embryonic axes during early developmental stages. However, in such seeds the carbohydrate metabolism is poorly understood. Thus, two major questions need to be answered to understand how low temperature affects the carbohydrate metabolism in perispermic seeds: 1) What are the spatial and temporal patterns of carbohydrate allocation in germinating seeds? and 2) How is the carbohydrate partitioning regulated?

To address these questions, soluble acid invertase, SS, SPS and α-amylase (EC 3.2.1.1) activities and sucrose, glucose, and fructose levels were analysed in perisperm, cotyledons and embryonic axes during early developmental stages of quinoa seedlings under low temperature.

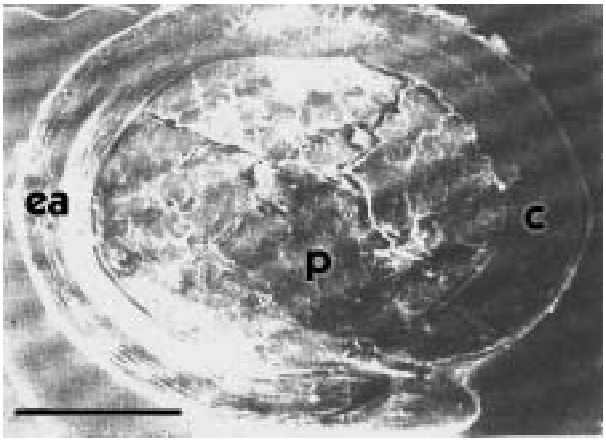

Figure 1. Transversal section of quinoa seed showing amylaceous perisperm (p), cotyledons (c) and embryonic axes (em). Bar = 1000 μm (In Gallardo et al. 1997).

Materials and Methods

Seed material

Quinoa seeds (*Chenopodium quinoa* Willd. cv. Sajama) were germinated on blotting paper in plastic boxes (28 × 20 cm) and incubated at 5 or 25 ± 1 °C in the dark. Sampling was performed at 6, 12, 24, 36, 48 and 72 h at 25 °C and at 12, 24, 36, 48, and 72 h at 5 °C. In both treatments, cotyledons and embryonic axes were separated and then weighed and kept at −20 °C until the analyses were performed.

Water Uptake

Twenty seeds were weighed and placed in Petri dishes (5 cm) containing a sheet of filter paper moistened with 1.5 mL of distilled water. After different periods of imbibition, seeds were removed, blotted dry with tissue paper, weighed, and returned to the dishes. The values of the water content, expressed in mg, correspond to the difference between wet seed weight and dry seed weight.

Radicle elongation

To determine the radicle elongation, fifty seeds were placed in Petri dishes (10 cm) containing a sheet of filter paper (Whatman 1), and moistened with 2.5 mL of distilled water. At different times twenty seeds were removed and its radicle measured with a digital vernier calliper and then placed in Petri dishes again.

Carbohydrate determination

Soluble sugars were extracted from the cotyledons and embryonic axes as follows: 500 mg of sample was homogenised in 2 mL of 80 % ethanol with a mortar and pestle. After heating the homogenate in a water bath at 75 °C for 10 min, the insoluble residue was removed by centrifugation at 5000×g for 10 min. The precipitate was re-homogenised with 2 mL of 80 % ethanol and centrifuged as described above. The supernatants were pooled and dried under a stream of hot air, and the residue was resuspended in 1 mL of water and desalted using an ion-exchange (Amberlite MB3) column.

Glucose was determined using a glucose-oxidase-peroxidase coupled assay according to Jorgensen and Andersen (1973). Total fructose was measured by the method of Roe and Papadopoulos (1954) and sucrose by the protocol of Cardini et al. (1955).

Enzyme extraction

All steps were carried out at 4 °C. For soluble acid invertase, SS and SPS extraction, cotyledons or embryonic axes were ground with a mortar and pestle in 3 volumes of extraction buffer containing 50 mmol/L sodium phosphate, pH 7.4, 1 mmol/L β-mercaptoethanol and 5 µmol/L $MnSO_4$. The homogenates were centrifuged for 15 min at 12,000×g and the pellet discarded. Because the extraction buffer did not contain either detergent, chelating and chaotropic agents or high concentrations of salt, the probability of extracting the cell wall invertase activity is very low. The supernatants were then dialysed against a sodium acetate buffer (10 mmol/L, pH 5.5) containing 1 mmol/L β-mercaptoethanol and were used for enzymatic activity determinations.

α-amylase extraction was performed from perisperm tissue in a similar way, except that the extraction buffer was in this case 100 mmol/L sodium acetate, pH 5.5 containing 1 mmol/L β-mercaptoethanol and 3 mmol/L $CaCl_2$. Dialysis was carried out against a sodium acetate buffer (10 mmol/L, pH 5.5) containing 1 mmol/L β-mercaptoethanol and 3 mmol/L $CaCl_2$.

Enzyme activities

Soluble acid invertase, was measured according to Prado et al. (1985). Briefly, 10 µL of extract was added to a reaction mixture containing 60 mmol/L sucrose, 200 mmol/L sodium acetate buffer (pH 5.5), 1 mmol/L β-mercaptoethanol, in a final volume of 100 µL. Released reducing sugars were estimated by Nelson's method (Nelson 1944). SS and SPS were measured according to Batta and Singh (1986). SS activity, in the synthesis direction, was assayed in 40 mmol/L Tris-HCl buffer (pH 8), 10 mmol/L $MgCl_2$, 1 mmol/L β-mercaptoethanol, 8 mmol/L UDPGlc, 16 mmol/L fructose and 20 µL of extract in a final volume of 100 µL. SPS activity was assayed in 40 mmol/L Tris-HCl buffer (pH 8), 10 mmol/L $MgCl_2$, 1 mmol/L β-mercaptoethanol, 8 mmol/L UDPGlc, 16 mmol/L fructose-6-phosphate, 8 mmol/L glucose-6-phosphate and 20 µL of extract in a final volume of 100 µL. The amount of product synthesised in both reactions was determined by the protocol of Cardini et al. (1955).

α-amylase activity in perisperm was measured as follows: 20 µL of extract was added to a reaction mixture containing 10 % soluble starch solution, sodium acetate buffer (200 mmol/L, pH 5.5), 1 mmol/L β-mercaptoethanol, 3 mmol/L $CaCl_2$ and water in a final volume of 100 µL. The reaction was initiated by adding the enzyme incubated for 30 min at 37 °C and stopped by adding 0.5 mL of Somogyi's cupro-alkaline reagent. Released reducing sugars were estimated by Nelson's method (Nelson 1944). Control reactions without substrate were run in parallel in order to evaluate the endogenous activity for all enzymes.

Assay conditions for linear correlation in the amount of enzymes and reaction time were determined in all tests.

Results

Water uptake and radicle elongation

As shown in Figure 2, at 25 °C, radicle protrusion occurred between 5 and 6 h, while at 5 °C it was between 9 and 10 h, showing a delay of approximately 4 h. However, seeds maintained under low temperature reach the same water content of control seeds within 48 h (data not shown).

Radicle length data are shown in Figure 3. The highest elongation was observed between 48–72 h. At 5 °C the radicle elongation was significantly inhibited along the experiment.

Enzyme activities

α-amylase activity in the perisperm of quinoa seeds at 25 °C and 5 °C is shown in Figure 4. Enzyme activity was higher at 25 °C than 5 °C during the time course of the experiment. It showed a strong increase up to 12 h and then it increased progressively until 36 h reaching 0.083 µmoles of reducing

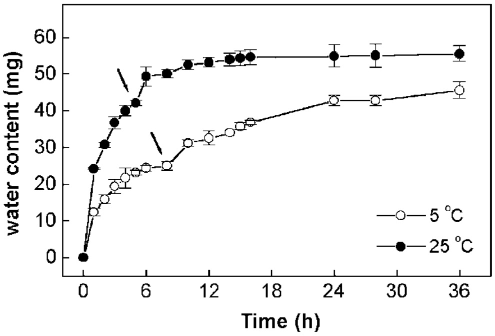

Figure 2. Time course of water uptake for quinoa seeds at 5 and 25 °C. The arrows indicate radicle protrusion. Bars on data points represent the mean of four replicates ± SD.

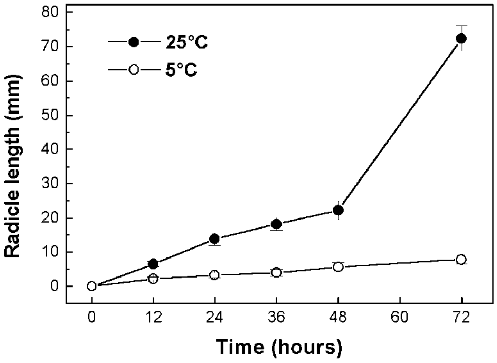

Figure 3. Radicle elongation profile at 5 and 25 °C. Bars on data points represent the mean of four replicates ± SD.

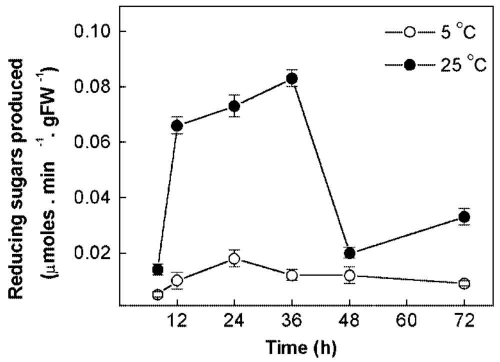

Figure 4. Time course of α-amylase activity measured in quinoa perisperm at 5 and 25 °C. Bars on data points represent the mean of four replicates ± SD.

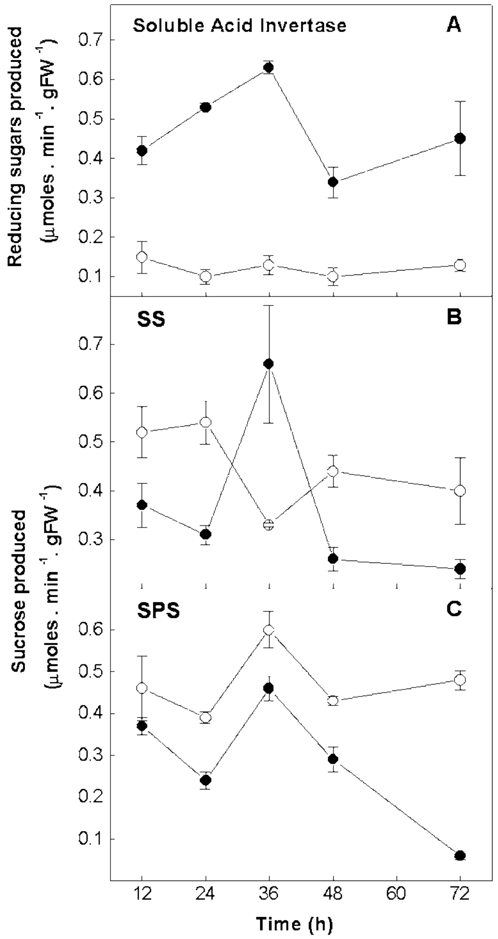

Figure 5. Time courses of soluble acid invertase, SS and SPS activities in quinoa cotyledons at 5 °C (open circle) and 25 °C (solid circle). Bars on data points represent the mean of four replicates ± SD.

sugars released (min^{-1} g FW^{-1}). From this point on, a decrease in α-amylase activity was observed. At 5 °C the enzyme activity did not show any significant changes along the experiment (Fig. 4).

To obtain information about sucrose metabolising enzymes, soluble acid invertase, SS and SPS were assayed in cotyledons and embryonic axes of quinoa seedlings at 25 and 5 °C. In cotyledons, soluble acid invertase activity was much higher at 25 °C than 5 °C (Fig. 5 A). The enzyme showed a linear increase up to 36 h reaching 0.63 µmol of reducing sugars released (min^{-1} g FW^{-1}) and then decreased to its initial level. At 5 °C the activity did not show any significant changes throughout the experiment (Fig. 5 A). SS activity, however, was higher at 5 °C than 25 °C except at 36 h. At 5 °C the highest value (0.54 µmol of sucrose produced min^{-1} g FW^{-1}) was reached at 24 h and it decreased afterwards. (Fig. 5 B). SPS activity was also higher at 5 °C than 25 °C (Fig. 5 C). The highest value (0.6 µmol of sucrose produced $min^{-1} \cdot$ g FW^{-1}) was observed at 36 h. At 25 °C SPS activity showed a similar pattern that 5 °C, but from 36 h a strong decrease was observed (Fig. 5 C).

In embryonic axes, invertase activity was initially much higher at 25 °C than 5 °C (Fig. 6 A). The activity showed a

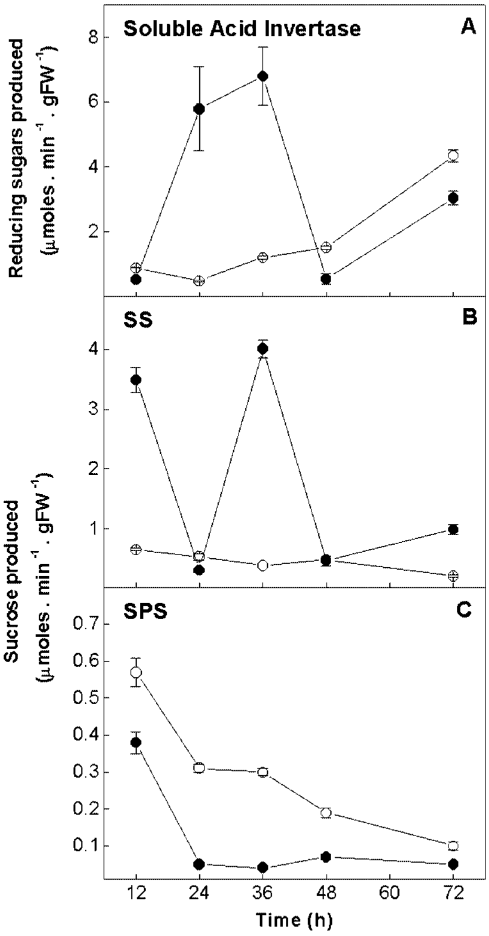

Figure 6. Time courses of soluble acid invertase, SS and SPS activities in quinoa embryonic axes at 5 °C (open circle) and 25 °C (solid circle). Bars on data points represent the mean of four replicates ± SD.

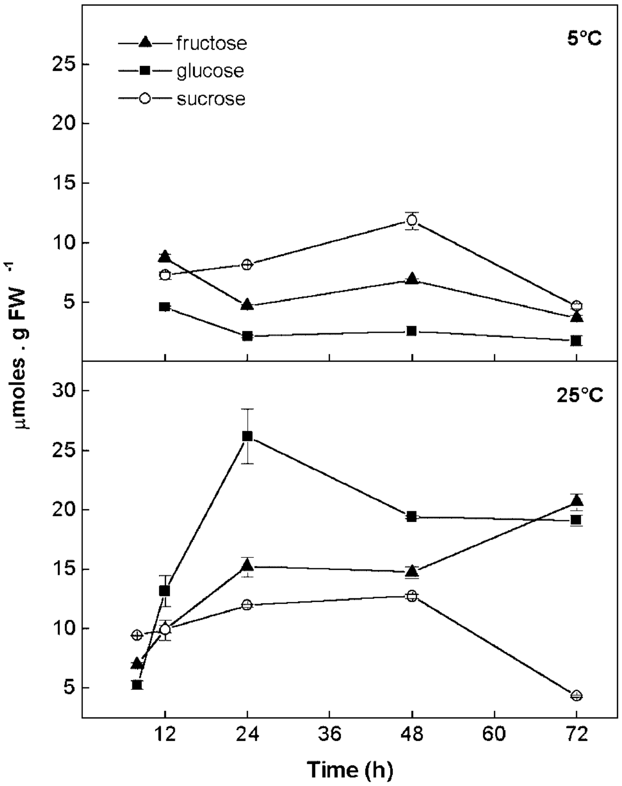

Figure 7. Changes in carbohydrates content in quinoa cotyledons. Bars on data points represent the mean of four replicates ± SD.

strong increase up to 36 h, reaching 6.86 μmol of reducing sugars released (min^{-1} g FW^{-1}) and then a sharp decrease was observed. At 5 °C invertase activity showed a slight increase until 48 h, followed by a steady increase up to 72 h reaching 4.39 μmol of reducing sugars released (min^{-1} g FW^{-1}) (Fig. 6 A). In a similar way, SS activity resulted much higher at 25 °C than at 5 °C and the highest value was reached at 36 h (4.02 μmol of sucrose produce min^{-1} g FW^{-1}). From this point on, SS activity sharply decreased and remained low (Fig. 6 B). At 5 °C SS activity was very low and it did not show any significant changes during the experiment (Fig. 6 B). SPS activity was higher at 5 °C than 25 °C (Fig. 6 C). The enzyme activity showed a similar pattern in both temperatures reaching the highest values at 12 h (0.57 and 0.38 μmol of sucrose produce min^{-1} g FW^{-1}, respectively), then, the activities showed a progressive decrease until the end of the experiment. It is important to highlight that SPS activity was always one magnitude of order less than SS and invertase activities.

Changes in carbohydrate levels

Concentrations of soluble carbohydrates in cotyledons and embryonic axes under low temperature showed changes during the experiment. In cotyledons the more abundant carbohydrate was sucrose, whose maximum value was 11.87 μmoles (g FW^{-1}) reached at 48 h (Fig. 7 upper panel). Fructose level was always higher than glucose and the fructose-to-glucose ratio was maintained without changes during the experiment. These results could imply that the rate of fructose metabolism is lower than glucose's. However, at 25 °C, glucose was the most abundant carbohydrate reaching 26.31 μmoles (g FW^{-1}) (Fig. 7 lower panel). In relation to sucrose levels only, small changes between both treatments were observed. Fructose content showed a similar value to sucrose until 48 h (6.3 μmoles g FW^{-1}) and then a decrease was observed until the end of the experiment (Fig. 7 upper panel).

In contrast to cotyledons, embryonic axes showed differences in sucrose levels between 5 °C and 25 °C (Fig. 8). Thus, at 5 °C the sucrose content between 12 and 24 h reached about 7.5 μmoles (g FW^{-1}) and then it decreased to nearly undetectable levels. Nevertheless, at 25 °C the sucrose content was almost non-detectable. In relation to glucose and fructose contents an inverse pattern to the sucrose one was observed at 5 °C where the lowest values were observed between 12 and 24 h (Fig. 8 upper panel). At 25 °C both hexoses were higher than at 5 °C (Fig. 8 lower panel), showing a strong increase up to 24 h and then declining up to 48 h. After this point, sugar levels remained unchanged.

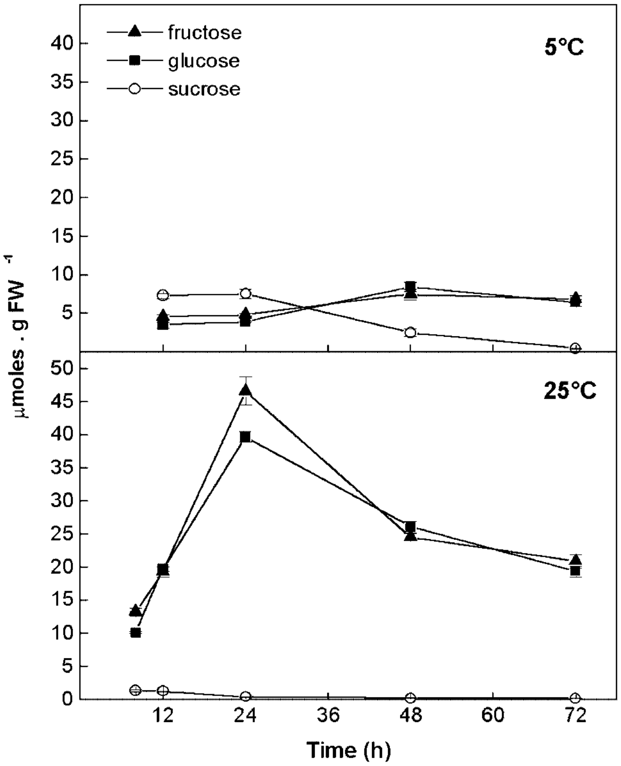

Figure 8. Changes in carbohydrates content in quinoa embryonic axes. Bars on data points represent the mean of four replicates ± SD.

Discussion

In a few plants, such as *Yuca*, coffee and quinoa seeds, the amylaceous perisperm represents the main exportable carbohydrate source (Burnouf-Radosevich 1988, Bewley and Black 1994). In seedlings of these species, cotyledons and embryonic axes represent the most important sink tissues and their growth is accompanied by changes in the sink strength of different organs. This physiological pattern is not static under the influence of environmental factors such as low temperature, which may act on carbohydrate partitioning (Roitsch 1999, Sowinski et al. 1999). Plant carbohydrates do not only function as metabolic resources and structural constituents of cells, but they also act as important regulators of various processes associated with the growth and development of plants (Ho et al. 2001).

Seed starch degradation and soluble carbohydrate exportation begins after radicle elongation occurs (Bewley and Black 1994). The starch metabolism is initiated by a hydrolytic reaction catalyzed by α-amylase enzyme. The emerging embryo in most endospermic seeds produces a great demand of carbohydrate to sustain the growth seedling and radicle elongation, which coincides with a high α-amylase activity (Sturm 1999). Nonetheless, in our study the highest α-amylase activity was observed at 25 °C before radicle elongation occurs (Fig. 3). This event might be important for the establishment of quinoa seedlings because quinoa seeds are characterised by a very high embryo development rate.

It is known that in amylaceous grains the concerted and simultaneous action of α-amylase and SPS enzymes is needed to produce a transportable carbon supply to support the high metabolism of the seedling (Nomura and Akazawa 1974). In spite of this, our results did not support this fact because in quinoa seeds exposed to low temperature, the α-amylase and SPS activities were not coordinated (Fig. 2). The changes observed in quinoa probably reflect metabolic differences in the germination process between endospermic and perispermic seeds.

Geigenberger and Stitt (1991) demonstrated that when phloem transport is inhibited, a diminished soluble acid invertase activity and unchanged sucrose levels were observed. In accord with these results we observed a similar trend in quinoa cotyledons at 5 °C (Fig. 7 upper panel). These authors propose that this efficient regulation is possible because sucrose metabolism is regulated by a rapid cycle in which sucrose is simultaneously synthesised and degraded. Owing to this cycle, the net flux through the sucrose pool responds very sensitively to the mechanism that modulates the unidirectional synthetic or degradation flux.

SPS activity has been correlated with sucrose accumulation as a response to low temperature (Guy et al. 1992). It is well known that exposure of tolerant plants to low temperature corresponds with an accumulation of soluble carbohydrates, e.g. sucrose and hexose-Ps (Hurry et al. 1995). Yet, it is now generally accepted that net sucrose synthesis can also occur in non-photosynthetic source tissues (e.g., germinating seeds) and it appears that SPS is the enzyme responsible for sucrose synthesis (Winter and Huber 2000).

Quinoa cotyledons at 5 °C and 25 °C contained high levels of sucrose-synthesizing and sucrose-degrading enzymes. These results could implicate that futile cycles of sucrose are active processes in quinoa cotyledons. Wang et al. (2000) postulated that a rapid cycling of sucrose could enhance sensitivity to respond to the metabolic flux and the carbon partitioning would be in favour of sucrose accumulation for counteracting the stress condition; however, no sucrose accumulation was observed in quinoa cotyledons.

In relation to embryonic axes exposed to low temperature, sucrose content decreased coincidently with a progressive increase in soluble acid invertase activity (Figs. 6 A and 7). These results could suggest an invertase adaptation and changes in carbohydrates allocation in response to low temperature. The low sucrose content and the high invertase level observed at 25 °C probably respond to the need of an energy supply to support the faster embryo growth. In this sense, Sturm (1999) demonstrated that a high hexose level is required as a driving force for cell elongation and the maintenance of cell osmotic pressure. In accordance to Sturm (1999), Pfiffer and Kutschera (1995) demonstrated that cell elongation in developing sunflower hypocotyls exhibits a close correlation between the rate of cell elongation and soluble acid invertase activity.

In quinoa embryonic axes the SPS activity was always very low compared to SS and invertase (Figs. 5 and 6). However, SS and invertase levels were very different between embryonic axes and cotyledons (one magnitude of order). This fact could be explained by a higher sink strength present in embryonic axes due to its faster elongation. Numerous studies support that invertase and SS are related to several physiological processes such as tissue growth, utilization and storage of sugars in sink organs (Sturm 1999, Druart et al. 2001, Nguyen-Quoc and Foyer 2001). The low SS level detected in embryonic axes at 5 °C could also correspond to a lower metabolic requirement of UDPGlc due to a slower growth rate imposed by low temperatures.

Our results indicate that in quinoa embryonic axes at 5 or 25 °C, an unidirectional flux to sustain radicle elongation process is occurring. However, the possibility that under our experimental conditions (darkness, without exogenous sugars supply) futile cycles are inhibited cannot be discarded.

Although it is well known that the water uptake rate is inhibited by low temperature, the delay of 4 h observed in water uptake at 5 °C (Fig. 2) is not related to the differences observed in carbohydrates' and enzymes' distribution patterns. In this context, we suppose that low temperatures, rather than water uptake are mainly responsible for the changes in carbohydrate and related enzyme activities observed in quinoa seedlings. It could be considered as one of the reasons for the high threshold quinoa exhibits towards cold and drought resistance.

Given the facts provided in this study, four points are evident. 1) Metabolism in germinating perispermic seeds would be different from the metabolism of endospermic seeds, 2) Sucrose futile cycles would be operating in cotyledons but not in embryonic axes of quinoa seedlings under our experimental conditions, 3) Low temperature might induce different regulatory mechanisms on invertase, SS and SPS enzymes in both cotyledons and embryonic axes of quinoa seedlings, and 4) Because no coincidences were found between the delays in water uptake and carbohydrates' and enzymes' distribution patterns observed at 5 °C; we conclude that it would be the low temperature rather than water uptake that is mainly responsible for the changes observed in carbohydrate and related enzyme activities.

To conclude, our study provides the basis for future studies and shows that quinoa is an appropriate model system to analyse the carbohydrate metabolism in perispermic seeds under stress.

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