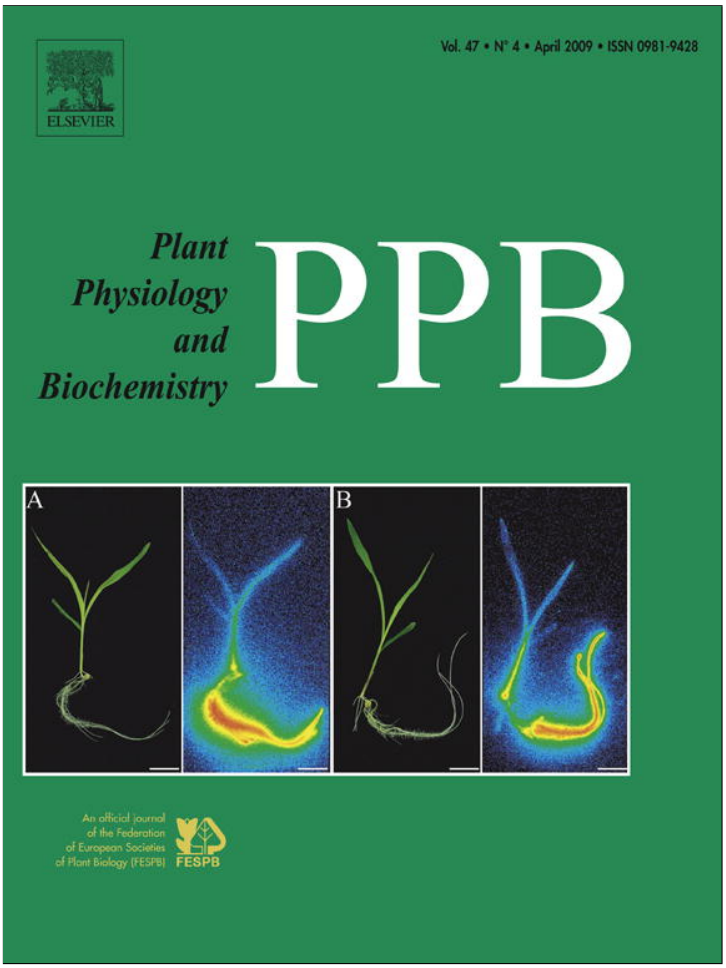

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Research article

Low-temperature effect on enzyme activities involved in sucrose–starch partitioning in salt-stressed and salt-acclimated cotyledons of quinoa (*Chenopodium quinoa* Willd.) seedlings

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ABSTRACT

The effect of low temperature on growth, sucrose–starch partitioning and related enzymes in salt-stressed and salt-acclimated cotyledons of quinoa (*Chenopodium quinoa* Willd.) was studied. The growth of cotyledons and growing axes in seedlings grown at 25/20 °C (light/dark) and shifted to 5/5 °C was lower than in those only growing at 25/20 °C (unstressed). However, there were no significant differences between low-temperature control and salt-treated seedlings. The higher activities of sucrose phosphate synthase (SPS, EC 2.4.1.14) and soluble acid invertase (acid INV, EC 3.2.1.25) were observed in salt-stressed cotyledons; however, the highest acid INV activity was observed in unstressed cotyledons. ADP-glucose pyrophosphorylase (ADP-GPPase, EC 2.7.7.27) was higher in unstressed cotyledons than in stressed ones. However, between 0 and 4 days the highest value was observed in salt-stressed cotyledons. The lowest value of ADP-GPPase was observed in salt-acclimated cotyledons. Low temperature also affected sucrose synthase (SuSy, EC 2.4.1.13) activity in salt-treated cotyledons. Sucrose and glucose were higher in salt-stressed cotyledons, but fructose was essentially higher in low-temperature control. Starch was higher in low-temperature control; however, the highest content was observed at 0 day in salt-acclimated cotyledons. Results demonstrated that low temperature induces different responses on sucrose–starch partitioning in salt-stressed and salt-acclimated cotyledons. Data also suggest that in salt-treated cotyledons source–sink relations (SSR) are changed in order to supply soluble sugars and proline for the osmotic adjustment. Relationships between starch formation and SuSy activity are also discussed.

1. Introduction

Seedling establishment is a critical process to plant growth, especially under adverse environmental conditions [7]. Within adverse conditions, soil salinity and low temperature are perhaps the more important stress factors that determine the geographic distribution of plants and their productivity [2]. Seedling injuries caused by these stresses generate a great damage to agriculture reducing the yield and quality of products [7]. During seedling establishment low temperature and salinity modifies several biological processes such as osmotic homeostasis, photosynthetic carbon fixation, carbon partitioning, carbohydrate and lipid metabolisms, and gene expression [7,15]. Seedlings adapt to salinity and low temperature by different mechanisms, including changes in morphological and developmental pattern as well as physiological and biochemical processes [13,16,26,31,34,40]. Adaptation is associated with maintaining osmotic homeostasis by metabolic adjustments that lead to the accumulation of metabolically compatible compounds such as carbohydrates, polyols, betaines and proline [7,44]. These organic osmolytes can accumulate to high concentrations without disturbing intracellular biochemistry [7]. Apart from their role in osmotic adjustment, compatible solutes also have osmoprotective functions. Due to their specific hydrophilic structure, they are capable of replacing water on the surfaces of proteins, protein complexes or membranes, thus preserving their biological functions [7]. Most compatible solutes also seem to play an important role in hydroxyl radical scavenging thus defending seedlings against oxidative damage, which is a common consequence of many abiotic stresses [27]. Sucrose is the more commonly accumulated free sugar in response to low temperature and salinity stresses [13,15,33]. Nevertheless, carbohydrate accumulation is not limited to only sucrose; lesser amounts of glucose, fructose, and another sucrose-derived oligosaccharide family

Abbreviations: Acid INV, soluble acid invertase; ADP-GPPase, ADP-glucose pyrophosphorylase; AMG, amyloglucosidase; G6PDH, glucose-6-phosphate dehydrogenase; PGM, phosphoglucomutase; PPFD, photosynthetic photon flux density; SPS, sucrose phosphate synthase; SuSy, sucrose synthase.

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have also been observed in salt and cold stressed plants [31,41]. Moreover, low temperature and salinity stresses often involve changes in starch content [14,20,40]. This fact is very important because in many seeds, including mono- and dicotyledonous species, starch is the main carbon reserve [30].

According to several studies seedling adaptation to saline and cold stresses also includes modifications of source–sink relations (SSR) and sucrose–starch partitioning [2,22,33]. Modification of SSR typically shifts the concentration of metabolites, mainly sucrose and starch, in the tissues. Sufficiency soluble sugars supply is known to activate various carbohydrate-consuming cell functions such as biosynthesis of polysaccharides and proteins, cell cycle, respiration, and nitrogen assimilation [17]. Sugars can also act as signal molecules and regulate gene expression of different physiological cycles [10,15,44]. However, despite the clear alterations in growth and photosynthetic metabolism that occur in response to salt and low-temperature stress, the exact nature of the changes in carbon metabolism that are induced by these stresses have not still been completely elucidated [7]. In this context, it has been found that salinity and low temperature induce synthesis of polyols (linear polyhydric sugar alcohols) and cyclitols (cyclic polyhydric sugar alcohols) from the more common storage carbohydrates such as starch and sucrose [13,14]. These observations have led to the suggestion that naturally occurring changes in the carbohydrate status may have an adaptive role in allowing seedlings to survive under saline and/or low-temperature conditions [12,31,38,41]. However, under field conditions stress does not generally come in isolation and many stresses act simultaneously. In response to these stress signals that cross- talk with each other, seedlings have developed diverse pathways for combating and tolerating them [15]. Thus, when different stresses co-occur seedling responses are usually unpredictable by a single factor of analyses, and so they are unique and cannot be directly extrapolated from the response of seedlings to each stress applied individually. In addition, the simultaneous exposure of seedlings to different abiotic stress conditions will result in the co-activation of different stress-response pathways, which can result in intensification, overlapping or reversal of stress effects [13]. Therefore, it is necessary to understand how seedlings respond to combined stress conditions.

Many studies have demonstrated quantitative and qualitative changes in the carbohydrate content of seedlings exposed to low temperature and salinity [33,43,45,51] but the literature available on combined effects of both stresses is scarce [13,38]. Consequently, it still remains unclear whether the positive effects of a particular carbohydrate are interchangeable between saline and low-temperature stress, or whether the carbohydrates fulfil specific roles under these stress conditions when they act simultaneously. The aim of this study was to investigate specificity of changes in SSR of quinoa cotyledons in relation to growth, proline, and sucrose–starch partitioning and related enzymes, as a consequence of simultaneous exposure of seedlings to salinity and low-temperature stresses. Quinoa, the world's potential new crop, was chosen as experimental material because it is a well adapted crop to adverse abiotic factors and exhibits a fast growth during seedling establishment [23]. Moreover, in the cotyledoneous, two- and five-leaf stage, quinoa has a high frost resistance with no damage at low temperatures; however, frost exposure during flower bud formation and anthesis has a serious negative effect [18].

2. Results

2.1. Growth measurements

Fig. 1 shows the growth of cotyledons and growing axes in stressed and unstressed quinoa seedlings. In salt-stressed and salt-acclimated by the fourth day cotyledon growth was already beginning to slow down but from the sixth day on further growth occurred. In unstressed and low-temperature control cotyledons a sustained increased during the experimental period was observed; however, it was higher in the first ones (Fig. 1A). Interestingly, from the sixth day there were no significant differences in the growth of salt-treated and low-temperature control cotyledons. Growing axis length was also higher in unstressed seedlings than in stressed and low-temperature ones, but there were no significant differences between stressed and low- temperature seedlings during the experimental period (Fig. 1B). In unstressed seedlings a delay in growth rate was observed from the ninth day, which was coincident with emergence of the first pair of leaves.

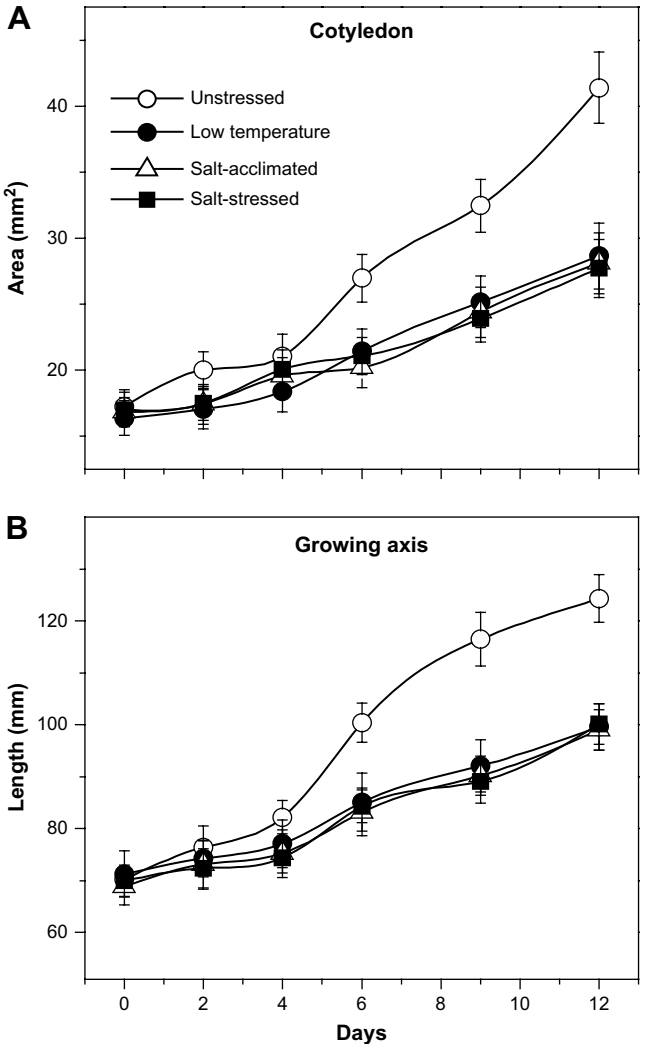

Fig. 1. Changes in cotyledon area (A) and growing axis length (B) during growth and development of quinoa seedlings. Cotyledon area corresponds to one pair. Values are means ± SD of three different experiments ($n = 20$).

2.2. Changes in enzyme activities

In our study, we examined the major regulatory enzymes involved in the carbon flux of seedlings exposed to low temperature and saline stresses (Fig. 2). Measurements of the activity of SPS exposed a low temperature showed different patterns in both salt-stressed and salt-acclimated cotyledons when compared with low-temperature and unstressed control cotyledons (Fig. 2A). The total

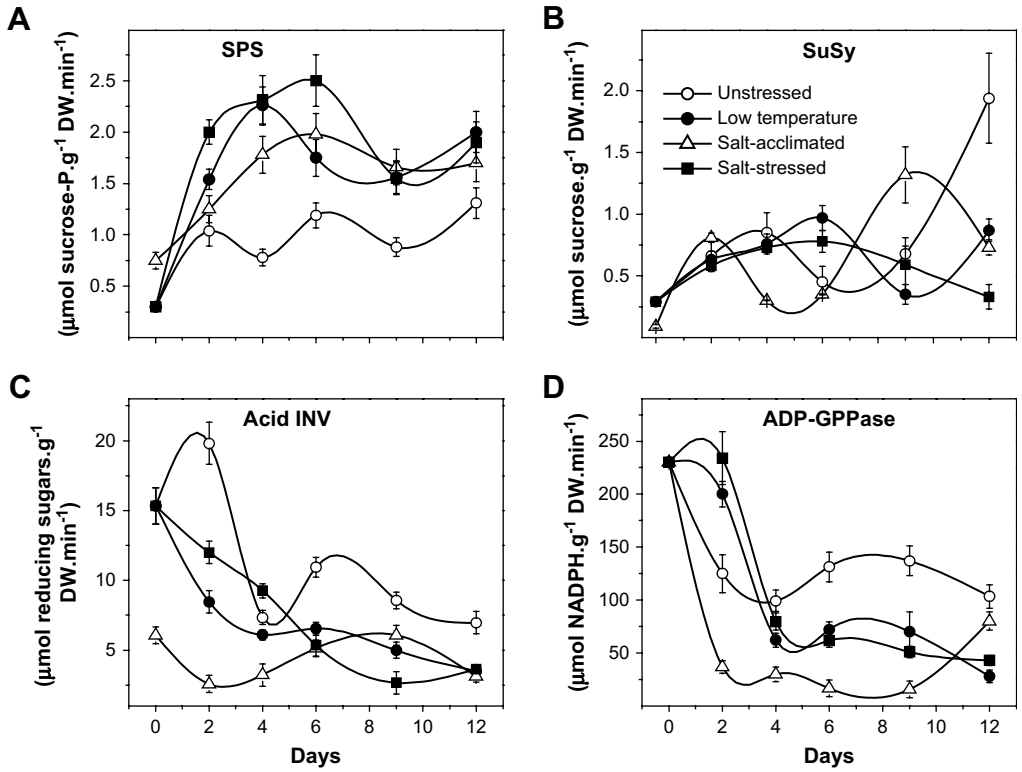

Fig. 2. Enzymatic activities in stressed and unstressed cotyledons of quinoa seedlings. (A) SPS, (B) SuSy, (C) acid INV, (D) ADP-GPPase. Values are means ± SD of three different experiments ($n = 4$).

activity of SPS was higher in salt-stressed cotyledons than in salt-acclimated and control ones. The SPS pattern of salt-stressed cotyledons showed a rapid initial increase up to 2 days followed by a slow increment reaching a maximum of 2.51 µmol g^{-1} DW min^{-1} up to 6 days. This maximum was nearly 2.5-fold higher than in unstressed cotyledons. After that, activity dropped sharply to a value of 1.50 µmol g^{-1} DW min^{-1} until 9 days, and then increased slowly to the end of the experiment. In salt-acclimated cotyledons the enzyme activity showed a progressive increase up to 6 days with a maximum value of 1.98 µmol g^{-1} DW min^{-1} (1.6-fold higher compared to unstressed cotyledons). From this point on, SPS activity only showed a slow decrease until the end of the experiment. In low-temperature control cotyledons the pattern of SPS activity also showed a rapid initial increase up to 4 days, reaching a maximum value of 2.28 µmol g^{-1} DW min^{-1} (2.2-fold higher than in unstressed cotyledons), followed by a progressive decrease until 9 days. After that, SPS activity showed a slow increment to the end of the experiment. The lowest SPS activity was observed in unstressed cotyledons but the pattern was, in general, similar to stressed ones. However, the time of onset was different, being earlier in unstressed cotyledons.

Fig. 2B shows the pattern of SuSy activity in both stressed and control cotyledons. Salt-stressed cotyledons showed a slow initial increase of SuSy activity reaching a maximum up to 6 days, and then gradually decreased until the end of the experiment. By contrast, in salt-acclimated cotyledons the SuSy activity showed a rapid increase until the second day and then rapidly decreased up to 4 days reaching a minimum value of 0.27 µmol g^{-1} DW min^{-1}. After that, SuSy activity showed a strong increment up to 9 days reaching a maximum of 1.30 µmol g^{-1} DW min^{-1}. From this point on, SuSy activity showed a pronounced decrease until the end of the experiment. In low-temperature control cotyledons the SuSy pattern also showed a progressive increment up to 6 days, reaching a maximum value of 0.98 µmol g^{-1} DW min^{-1}, and thereafter strongly decreased. After reaching a minimum value of 0.44 µmol g^{-1} DW min^{-1} at the ninth day, the SuSy activity showed a rapid recovery until 12 days (end of the experiment). SuSy pattern did not show great variations between low-temperature control and salt-stressed cotyledons up to 9 days, but from this point on it was different. SuSy activity in unstressed cotyledons did not show significant variations with respect to stressed ones up to 9 days, but increased strongly, reaching a maximum of 1.98 µmol g^{-1} DW min^{-1} (2.0-fold higher compared to stressed cotyledons) up to 12 days. It is interesting to point out that both low-temperature and unstressed controls showed between 9 and12 days a strong increase in SuSy activity, while salt-stressed and salt-acclimated activity continuously declined.

Soluble acid INV activity was significantly higher in unstressed cotyledons than in stressed ones (Fig. 2C). The highest value, 19.6 µmol g^{-1} DW min^{-1}, was observed at the second day and then a continuous decrease occurred. In stressed cotyledons, the activity of soluble acid INV between 0 and 6 days was higher in salt-stressed than in salt-acclimated and low-temperature cotyledons. Acid INV pattern in salt-stressed cotyledons showed a progressive decrease up to 9 days, reaching a minimum value of 2.43 µmol g^{-1} DW min^{-1}, and then practically remained unchanged until the end of the experiment. By contrast, in salt-acclimated cotyledons acid INV pattern showed a strong initial decrease until the second day and then increased gradually up to 9 days, reaching a value of 5.64 µmol g^{-1} DW min^{-1}. From this point on, soluble acid INV activity showed a progressive decrease until the end of the experiment. During the early development (0–2 days) acid INV activity of salt-stressed cotyledons was nearly 3.6-fold higher compared to salt-acclimated ones. Enzyme pattern in low-temperature control showed a rapid initial decrease until 4 days followed by a slow increment up to 6 days. After that, acid INV activity decreased until

the end of the experiment. In general, salt-acclimated, low-temperature and unstressed acid INV patterns were similar, but the time of onset was not. This was observed earlier in salt-acclimated cotyledons. Acid INV temporal pattern of salt-stressed cotyledons was different. It is interesting to point out that acid INV activity was of a higher magnitude than SuSy activity in both stressed and unstressed cotyledons.

The pattern of ADP-GPPase activity is shown in Fig. 2D. In salt-stressed cotyledons enzyme activity between 0 and 2 days did not show significant changes but decreased sharply until 4 days, reaching a minimum value of 80.0 μmol g^{-1} DW min^{-1}. From this point on, ADP-GPPase activity showed a slow decrease until the end of the experiment. By contrast, in salt-acclimated cotyledons ADP-GPPase activity between 0 and 2 days showed an initial sharp decrease, reaching a minimum value of 35.8 μmol g^{-1} DW min^{-1} up to 2 days, and then remained relatively unchanged until 9 days. After that, ADP-GPPase activity showed a pronounced increment up to 12 days. ADP-GPPase activity in low-temperature control between 0 and 4 days also showed a strong decrease and thereafter a slow increment up to 9 days was observed. Then, enzyme activity showed a progressive decrease until the end of the experiment. Although ADP-GPPase activity in unstressed cotyledons also showed a decrease until 4 days it was less pronounced than in stressed cotyledons, and thereafter a slow increase up to 9 days was observed. From this point on, ADP-GPPase activity decreased until the end of the experiment. Between 4 and 12 days the ADP-GPPase activity was higher in unstressed cotyledons than in stressed ones, but the highest value (235.9 μmol g^{-1} DW min^{-1}) was observed in salt-stressed cotyledons. For all cotyledons, ADP-GPPase activity without 3-phosphoglycerate was significantly lower than in the presence of 3-phosphoglycerate (data not shown).

2.3. Changes in soluble sugars, starch and proline contents

The endogenous content of soluble carbohydrates (sucrose, glucose, fructose) and starch was measured in stressed and unstressed quinoa cotyledons (Fig. 3). Soluble sugars and starch showed different patterns in both salt-stressed and salt-acclimated cotyledons when compared with low-temperature and unstressed controls, respectively. Results showed that sucrose content was higher in salt-stressed cotyledons than in salt-acclimated, low-temperature and unstressed ones (Fig. 3A). Of interest, in unstressed cotyledons the content of sucrose was nearly 8.5-fold lower than in stressed ones. In salt-stressed cotyledons sucrose pattern showed a rapid initial increment, reaching between 2 and 4 days a maximum content of 34.50 mg g^{-1} DW. From this point on, sucrose content remained constant up to 6 days and then decreased slowly until the end of the experiment. In salt-acclimated cotyledons the sucrose content showed a gradual increment up to 4 days, reaching a maximum value of 28.41 mg g^{-1} DW, and then remained constant until 6 days. After that, sucrose content showed a progressive decrease. Sucrose content of low-temperature control showed an initial similar trend, reaching a maximum value of 33.70 mg g^{-1} DW up to 4 days. From this point on, sucrose content showed a progressive decrease up to 9 days and then increased again until the end of the experiment . By contrast, in unstressed cotyledons the sucrose content did not show significant variations during the experimental period. Glucose content showed a different pattern between control and treated cotyledons but the highest value was observed in salt-stressed ones. There was a sustained increase up to 4 days in both salt-stressed and salt-acclimated cotyledons, but this was stronger in the former, reaching a maximum value of 46.36 mg g^{-1} DW. After that, glucose content gradually decreased until the end of the experiment (Fig. 3B). Regarding unstressed cotyledons the glucose content increased up to the second day, reaching a maximum of 29.93 mg g^{-1} DW, and then strongly decreased until 4 days, reaching a minimum value of 5.1 mg g^{-1} DW. From this point on, glucose content remained practically unchanged up to 9 days and then increased until the end of the experiment. At 6 days, glucose content of low-temperature control showed a similar trend to salt-treated cotyledons; however, from this point a sustained increment reaching a maximum value of 34.2 mg g^{-1} DW at 12 days was observed (Fig. 3B). Fructose was essentially higher in low-temperature control than in salt-stressed and unstressed cotyledons (Fig. 3C). Fructose pattern was similar in both low-temperature and salt-treated cotyledons, reaching a maximum content up to 4 days (25.11 mg g^{-1} DW, 25.11 mg g^{-1} DW, 17.42 mg g^{-1} DW for low-temperature, salt-acclimated and salt-stressed cotyledons, respectively). From this point on, fructose content decreased in all treatments; however, in low-temperature control from the ninth day until the end of the experiment a sustained increment was observed. Although the fructose pattern of unstressed cotyledons showed a similar trend to stressed ones, the maximum content was observed earlier.

Starch content up to 6 days was higher in low-temperature and salt-treated cotyledons compared to unstressed ones (Fig. 3D). Regarding the starch pattern, in salt-acclimated cotyledons between 0 and 4 days a strong decrease was observed and thereafter remained practically unchanged until the end of the experiment. By contrast, in salt-stressed cotyledons starch content showed a progressive decrease during the experimental period only. Starch content of unstressed cotyledons showed a similar pattern to salt-acclimated ones but the initial decrease was observed between 0 and 2 days. In low-temperature control starch content showed a strong increment up to 2 days, reaching a maximum value of 200.0 mg g^{-1} DW, and then continuously declined until the end of the experiment (Fig. 3D).

Proline content was higher in salt-treated cotyledons than in unstressed and low-temperature controls. However, between 0 and 6 days the highest value was observed in salt-acclimated seedlings (Fig. 4). Proline pattern in salt-stressed cotyledons showed a low content up to 4 days and then strongly increased until the end of the experiment, reaching a maximum of 9.67 mg g^{-1} DW (approximately 9.5-fold higher compared to unstressed control). Salt-acclimated cotyledons showed a similar trend but the increment from the fourth day was slower. In both low-temperature and unstressed controls proline content was very low and stayed practically unchanged during the experimental period. However, in low-temperature control between 9 and 12 days a slight increment was observed.

3. Discussion

Chenopodium quinoa is a halophyte species that has epigeal-type cotyledons with a very low carbon reserve [36]. During the pre-emergence phase of germination, perisperm is the unique carbohydrate source for quinoa growth [41]; however, the subsequent dry weight increment, which begins immediately after emergence and several days before the first true leaves are unfolded, is indicative of a considerable photosynthetic contribution by cotyledons [40]. Quinoa cotyledons expand substantially, develop chlorophyll and are efficient photosynthetic organs [34,40]. In this context, photosynthate from cotyledons determines the seedling establishment, and then the growth rate of seedlings depends upon sucrose–starch partitioning [32,42]. Seedling growth is a critical process whose functionality is strongly affected by adverse environmental factors, especially drought, salinity and low temperatures, which affect the source–sink relations (SSR) [15,22,24,32]. Under field conditions stress does not generally come in isolation and many stresses act simultaneously [7,44]. Therefore responses

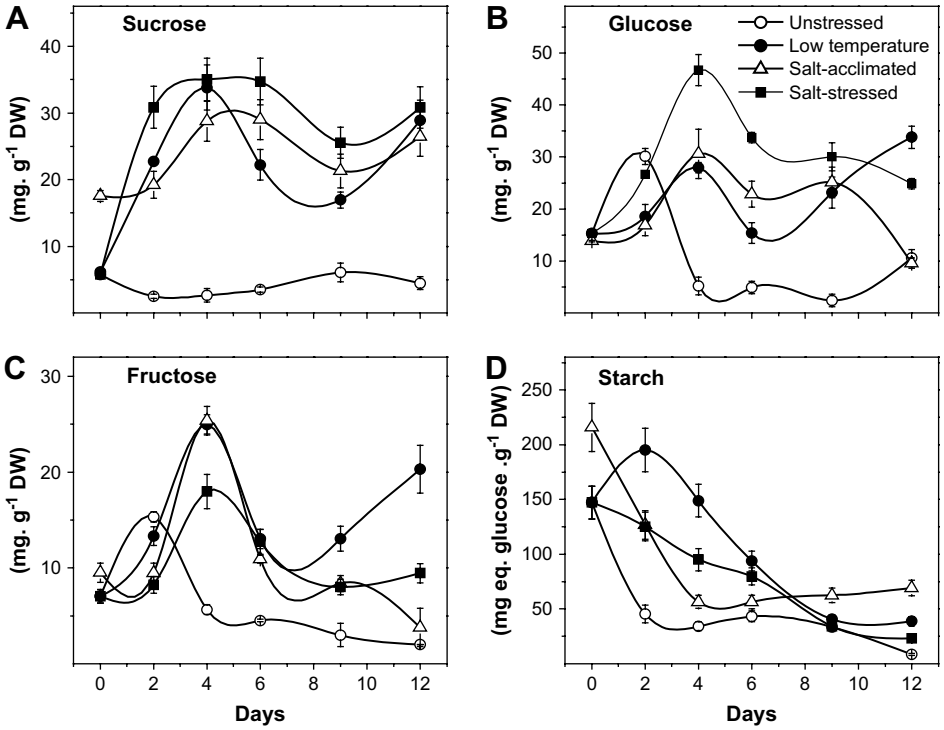

Fig. 3. Evolution of soluble sugars and starch contents in stressed and unstressed cotyledons of quinoa seedlings. (A) sucrose, (B) glucose, (C) fructose, (D) starch. Values are means ± SD of three different experiments ($n = 4$).

to combined stresses are unique and cannot be directly extrapolated from responses to each stress applied individually [13]. Although it is difficult to determine that processes are more severely affected by simultaneous stresses, differential responses generating complex effects have been communicated [15,44]. Under low temperature and salinity the photosynthate is used to support crucial processes such as growth, maintenance and osmotic adjustment [13,34,42], and so responses to these stresses can be interpreted based on changes in allocation of the photosynthetic fixed carbon [48]. In order to gain an insight into the physiological changes occurring during simultaneous stresses, we studied the effect of low temperature and salinity on growth and sucrose–starch partitioning in cotyledons of quinoa seedlings. The growth of cotyledons and shoot–root axis was affected under low temperature when compared to seedlings growing at higher temperature (unstressed), but there were no differences between salt-treated (salt-acclimated, salt-stressed) and low-temperature control seedlings (Fig. 1A,B). However, we observed important changes in the cotyledonary carbohydrate status between salt-treated and low-temperature and unstressed controls. The high sucrose accumulation observed in salt-treated cotyledons, principally in salt-stressed ones, could imply that in these cotyledons the metabolic carbon flux was mainly used for sucrose accumulation, necessary to counteract the osmotic imbalance imposed by salt (Fig. 3A). In agreement with this assumption in unstressed cotyledons, sucrose content was significantly lower than in salt-treated ones. The activity of SPS in salt-treated and low-temperature control cotyledons was higher than in unstressed ones, but the highest value was observed in salt-stressed cotyledons (Fig. 2A). Thus, SPS activity appears to be positively correlated with sucrose synthesis and our results agree with those reported by Guy et al. [16] and Djanaguiraman et al. [11], who demonstrated increments of SPS activity and sucrose in low-temperature-stressed and salt-acclimated plants. Low-temperature stress and salt acclimation also lead to accumulation of large pools of free hexoses (glucose, fructose) and enhancement the photosynthetic carbon metabolism [11,25,31,41]. Then changes in enzymes involved in sucrose cleavage are expected. Although our results demonstrated that there exists a good correlation between acid INV activity and sucrose hydrolysis in salt-treated and low-temperature control cotyledons, the highest enzyme activity was observed in unstressed ones (Fig. 2C). The high level of acid INV detected in unstressed cotyledons can be explained by the highest metabolic demand of

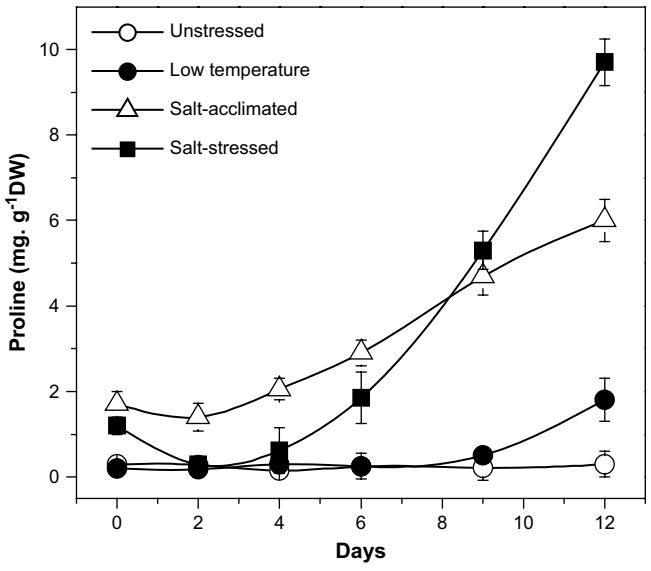

Fig. 4. Evolution of proline content in stressed and unstressed cotyledons of quinoa seedlings. Values are means ± SD of three different experiments ($n = 4$).

free hexoses necessary to sustain a more active growth observed in these seedlings. In agreement with this assumption, glucose and fructose were lower in unstressed cotyledons. Likewise, this was coincident with findings of Kingston-Smith et al. [21], who postulated that in growing leaves a low level of hexoses is necessary to maintain a respiratory substrate without risking the metabolic activity being perturbed by large changes in photoassimilate abundance in nearby cells. On the other hand, the relatively high acid INV activity observed between 0 and 6 days in salt-stressed cotyledons could be explained by a higher demand of soluble sugars for osmotic adjustment. This conclusion agrees with the highest glucose content observed in salt-stressed cotyledons (Fig. 3B). In addition, previous results obtained in our laboratory have demonstrated that saline stress induces accumulation of glucose rather than fructose in embryonic axes of quinoa seedlings [41]. Although SuSy is also involved in sucrose metabolism no correlation with its content was observed, so we postulated that acid INV plays a more active role in the growth of quinoa seedlings. This hypothesis agrees with findings of Pfiffer and Kutschera [33], who have demonstrated that cell elongation in developing sunflower hypocotyls exhibits a close correlation with acid INV activity.

In photosynthetic tissues sucrose–starch partitioning is the key process to control SSR [22]. Starch production depends upon the concerted activity of several enzymes, but the activity of ADP-GPPase is of prime importance in its synthesis [2,3]. Thus, we also examined the effect of low temperature on ADP-GPPase activity and starch content of salt-stressed and salt-acclimated quinoa cotyledons. Quinoa has no amylaceous cotyledons but they are capable of producing photosynthetic sugars a few hours after emergence occurs [40]; therefore early changes in SSR and sucrose–starch partitioning are expected to occur. Although there were no significant differences between salt-stressed and low-temperature control ADP-GPPase activities, the highest starch content was observed in low-temperature control cotyledons (Figs. 2D and 3D). Leaf starch accumulation induced by low temperature has also been described for other species such as *Oryza sativa*, *Helianthus annuus*, *Lycopersicon esculentum* and *Cucumis sativus*, among others [26,27]. For these species it has been proposed that decreases in the flux of carbon from source leaves produced by low temperature may indicate a low sink demand as well as a restriction of phloem transport itself by cold. This assumption can also be applied to 5/5 °C growing control of quinoa cotyledons; however, on the basis of our results, it can be suggested that the lowest content of starch observed in both salt-stressed and salt-acclimated cotyledons is due to changes in carbon allocation to produce free proline to counteract the saline stress. This supposition agrees with the great difference in proline content observed between salt-treated and low-temperature control (Fig. 4). Also, our results showed an early proline accumulation in salt-acclimated cotyledons, probably due to a previous salt inductor effect. This assumption agrees with previous studies that demonstrated an increase in the level of proline in germinating seeds under salinity [49]. Also, we observed from the fourth day a pronounced decrease in fructose and glucose content in both salt-stressed and-acclimated cotyledons that was coincident with the strong proline increment observed in these cotyledons (Figs. 3B,C and 4). We believe that this finding can also be attributed to a change in the carbon flux to build the amino acid carbon skeleton [49]. However, the low starch content observed in unstressed cotyledons can be explained by the faster carbon investment for seedling growth.

It has also been suggested that starch synthesis in cotyledons can represent the formation of a secondary pool of carbon to prevent the increase of sugar concentrations to inhibitory levels [42]. Thus, starch synthesis may be a reflection of the need to produce an internal metabolic sink for soluble sugars in the cotyledons. Many studies have undoubtedly demonstrated that in photosynthetic tissues the synthesis of ADPG, through ADP-GPPase activity, takes place in chloroplasts, while in non-photosynthetic tissues this occurs outside the plastids [3,37]. However, in these two isoforms of ADP-GPPase: plastidial and extra-plastidial (cytosolic) coexist, whereas in the photosynthetic tissues a plastidial form only is present [3,8,30]. Baroja-Fernández et al. [4] demonstrated that chloroplast is able to incorporate ADPG from cytosol and to synthesise starch. They have proposed an alternative pathway to chloroplastidic starch biosynthesis based on this finding and the SuSy capacity to produce ADPG from sucrose and ADP in the cytosol, instead of chloroplastidic ADP-GPPase activity [28]. Nevertheless, it has been suggest that in this pathway some ADPG produced in the chloroplast by ADP-GPPase activity is exported to the cytosol before it is imported again to plastid [4]. Baroja-Fernandez et al. [5] also demonstrated a positive correlation between SuSy activity and starch synthesis in non-photosynthetic potato tissues. However, in maize root tips no positive correlation was observed, it being demonstrated that in this tissue starch is produced from ADPG synthesised in the plastid by ADP-GPPase activity and not in the cytosol by SuSy enzyme [1]. In agreement with this finding, our results did not show a positive correlation between SuSy activity and starch content in both stressed and unstressed cotyledons (Figs. 2B and 3D). Therefore we considered that in quinoa cotyledons SuSy activity together with UDP-GPPase and hexose-P isomerase activities acts to maintain a sizeable pool of sucrose, UDPG and hexose-P, which is important in providing abundant substrates for metabolic pathways, but not in the synthesis of the transitory starch. Nevertheless, further studies are needed to clarify this question.

In conclusion, although several works have demonstrated that abiotic stresses induce changes of enzyme activities related to sucrose and starch metabolism, those referring to the combined effect of two or more stress factors are very scarce. Thus, the impact of simultaneous stresses on seedling establishment and growth remains unclear and needs further clarification. Results obtained in the present study provide interesting data for future studies on this topic.

4. Materials and methods

4.1. Plant material

Quinoa seeds (*Ch. quinoa*) cv. Sajama were germinated in plastic boxes (28 × 20 × 5 cm) containing vermiculite moistened with a fourth-strength Hoagland solution, under 12 h photoperiod, at 25/20 °C (light/dark) temperature regime, 70% relative humidity and photosynthetic photon flux density (PPFD) of 190 μmol photons m^{-2} s^{-1}. After 4 days, when cotyledons had expanded, quinoa seedlings were shifted to a 5/5 °C (light/dark) temperature regime, with a photoperiod and irradiance similar to the above described. Saline treatment was performed by supplying a mineral solution containing 200 mM NaCl. Seedlings growing at 25/20 °C without NaCl and shifted to 5/5 °C in presence of 200 mM NaCl were considered as salt-stressed seedlings, while those growing at 25/20 °C with NaCl and shifted to 5/5 °C in the presence of 200 mM NaCl were considered as salt-acclimated seedlings. Seedlings grown with and without NaCl had a similar morphology and developmental state. Seedlings growing at 25/20 °C and shifted to 5/5 °C without NaCl were used as low-temperature control stress, whereas seedlings maintained at 25/20 °C without NaCl during the experimental period were considered as unstressed control plants. Cotyledons for analyses were harvested 8 h after light was turned

on at 0, 2, 4, 6, 9, and 12 days from the beginning of 5/5 °C growth period, and stored at −20 °C until analyses were performed.

4.2. Growth measurements

Cotyledon growth was measured as increase in area of one pair. The area was defined as length × width. This value, although not the exact area of the cotyledon, is proportional to its area. Growing axis (hypocotyl and root) growth was measured as increase in length. Cotyledon and growing axis measurements were performed using a digital calibre (Hitachi, Japan) and a plastic ruler (accuracy ±0.5 mm), respectively. Twenty pairs of cotyledons and growing axes from different seedlings were selected at random for each measurement. After fresh weight (FW) determination, the dry weight (DW) was determined by drying of cotyledons at 70 °C for 3 days.

4.3. Soluble sugars, proline, and starch determinations

Soluble sugars (sucrose, glucose, fructose) were extracted from 0.5 g FW of cotyledons by homogenisation in 2 ml of 80% (v/v) ethanol with a mortar and pestle. After heating the homogenate in a water bath at 75 °C for 10 min, the insoluble fraction was removed by centrifugation at 5000 × *g* for 10 min. The precipitate was homogenised twice with 2 ml of 80% (v/v) ethanol and centrifuged again. Supernatants were pooled and dried under a stream of hot air, and then the residue was resuspended in 1 ml of distilled water and desalted by filtration through an ion-exchange column (Amberlite MB3, BDH, England). Sucrose was determined by the protocol of Cardini et al. [9] and fructose by the method of Roe and Papadopoulos [39]. Glucose was determined using a glucose oxidase–peroxidase coupled assay according to Jorgensen and Andersen [19]. Soluble sugar contents were expressed as mg (sucrose, glucose, fructose) g^{-1} DW. Aliquots of the supernatant without desalting were used for proline determination according to the Ting and Rouseff [50] procedure.

For starch determination, the insoluble fraction remaining after ethanolic extraction was resuspended in 2 ml of 2.5 M NaOH and boiled for 5 min to gelatinise the starch. After cooling the suspension pH was adjusted to 4.5 using 2 M HCl, and then the resulting gelatinised starch was hydrolysed with buffered amyloglucosidase (AMG, EC 3.2.1.3) (15 UI ml^{-1} of amyloglucosidase from *Rhizopus* mould, in 0.1 M sodium acetate buffer, pH 4.5). After 10 min at 50 °C, aliquots were taken and the soluble reducing sugars released were assayed by Nelson's method [29]. Starch content was expressed as (mg glucose equivalent released) g^{-1} DW.

4.4. Extraction and assay of sucrose phosphate synthase (SPS), sucrose synthase (SuSy), soluble acid invertase (acid INV) and ADP-glucose pyrophosphorylase (ADP-GPPase)

SPS and SuSy activities were measured as described previously by Batta and Singh [6] with minor modifications. Briefly, cotyledons (1 g FW) were homogenised in a chilled mortar and pestle with 3 ml of extraction buffer (100 mM Tris–HCl buffer (pH 7.6), 10 mM $MgCl_2$, 5 mM EDTA, and 1 mM β-mercaptoethanol). The homogenate was centrifuged at 15,000 × *g* for 15 min at 4 °C, and the supernatant dialysed for 3 h against 10 mM Tris–HCl buffer (pH 8.0), containing 10 mM $MgCl_2$, and 1 mM β-mercaptoethanol. The dialysed extract was used as a source of crude enzyme for assaying SPS and SuSy activities. SPS activity was assayed in the direction of sucrose–phosphate synthesis by incubating a reaction mixture (100 μl) containing 40 mM Tris–HCl buffer (pH 8.0), 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 8 mM UDPG (uridine 5′-diphosphoglucose), 16 mM fructose-6-phosphate, 8 mM glucose-6-phosphate, and 20 μl of enzyme extract. SuSy activity was assayed in the direction of sucrose synthesis by incubating a reaction mixture (100 μl) containing 40 mM Tris–HCl buffer (pH 8.0), 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 8 mM UDPG, 16 mM fructose, and 20 μl of enzyme extract. In both assays, mixtures were incubated at 37 °C for 30 min. The amount of product (sucrose-P or sucrose) synthesised was determined by the Cardini et al. [9] procedure. The enzyme activities were expressed as μmol sucrose-P or sucrose synthesised g^{-1} DW min^{-1}.

Acid INV activity was measured as described previously by Prado et al. [35] with minor modifications. Briefly, cotyledons (1 g FW) were homogenised in a chilled mortar and pestle with 3 ml of extraction buffer (50 mM sodium phosphate buffer (pH 7.4), 1 mM β-mercaptoethanol and 5 μM $MnSO_4$). The homogenate was centrifuged at 15,000 × *g* for 15 min at 4 °C, and the supernatant dialysed for 60 min against 10 mM sodium acetate buffer (pH 5.5), containing 1 mM β-mercaptoethanol. The dialysed extract was used as a source of crude enzyme for assaying acid INV activity. Because the extraction medium did not contain detergent, chelating and chaotropic agents or high salt concentrations; the probability of extracting the cell wall invertase activity was very low. Acid INV activity was assayed by incubating a reaction mixture (100 μl) containing 200 mM sodium acetate buffer (pH 5.5), 60 mM sucrose, 1 mM β-mercaptoethanol, and 10 μl of enzyme extract. The mixture was incubated at 37 °C for 30 min, and the released reducing sugars were determined by Nelson's method [29]. The enzyme activity was expressed as μmol reducing sugars produced g^{-1} DW min^{-1}.

ADP-GPPase was extracted following a modified Sowokinos [46] method. Briefly, cotyledons (1 g FW) were homogenised in a chilled mortar and pestle with 3 ml of extraction buffer (100 mM Tris–HCl buffer (pH 7.6), 10 mM $MgCl_2$, 5 mM EDTA, 1 mM β-mercaptoethanol, and 5 μM $MnSO_4$). The homogenate was centrifuged at 15,000 × *g* for 15 min at 4 °C, and the supernatant dialysed for 3 h against a 10 mM Tris–HCl buffer (pH 8.0), containing 10 mM $MgCl_2$ and 1 mM β-mercaptoethanol. ADP-GPPase activity was determined measuring NADPH production according to Sowokinos and Varns [47] procedure. ADP-GPPase activity was carried out in two steps.

First step. Reaction mixture (500 μl) contained 40 mM HEPES–NaOH buffer (pH 8.0), 5 mM $MgCl_2$, 10 mM ADPG (adenosine 5′-diphosphoglucose), 20 mM 3-phosphoglycerate, 10 mM sodium pyrophosphate, and 100 μl of enzyme extract was incubated at 37 °C for 20 min, and then boiled for 3 min. After centrifuging at 5000 × *g* for 5 min, the pellet was discarded.

Second step. Reaction mixture (1 ml) contained 40 mM HEPES-NaOH buffer (pH 7.4), 12 mM NADP, 2.25 UI phosphoglucomutase (PGM, EC 2.7.5.1), 3 UI glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), and 450 μl of first step supernatant. NADPH production was monitored at 340 nm and 37 °C using a double beam spectrophotometer (Hitachi U-2800A, Japan). Absorbance values were recorded until loss of initial linear reaction rate occurred. Substrates and coupled assay components were in excess, so that NADPH production rate was linear to enzyme concentration and time. All assays were run with controls and no ADPG to detect any NADPH formation non-dependent on ADP-GPPase. The enzyme activity was expressed as (μmol NADPH produced) g^{-1} DW min^{-1}.

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