Review

Proline: A Reliable Biochemical Marker of Plant Abiotic Stress Tolerance?

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Review

Proline: A Reliable Biochemical Marker of Plant Abiotic Stress Tolerance?

Delia Maria Luca [1], Marius-Nicuşor Grigore [2] and Oscar Vicente [3,*]

[1] Faculty of Biology, "Alexandru Ioan Cuza" University, 20A Carol I Bd, Iasi 700506, Romania
[2] Doctoral School of Biology, "Alexandru Ioan Cuza" University, 20A Carol I Bd, Iasi 700506, Iasi, Romania
[3] Institute for the Conservation and Improvement of Valencian Agrodiversity (COMAV). Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain
* Correspondence: ovicente@upvnet.upv.es

Abstract

Climate change is placing global agriculture under growing pressure, as plants must withstand extreme environmental conditions such as drought and high salinity, both inducing osmotic and oxidative stress. As part of their survival strategies, plants accumulate protective molecules (osmolytes), including the amino acid proline. For decades, plant biology has largely assumed that high proline accumulation under stress signals strong stress tolerance. However, this review challenges that "proline-centric" perspective. Analyses across a wide range of plant species reveal a more complex picture. Stress-induced proline accumulation is not universal: in some species, proline levels remain relatively unchanged, with other metabolites acting as functional osmolytes, or increase only in response to artificially applied severe stress conditions. Even when proline increases, its absolute concentrations may be too low to contribute significantly to osmotic adjustment. Nevertheless, proline may still be involved in stress tolerance mechanisms through its additional roles, detoxifying reactive oxygen species (ROS), directly stabilising proteins or acting as a stress signalling molecule. Comparative analyses of genetically related taxa with varying degrees of stress tolerance sometimes show negative correlations between proline accumulation and tolerance, with higher proline concentrations measured in the most sensitive genotypes. Overall, the evidence indicates that proline's role in plant survival is highly context-dependent and strongly influenced by genetic background and must therefore be evaluated on a case-by-case basis. Distinguishing whether proline acts as an adaptive defence or merely as a biochemical marker of physiological strain is essential for accurately assessing plant stress tolerance.

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1. Introduction

It is well established that climate change intensifies the impact of environmental factors generating abiotic stress in plants—such as salinity, drought, heatwaves, or heavy rainfall and flooding—to the detriment of crop yields, food security, and the biodiversity of wild species [1,2]. To survive these effects, plants have evolved a complex, multi-layered defence strategy. This strategy involves the tight regulation of ion transport and homeostasis, particularly through mechanisms that restrict the entry of sodium (Na^+) and chloride (Cl^-) ions into the transpiration stream or sequester them safely within the vacuole to prevent cytoplasmic toxicity [3]. At the same time, plants activate antioxidant systems including both enzymatic components—for example, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) or glutathione reductase (GR)—and antioxidant metabolites, such as glutathione, ascorbate, and phenolic compounds, to neutralise the reactive oxygen species (ROS) generated by metabolic imbalances [4,5].

However, a plant's ability to maintain water uptake and limit cellular dehydration under hyperosmotic conditions largely depends on osmotic adjustment. This process involves the active synthesis and intracellular accumulation of low-molecular-weight organic compounds known as compatible solutes or osmolytes. These compounds form a chemically diverse group, including quaternary ammonium compounds, sugar alcohols, soluble sugars and amino acids [6].

Amongst these compatible solutes, the amino acid proline (Pro) occupies a distinctive—and often controversial—position in plant stress physiology. Pro accumulation is one of the most widely reported metabolic responses to abiotic stress and has been documented across a broad phylogenetic range, from marine invertebrates [7] to herbaceous crops [8], cereals [9], ornamentals [10], halophytes [11,12] or forest trees [13,14]. The role of Pro in mediating plant responses to abiotic stress, particularly drought and salinity, has therefore been a cornerstone of plant physiological research for decades, and it is widely accepted that Pro contributes to stress tolerance mechanisms. Nevertheless, a critical examination of the literature reveals considerable ambiguity and, in some cases, contradictory interpretations. Many studies infer that Pro acts as a primary osmolyte solely on the basis of its increase under stress, often without considering that the amount of proline accumulated, while statistically significant compared to the non-stressed control, may not be physiologically significant for osmotic adjustment. The assumption that Pro is directly involved in the mechanisms of stress tolerance in plants is so widely accepted that in many publications Pro is the only potential osmolyte quantified, disregarding the possible contribution of additional compatible solutes, such as soluble sugars, sugar alcohols or glycine betaine. The additional roles of Pro in the responses of plants to abiotic stress, as “osmoprotectant” or low-molecular-weight chaperon, directly stabilising proteins and other macromolecules under stress conditions, as ROS scavenger, or as a stress signalling molecule [15,16], make the assessment of its contribution to stress tolerance even more difficult.

Notwithstanding the relevance of other stress responses, such as the control of ion transport or the activation of antioxidant systems, in the mechanisms of tolerance, this review focusses specifically on the role of Pro. We attempt to critically evaluate the functional significance of Pro accumulation across diverse taxa, including crops, forest trees, ornamentals, halophytes and invasive species, to address persistent uncertainties regarding its role in stress tolerance. Based on published data, including work from of our groups during the last decade, we challenge the prevailing “proline-centric” paradigm by examining key factors frequently overlooked in the literature: the functional specificity of Pro compared with alternative osmolytes, the physiological relevance of absolute concentration thresholds required for osmotic adjustment, and the observation, when comparing different related taxa, of negative correlations between the stress-induced Pro accumulation and their relative stress tolerance. By synthesising available evidence—including cases where Pro biosynthesis is triggered only under extreme stress conditions—this review proposes a more nuanced framework for distinguishing Pro accumulation as a passive biomarker of stress from its role as a functional adaptive stress tolerance mechanism. We focus primarily on studies showing the accumulation of Pro in response to drought, high salinity or other abiotic stressors, and its effects on osmotic adjustment and stress tolerance, not considering other experimental approaches, such as the modification of endogenous Pro concentrations in transgenic plants, which can also support its functional role in tolerance mechanisms. Also, the additional functions of Pro in plant metabolism, growth and development, reviewed, for example, in [17] or [16], are outside the scope of the present article.

2. Proline as a Bona Fide Functional Osmolyte

In many species, the functional role of Pro in abiotic stress tolerance mechanisms is strongly supported by substantial increases in concentration in response to the applied stress treatments, reaching levels high enough to significantly contribute to osmotic adjustment, helping avoid or reduce cellular dehydration. It should be mentioned here that Pro (or any other functional osmolyte) primarily accumulates in the cytoplasm, which represents only a fraction of the cell volume; this should be considered when assessing the osmotic effect of Pro concentrations, which should be expressed in terms of sample dry weight (or tissue water content). However, in many publications Pro contents (and other parameters) are referred to fresh weight, which may hamper the assessment of the actual Pro concentrations. Since the stress treatments generally cause cellular dehydration, differences between Pro levels in control and stressed plants, if expressed in FW terms, may be partly due to differential water loss in the samples.

In this section, we will describe and briefly discuss some of the results reported in hundreds of publications that provide the basis for the generally accepted view of Pro as a bona fide functional osmolyte.

In four Mediterranean Limonium species, Pro and fructose were identified as the major functional osmolytes responsible for osmotic adjustment, playing a more significant role than other osmoprotectants such as glycine betaine (GB) or sucrose [18]. Upon exposure to 800 mM NaCl, Pro accumulation increased significantly but unevenly across all species, with L. virgatum showing the most pronounced response, reaching nearly 470 μmol g^{-1} DW (a 25-fold increase over the non-stressed control), followed by L. girardianum and L. narbonense. On the other hand, in L. santapolense Pro increased only 5-fold, up to about 100 μmol g^{-1} DW, a concentration still high enough to contribute significantly to osmotic adjustment (Figure 1), although not so much as fructose, which accumulated up to nearly 1 mmol g^{-1} DW in L. santapolense and L. narbonense, about double concentration than in the other two species [18].

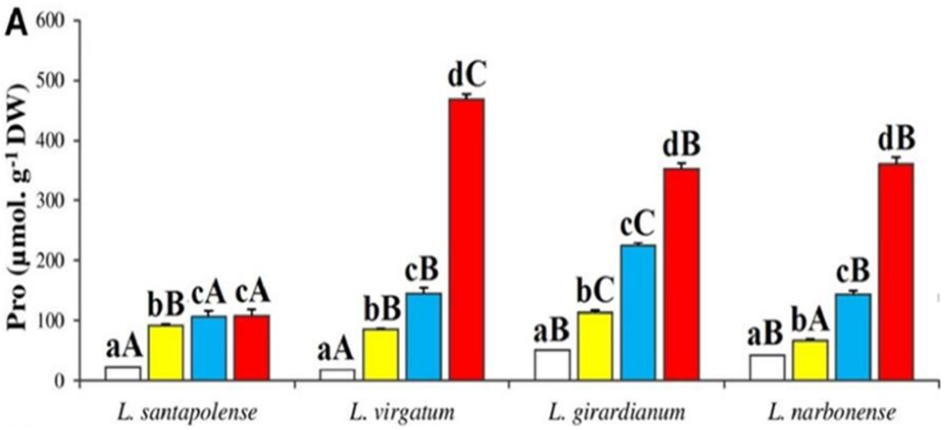

Figure 1. Proline (Pro) content in leaves of four Limonium species after one month of salt treatments at the indicated NaCl concentrations. The values shown are means ± SE (n = 5). Different lowercase letters above the bars indicate significant differences between treatments for each species, and different capital letters indicate significant differences between species for each treatment, according to Tukey's test ($\alpha = 0.05$). Adapted from [18].

In another Limonium species, L. augustebracteatum (Figure 2), Pro accumulation increased linearly by up to 48-fold (~760 μmol g^{-1} DW) in the presence of 800 mM NaCl. Notably, this response was absent under water stress conditions, suggesting that in this species, Pro biosynthesis and accumulation serve as a specialised adaptive mechanism for salinity tolerance but not a general defence against drought. On the other hand, glycine betaine also accumulated to high concentrations, in this case in response to both increased salinity and a water deficit treatment [12]. It should be noted that GB concentration is relatively high in the non-stressed control, suggesting a constitutive mechanism of defence against stress, which is characteristic of GB-accumulating taxa (see below data for Salicornia species). Moreover, the simultaneous accumulation of both osmolytes to similar levels is inconsistent with the notion proposed by some authors that species that use Pro as the major functional osmolyte are poor GB accumulators, and vice versa (e.g., [19]), which holds true only for some species or genera. In fact, Limonium species are a good example of plants that use a diverse array of compatible solutes in their responses to stress [20].

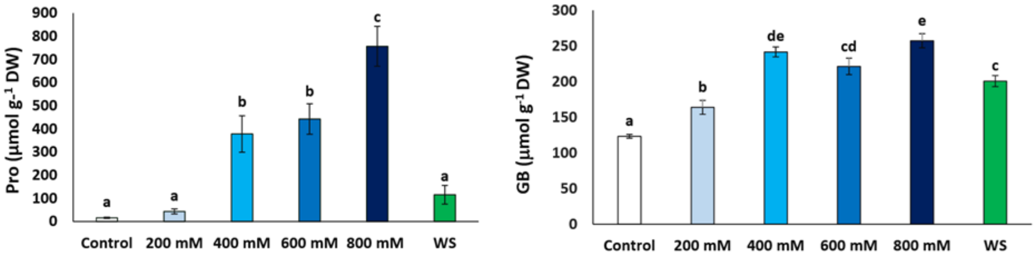

Figure 2. Proline (Pro) and glycine betaine (GB) contents in leaves of Limonium augustebracteatum plants after one month of treatment with the indicated NaCl concentrations or one month of water stress (WS, complete withholding of irrigation). Means ± SE, n = 6. Different lowercase letters above the bars indicate significant differences between treatments, according to Tukey's test ($\alpha = 0.05$). Adapted from [12].

Contrary to the previous example, in the halophyte Sesuvium portulacastrum severe water deficit (25% field capacity) induced a progressive Pro accumulation, reaching ~300 µmol g^{-1} DW after one month of treatment, which represents a 3-fold increase over control values. Interestingly, the concentration of mineral ions (Na^+, K^+, Cl^-) also increased in leaves of water-stressed plants, contributing alongside Pro to osmoregulation [21].

Proline accumulation in response to salt stress is not limited to salt-tolerant taxa such as the halophytes mentioned above. Seedlings from seven Picea Abies (Norway spruce) populations from the Romanian Carpathian Mountains were treated for six weeks with increasing NaCl concentrations, and several biochemical stress markers were determined, including Pro contents in the needles [13]. Salt stress triggered a general and significant increase in needle Pro concentrations, observable even at the lowest tested concentration (75 mM NaCl). The magnitude of this response varied quantitatively amongst populations, but the maximum Pro levels were roughly similar, ranging from 80 to 120 µmol g^{-1} DW under severe stress (300 mM NaCl), sufficient in all cases to contribute significantly to osmotic adjustment in the salt-stressed seedlings (Figure 3). No differences in salt tolerance were observed amongst the seven provenances, which was expected since the habitats where the trees grow do not include saline environments that could have induced the selection of more tolerant genotypes. The positive correlation between Pro accumulation and salinity intensity was confirmed by Principal Component Analysis, supporting the role of Pro as a general stress marker. However, the authors noted that this correlation was statistically less significant than that observed for other variables, such as total phenolic compounds or sodium (Na^+) and potassium (K^+) contents, which should be considered as more reliable biomarkers of salt stress in this species [13].

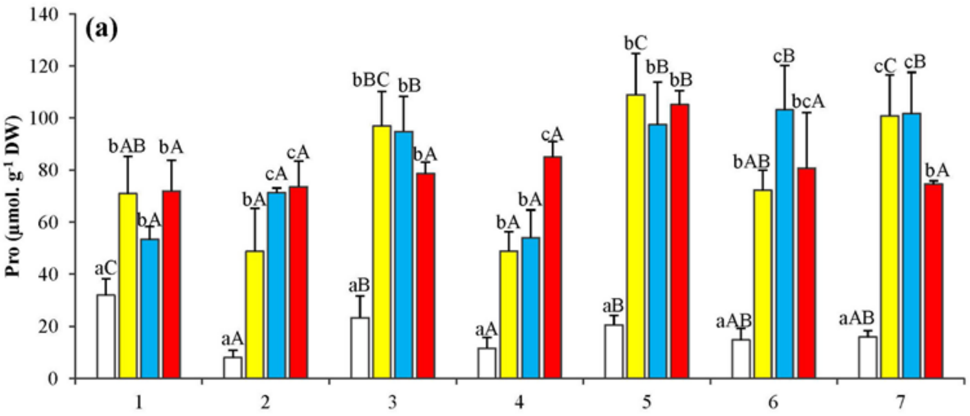

Figure 3. Variation of Pro (Pro) content in seedlings from seven Romanian Carpathian populations of Picea Abies, after 6-week treatments with the indicated NaCl concentrations. Data are means ± SD (n = 3). For each population, different lowercase letters above the bars indicate significant differences between treatments, and

capital letters indicate significant differences between populations undergoing the same treatment, according to the Tukey test ($\alpha = 0.05$). Adapted from [13].

Even in those cases where Pro contents increase in response to stress, reaching concentrations compatible with a relevant osmotic role, large quantitative differences can be observed across species. For example, six ornamental species of different genera (Bidens pilosa, Centaurea cyanus, Echinacea purpurea, Limonium sinuatum, Lobularia maritima, and Oenothera biennis) were subjected to two levels of water deficit for one month. For all species, the severe water stress treatment (complete withholding of irrigation) caused a significant accumulation of Pro compared with the non-stressed controls, ranging from a 5.3-fold increase in O. biennis to a massive 73-fold in L. sinuatum; maximum absolute Pro concentrations also varied widely, from less than 100 μmol g^{-1} DW in B. pilosa and O. biennis, to more than 300 μmol g^{-1} DW in L. sinuatum. Oenothera biennis consistently exhibited the lowest Pro concentrations across all treatments, which appear to be compensated in terms of osmoregulation by a relatively higher accumulation of soluble sugars [22].

The severity of the applied stress treatment can also serve as a critical threshold that triggers specific responses. Under moderate salinity (100 mM NaCl), Portulaca oleracea relied solely on stomatal regulation to manage water deficit, maintaining stable Pro and malondialdehyde (MDA, a reliable oxidative stress marker) levels, comparable to those of the non-stressed controls. However, exposure to severe salinity (300 mM NaCl) triggered a distinct shift in strategy: stomatal conductance decreased early on, followed by a transition from C4 photosynthesis to Crassulacean Acid Metabolism (CAM) to preserve carbon fixation. Crucially, this high-salinity level induced a large (ca. 11-fold) increase in leaf Pro contents, reaching 32.12 μmol g^{-1} FW by day 22 [23]; this concentration easily exceeds 300 μmol g^{-1} in terms of dry weight, considering the highly hydrated, succulent nature of this species' leaves. At these high concentrations, Pro can efficiently drive water uptake, supporting the authors' conclusions regarding its primary functional role in osmotic adaptation to high salinity [23].

Similarly, in pepper (Capsicum annuum) seedlings, Pro levels increased in response to eight-day treatments under different stress conditions (drought, salinity, heavy metals) [24]. Interestingly, inoculation with plant growth-promoting Bacillus amyloliquefaciens bacteria under salt stress further enhanced Pro accumulation to a peak of 9.74 mg g^{-1} DW (approximately 85 μmol g^{-1} DW). At this elevated concentration, Pro represented a primary driver for osmotic adjustment, allowing the plants to maintain high chlorophyll and sugar levels while effectively reducing oxidative stress markers like hydrogen peroxide [24].

In some cases, Pro accumulation in response to salt stress depends on the nature of the toxic ion. For example, in Tagetes minuta seedlings grown in the presence of NaCl or KNO_3, the accumulation of Pro revealed a distinct, ion-specific physiological response to salinity. While both treatments required osmotic adjustment, exposure to NaCl triggered a significantly more aggressive defence, resulting in a ca. 6-fold increase in Pro content, reaching 1350 μmol g^{-1} FW, compared to only a 3-fold increase under equimolar KNO_3 concentrations. This difference suggests that the higher toxicity of NaCl requires a substantially higher concentration of compatible solutes not only to achieve effective osmoregulation, but also to ensure cell protection against NaCl-induced damage. Alongside Pro, the authors measured other potential osmolytes, noting significant parallel accumulations of soluble proteins and soluble sugars (specifically glucose, mannose, and xylose-arabinose), which further contribute to osmotic adjustment [25].

Several studies carried out in different plant species also addressed the molecular mechanisms leading to Pro accumulation in response to salt, water deficit or other abiotic stress conditions. These experiments generally showed the stress-induced transcriptional activation of the gene encoding pyrroline-5-carboxylate synthetase (P5CS), the enzyme catalysing the rate-limiting step in the synthesis of Pro from glutamic acid, as well as the inhibition of Pro degradation due to the reduced activity of Pro dehydrogenase (ProDH). The activation of Pro biosynthesis and inhibition of its catabolic pathway has been demonstrated in litchi fruits subjected to chilling stress [26], salt-stressed

Arabidopsis thaliana plants [27,28], or in tomato under severe drought conditions [29], to give only a few examples.

3. Proline is not Involved in Osmotic Adjustment Under Stress

Proline accumulation is frequently labelled as a ubiquitous stress response, and quite often published reports highlight the relative stress-induced increase in Pro concentration, rather than its absolute value, to support a functional role of this compound in the stress tolerance mechanisms. However, in many plant species, abiotic stress simply does not trigger a significant increase in Pro contents or, even if it does, the maximum concentrations reached are still too low to contribute to cellular osmotic balance. For example, a study by Mircea et al. [30] analysed the drought stress responses of four potentially invasive ornamental grass species (Cymbopogon citratus, Cortaderia selloana, Pennisetum alopecuroides and P. setaceum), showing that water deficit did not trigger any increase in Pro contents in C. selloana and P. alopecuroides, whereas C. citratus and P. setaceum exhibited significant increases of 2.3-fold and 3.6-fold, respectively, under severe water stress conditions (Figure 4). However, the absolute Pro concentrations remained remarkably low in all cases (below 8 µmol g^{-1} DW), levels that are insufficient to provide any substantial osmotic effect. Basically, this study indicated that drought tolerance depends to a large extent on the active transport of Na^+ and K^+ cations to the aerial part of the plants, contributing to osmotic adjustment in all four species and, in the case of the most tolerant P. alopecuroides, also on the increasing root K^+ concentration under water deficit conditions [30].

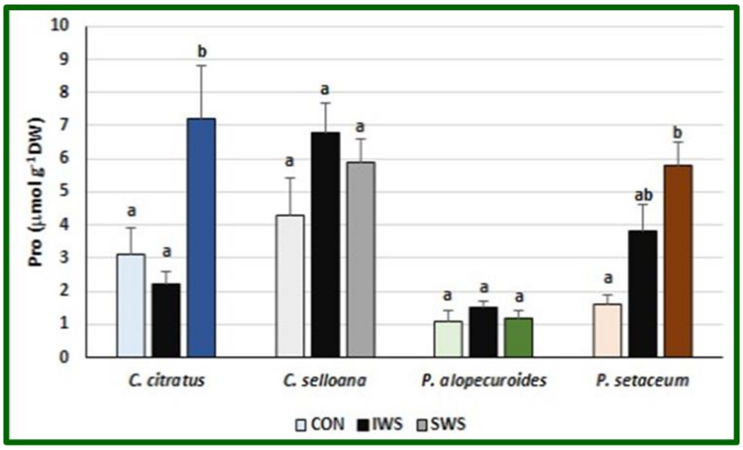

Figure 4. Effect of one-month water stress treatments on the shoot contents of proline (Pro) in Cymbopogon citratus, Cortaderia selloana, Pennisetum alopecuroides and P. setaceum. CON: control, well-watered plants; IWS: intermediate water stress (plants watered with half the volume than control plants); SWS: severe water stress (plants not watered at all). Values shown are means ± SE (n = 5). Different lowercase letters indicate significant differences between treatments for each species, according to the Tukey test ($p < 0.05$). Adapted from [30].

A similar lack of response has been observed in one-year-old *Abies alba* (silver fir) seedlings, where Pro levels were low (about 15 µmol g^{-1} DW) under control conditions and did not vary significantly after one month of water stress. Salt treatments, up to 300 mM NaCl, induced a modest increase, less than twofold, in Pro contents, not enough to contribute significantly to osmotic adjustment. These results indicate that Pro plays no functional role in the stress responses of this species, which relies instead on the accumulation of high levels of soluble sugars to respond to drought and high salinity stress [31]. Interestingly, *A. alba* seedlings exhibit relatively high (~ 400 µmol g^{-1} DW) Na^+ concentrations in their needles under both control and water-stress conditions, suggesting a constitutive mechanism of stress tolerance. As long as it does not reach toxic levels in the cytosol, Na^+ can contribute to osmotic balance as a ‘cheap’ osmoticum since, in terms of energy

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consumption, ion transport will be less demanding than the *de novo* synthesis of organic osmolytes [32].

This strict reliance on alternative osmolytes rather than Pro is observed across a broad spectrum of Mediterranean halophytes. A field study in salt marshes of La Albufera natural park, close to the city of Valencia (Spain), included the quantification of Pro and GB in Sarcocornia fruticosa, Inula crithmoides, and Plantago crassifolia plants along several seasons in 2009 and 2010 [33]. Even during the most stressful sampling period, summer 2009 (showing the highest soil electric conductivity, Na^+ and Cl^- soil concentrations, and evapotranspiration; and the lowest accumulated rainfall and soil moisture), the absolute levels of accumulated Pro were about 2 µmol g^{-1} DW in S. fruticosa and 3 µmol g^{-1} DW in I. crithmoides, and even lower for P. crassifolia (Figure 5). On the other hand, GB represents the functional osmolyte in the two first species, reaching concentrations 250 or 100-fold higher than those of Pro in S. fruticosa and I. crithmoides, respectively (Figure 5). Neither Pro nor GB appear to contribute significantly to stress responses in Plantago crassifolia under natural conditions; in fact, sorbitol has been identified as the major functional osmolyte in the genus Plantago (see below).

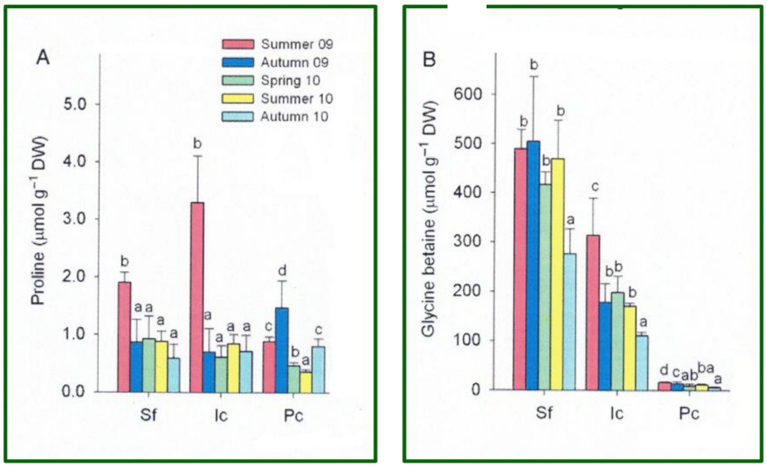

Figure 5. Seasonal variation in proline (left panel) and glycine betaine (right panel) contents (µmol g^{-1} DW), in Sarcocornia fruticosa (Sf), Inula crithmoides (Ic) and Plantago crassifolia (Pc) plants, collected in the indicated seasons from salt marshes in "La Albufera" Natural Park, Valencia (Spain). Values are means ± SD (n = 5). In each panel, different lowercase letters indicate significant differences between samplings for each taxon (α = 0.05). Adapted from [33].

The above data are supported by experiments in which three Salicornia species (S. bigelovii, S. europaea, and S. persica), closely related to Sarcocornia fruticosa, were subjected to increasing salt concentrations (up to 500 mM NaCl) for one, three or eight days, under controlled greenhouse conditions [34]. The salt treatments triggered increases in shoot Pro contents in all three species, in a time- and concentration-dependent manner, with small species-specific differences in the Pro levels reached and the accumulation kinetics (Figure 6). In any case, under the strongest stress conditions tested (eight days in the presence of 500 mM NaCl), Pro accumulated to only 2-3 µmol g^{-1} DW, between 4- and 6-fold higher than in the non-stressed controls (Figure 6). The applied salt treatments also induced a significant accumulation of glycine betaine (GB), with small, generally non-significant differences between species, but reaching maximum concentrations of ca. 250 µmol g^{-1} DW; that is, about 100-fold higher than those of Pro. It should be noted that, as indicated before for Limonium augustebracteatum, background levels of GB remained relatively high, over 100 µmol g^{-1} DW, in the absence of salt, resulting in a salt-induced relative increase of only about 2-fold (Figure 3).

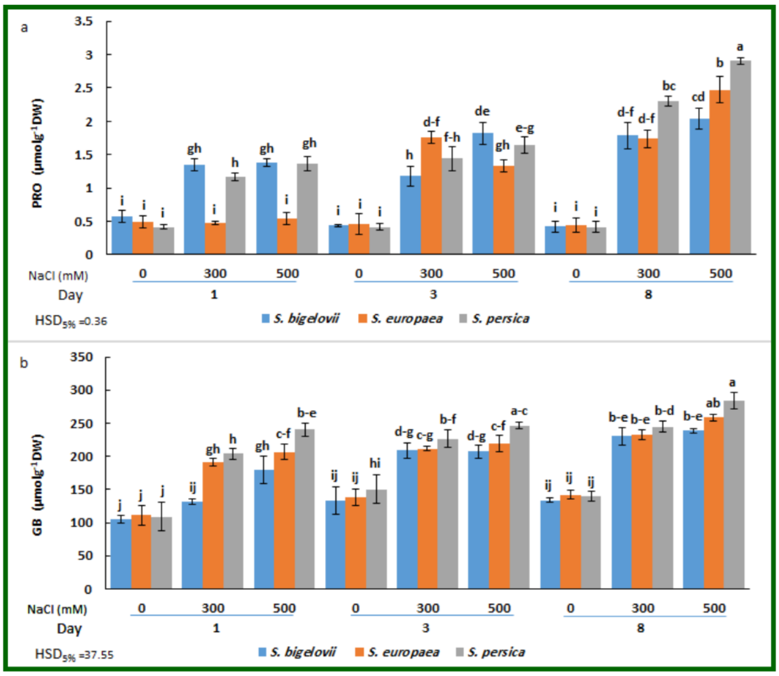

Figure 6. Effects of salt treatments (0, 300, and 500 mM NaCl) on proline (PRO) (a) and glycine betaine (GB) (b) shoot contents of three Salicornia species (S. bigelovii, S. europaea, and S. persica), at three sampling times (1, 3, and 8 days after starting the treatments). The values are means ± SD (n = 3). Different lowercase letters over the bars indicate significant differences between mean values, according to the Tukey test ($p < 0.05$). Taken from [34].

In two Tamarix species, T. aphylla and T. jordanis, Pro levels increased 5- to 9-fold under combined salt and drought stress [35]. However, the authors calculated that its actual contribution to the cell’s osmotic potential was only about 0.03 MPa, falling drastically short of the 0.2 to 0.4 MPa required for cytosolic osmoregulation. Consequently, they concluded that soluble sugars act as the primary functional osmolytes, while proline serves merely as a secondary osmoprotectant, ROS scavenger, and temporary nitrogen sink.

There are many other published studies reporting large relative increases in Pro concentrations in different plant species under varied abiotic stress conditions, representing a reliable marker of the level of stress affecting the plants but without reaching absolute concentrations high enough to contribute substantially to osmoregulation under stress. For example, a ca. 14-fold rise in Pro levels has been reported in peanut (Arachis hypogaea L.) plants subjected to a combined salt and drought stress treatment, or a 7-fold increase in response to the salt treatment applied independently [36]. Similarly, in sweet pepper (Capsicum annuum L.) seedlings, a direct relationship was found between the electrical conductivity (EC) of the nutrient solution and Pro levels, which increased gradually up to 15.6-fold when comparing the 17.4 dS m^{-1} and 2.9 dS m^{-1} treatments [37].

4. Comparative Studies in Genetically/Taxonomically Related Taxa and Correlation with Their Relative Tolerance

For those—many—plant species for which application of a particular stress results in a significant increase in Pro contents, there is generally a positive correlation between the intensity of the stress and the concentration of accumulated Pro, supporting its use as an abiotic stress biomarker. However, this does not mean that Pro is directly involved in the mechanisms of tolerance, even though this is often assumed in the literature. On the other hand, evaluating Pro accumulation in response to specific stress treatments across genetically or taxonomically related taxa showing different degrees of tolerance, represents a useful approach to assess the possible functional role of Pro in those tolerance mechanisms. When comparing the responses of several related genotypes, such

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as different populations, cultivars of subspecies of a particular species, or different species of the same genus, correlation of Pro contents with their relative degree of tolerance may reveal a profound divergence in Pro functional role. The correlation could be positive, with Pro reaching higher levels in the most tolerant genotypes, supporting its direct involvement in tolerance mechanisms. On the contrary, Pro concentration could be relatively higher in the most sensitive genotypes, which would be more stressed than tolerant ones under the same conditions; in this case, Pro is simply a stress biomarker but probably does not contribute substantially to tolerance.

4.1. Positive Correlations: High Pro Accumulation as a Determinant of Tolerance

In several plant genera, Pro accumulation in response to stress can be used to directly distinguish stress-tolerant species from their sensitive relatives. Comparative studies on Juncus species effectively revealed a positive correlation between Pro accumulation and stress tolerance, distinguishing two halophytes, J. maritimus and J. acutus, growing in saline and arid natural environments, from J. articulatus a species adapted to non-saline, humid habitats. Under high salinity conditions (400 mM NaCl), Pro content in the halophytes surged dramatically, increasing between 60- and 70-fold (on a tissue water basis) compared to non-stressed controls, reaching concentrations of ca. 120 μmol g^{-1} DW after eight weeks of treatment. In contrast, the sensitive J. articulatus exhibited a weak response, with maximum Pro levels remaining below 10 μmol g^{-1} DW under the same conditions [11]. A similar pattern was observed under water stress (eight weeks without irrigation), where the halophytes again demonstrated a robust response, reaching Pro levels over 100, or close to 200 μmol g^{-1} DW, in J. acutus and J. maritimus, respectively, whereas Pro contents below 10 μmol g^{-1} DW were measured again in J. articulatus. Pro is not the only osmolyte contributing to counteract the osmotic stress caused by high salinity or water deficit in Juncus species. Sucrose levels also increase significantly in response to both treatments, but without the stark differences observed for Pro as it accumulated similarly across the three species, with maximum concentrations between 150 and 250 μmol g^{-1} DW. These results strongly support Pro accumulation as a key mechanism of stress tolerance in Juncus [11].

Similar responses to salt stress have been reported when comparing two salt-tolerant members of the genus Plantago, P. crassifolia and P. coronopus, with the congeneric glycophyte P. major [38]. As mentioned before, sorbitol is the identified functional osmolyte in this genus. Background sorbitol levels in the absence of stress are already quite high and increase with the increase of NaCl concentration in irrigation water, but to a similar extent in the three analysed species, reaching maximum values between 1500 and 2000 μmol g^{-1} DW (Figure 7). Therefore, although sorbitol is clearly the major compatible solute responsible for osmotic adjustment under stress, its accumulation patterns cannot explain the differences in salt tolerance. Pro accumulation, on the other hand, is a distinctive response to high salinity triggered specifically in the halophytes. As determined in the field (Figure 5) background Pro levels are extremely low, below 2 μmol g^{-1} DW, concentration that does not increase upon controlled salt treatments up to 400 mM NaCl. However, Pro levels rise significantly at higher salinities, but only in the halophytes, up to 30 μmol g^{-1} DW in P. crassifolia and to ca. 45 μmol g^{-1} DW in P. coronopus (Figure 8) [38]. The positive correlation between Pro contents and the relative salt tolerance of these species suggests its direct participation in the mechanisms of tolerance. Since the contribution of Pro to osmotic balance is rather modest, compared to that of sorbitol, Pro function must be mostly based on its additional roles as an osmoprotectant, ROS scavenger and signalling molecule. It should be also noted that the salt concentrations that trigger Pro accumulation in the halophytes in the controlled treatments are higher than those that the plants must normally withstand in their natural habitats. In this genus, Pro biosynthesis can be considered as a “security switch”, which would be activated under natural conditions only as a response to an extreme, probably temporary increase in soil salinity, which is not unlikely in the current climate change scenario.

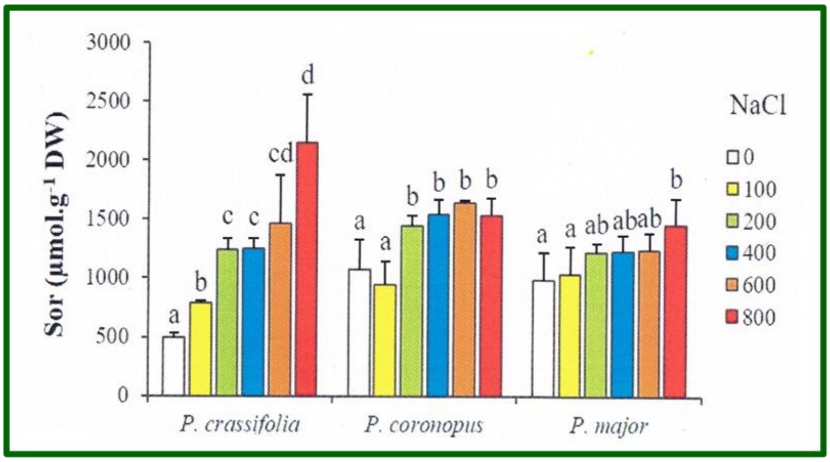

Figure 7. Sorbitol (Sor) leaf contents after four weeks of treatment with the indicated NaCl concentrations, in the three selected Plantago species (means ± SD, n = 5). Different lowercase letters indicate significant differences between treatments, for each species, according to Tukey test ($\alpha = 0.05$). Taken from [38].

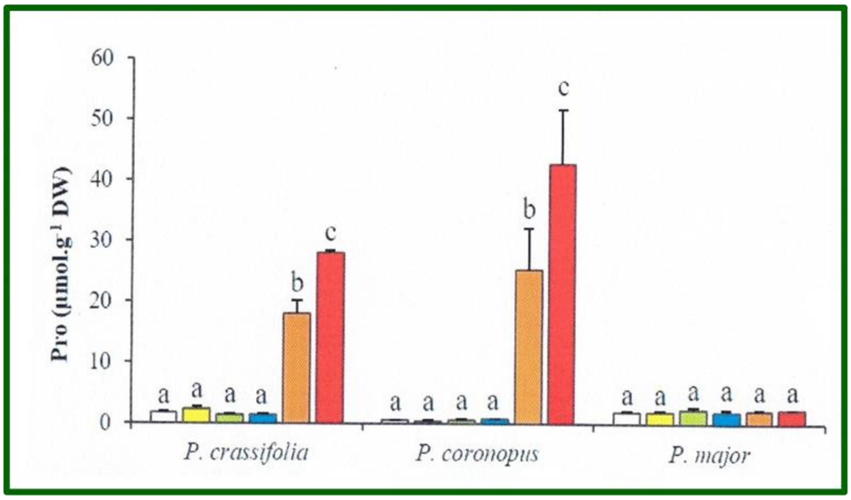

Figure 8. Proline (Pro) levels in the selected Plantago species, after four weeks of treatment with the NaCl concentrations indicated in Figure 7 (means ± SD, n = 5). Different lowercase letters within each species indicate significant differences between treatments, according to Tukey test ($\alpha = 0.05$). Taken from [38].

The analysis of the responses to water deficit stress of four Mediterranean Limonium species, L. santapolense, L. virgatum, L. girardianum, and L. narbonense, revealed that all are relatively resistant to drought, partly based on constitutive mechanism such as the active transport to the shoots of mono and divalent ions (Na^+, Cl^-, K^+, Mg^{2+}, Ca^{2+}), which showed significantly higher concentrations in leaves than in roots even in non-stressed control plants, and would contribute to osmotic adjustment under drought conditions. However, L. santapolense, a narrow endemism from the south of the Alicante province, in the Spanish Mediterranean coast, was the species that showed the most strongly induced responses to the water stress treatment, including a much higher relative increase in leaf Pro contents, which reached ca. 120 µmol g^{-1} DW, compared to the other three species (Figure 9). Higher accumulation of fructose, and the stronger activation of antioxidant enzymes (particularly superoxide dismutase and ascorbate peroxidase), were also observed for this species. This differential behaviour of L. santapolense, confirmed by Principal Component and cluster analyses, agrees with the fact that it grows in a more arid environment than the natural habitats of the other three taxa [39].

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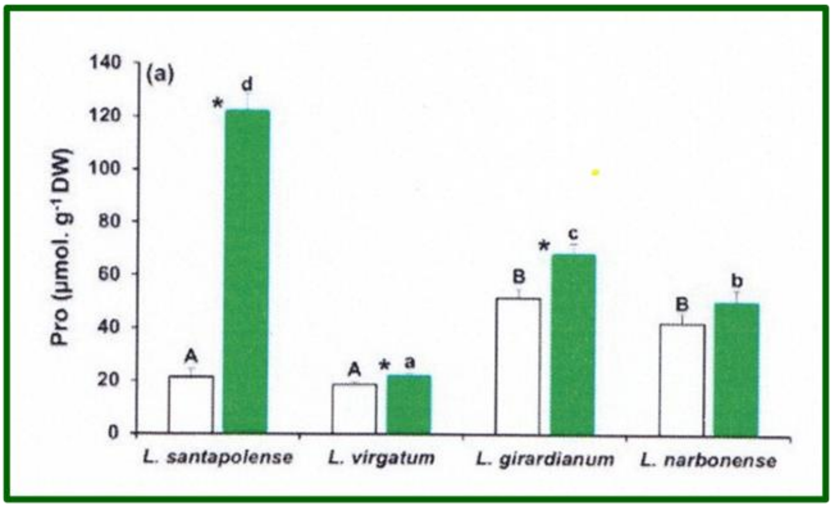


Figure 9. Proline (Pro) leaf contents in the indicated Limonium species, after one month of water stress treatment (complete withholding of irrigation, green bars) and in the control, well-watered plants (white bars). The values shown are means ± SE (n = 5). Different letters above the bars indicate significant differences between species, for control (capital letters) and water-stressed (lowercase letters) plants. Asterisks denote significant differences between treatments for each species, according to Tukey's test ($\alpha = 0.05$). Adapted from [39].

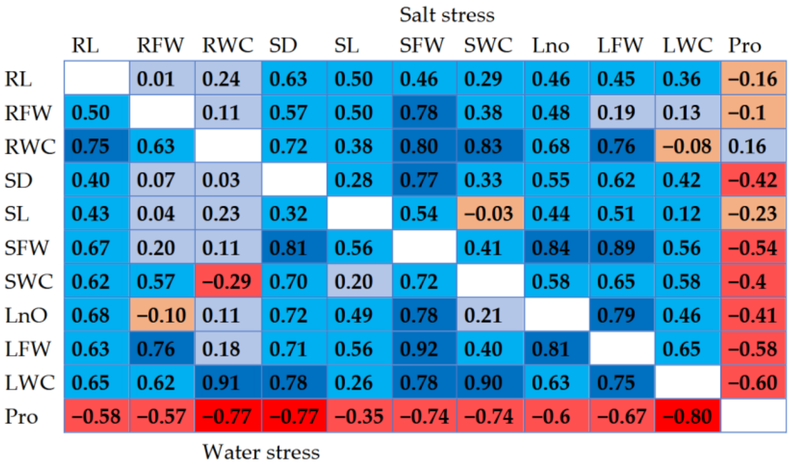
This positive correlation between Pro accumulation under stress and the relative tolerance of different related genotypes extends to cultivars of agricultural crops. For example, the exposure to 60 mM NaCl of different potato (Solanum tuberosum) cultivars induced Pro accumulation in leaf and stem tissues, with the highest concentrations localised in the stem. Notably, the salt-tolerant cultivar ″Desiree″ accumulated approximately three times more Pro in its stem than the sensitive cultivar ″Mozart″, due to a twofold increase in the expression of the biosynthetic gene P5CS1, a response lacking in ″Mozart″[40]. In Tunisian tomato (Solanum lycopersicum) genotypes subjected to a seven day salt treatment, the salt-tolerant cultivar ″San Miguel″ showed higher Pro contents in roots and leaves than the salt-sensitive cultivar ″Mouna HF1″, so that Pro accumulation was considered a suitable marker for salt tolerance that could be applied in breeding programmes [41]. Also, in maize (Zea mays) seedlings, the tolerant W23/M14 genotype maintained naturally higher baseline Pro levels (19.5 μg g^{-1} DW) than the sensitive ″Şafak″ genotype (8.6 μg g^{-1} DW). [42].

4.2. Negative Correlations: Pro Accumulation as a Symptom of Stress Severity

A substantial subset of comparative literature reveals that robust Pro synthesis frequently correlates negatively with stress resilience. In these contexts, massive Pro accumulation is not a proactive defence mechanism, but rather a symptom of cellular damage due to high stress sensitivity.

This negative correlation is documented, for example, by several studies on Phaseolus. Three cultivars of common bean (P. vulgaris) and one of runner bean (P. coccineus) were subjected to increasing salt concentrations, up to 150 mM NaCl, for three weeks. Determination of growth parameters allowed to establish that the common bean cultivar "Maxidor" was the most salt-tolerant, followed by the runner bean "Moonlight", whereas "The Prince" was the most sensitive. Leaf Pro contents were low and similar in non-stressed plants of all cultivars and increased in the salt-treated plants up to > 40 µmol g^{-1} DW in "The Prince", but only to ca. 10 µmol g^{-1} DW (about 4-fold over control values) in "Maxidor". These results suggested that Pro is not directly involved in the mechanisms of salt tolerance in these cultivars, which depend instead on blocking Na^+ (and Cl^-, to a lesser extent) transport from roots to shoots, myo-inositol accumulation in leaves and, at high external salinities, activation of K^+ transport to the leaves [43]. A similar pattern was maintained under water stress conditions, where the most tolerant cultivar, again "Maxidor", showed the lowest Pro accumulation [44].

The above results prompted a wider analysis of 47 P. vulgaris genotypes, including local landraces, commercial cultivars, and experimental lines, from Spain (23), Colombia (19) and Cuba (5), which were subjected to two or three weeks of salt stress (150 mM NaCl), or water stress (complete withholding of irrigation) treatments. For all genotypes, leaf Pro contents increased significantly in response to both stresses. Pro showed strong negative correlations with all determined growth parameters, especially with leaf/stem fresh weight and water content, as confirmed by PCA loading plots, with higher Pro concentrations unequivocally linked to stronger growth inhibition and stress sensitivity. Figures 10 and 11 show the heatmap of Pearson correlation coefficients and the PCA loading plots, respectively, for the two-week stress treatments. These results support the use of Pro content determination as a simple, rapid and reliable marker for large-scale screening to exclude sensitive cultivars in breeding programmes for drought and salt tolerance in Phaseolus [8].



Salt stress

	RL	RFW	RWC	SD	SL	SFW	SWC	Lno	LFW	LWC	Pro
RL		0.01	0.24	0.63	0.50	0.46	0.29	0.46	0.45	0.36	−0.16
RFW	0.50		0.11	0.57	0.50	0.78	0.38	0.48	0.19	0.13	−0.1
RWC	0.75	0.63		0.72	0.38	0.80	0.83	0.68	0.76	−0.08	0.16
SD	0.40	0.07	0.03		0.28	0.77	0.33	0.55	0.62	0.42	−0.42
SL	0.43	0.04	0.23	0.32		0.54	−0.03	0.44	0.51	0.12	−0.23
SFW	0.67	0.20	0.11	0.81	0.56		0.41	0.84	0.89	0.56	−0.54
SWC	0.62	0.57	−0.29	0.70	0.20	0.72		0.58	0.65	0.58	−0.4
LnO	0.68	−0.10	0.11	0.72	0.49	0.78	0.21		0.79	0.46	−0.41
LFW	0.63	0.76	0.18	0.71	0.56	0.92	0.40	0.81		0.65	−0.58
LWC	0.65	0.62	0.91	0.78	0.26	0.78	0.90	0.63	0.75		−0.60
Pro	−0.58	−0.57	−0.77	−0.77	−0.35	−0.74	−0.74	−0.6	−0.67	−0.80	

Water stress

Figure 10. Heatmap of Pearson moment correlation coefficient (r) between the analysed traits in Phaseolus vulgaris genotypes submitted to two weeks of water (no irrigation) and salt (150 mM NaCl) stress treatments. Dark blue denotes high positive correlation, dark red high negative correlation. Abbreviations: RL, root length; RFW, root fresh weight; RWC, root water content; SD, stem diameter; SL, stem length; SFW, stem fresh weight; SWC, stem water content; Lno, leaf number; LFW, leaf fresh weight; LWC, leaf water content; Pro, proline content. Adapted from [8].

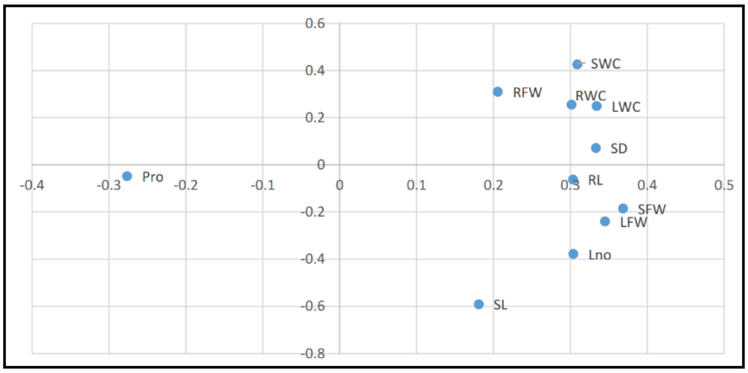


Figure 11. Loading plot of the principal component analysis (PCA) conducted with the analysed traits, in P. vulgaris cultivars subjected to two weeks control, water deficit and salt stress treatments. 56.5% and 13.6% of the total variability are explained by the first (x-axis) and the second (y-axis) components, respectively. Abbreviations as in Figure 10. Adapted from [8].

Similar negative correlations have been observed in the analysis of nine accessions of “Ardhaoui” barley landraces from different regions of Tunisia, subjected to water stress by completely stopping irrigation for three weeks. Large increases in Pro levels were measured in all accessions, although with wide quantitative differences as Pro concentrations ranged from nearly 150

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to about 600 µmol g^{-1} DW. These high values indicated that Pro must contribute to osmoregulation under drought, together with soluble sugars, which also increased in the water-stressed plants. However, the most tolerant accession 2, from El May Island, exhibited the lowest Pro levels, whereas the highly susceptible accession 4, from Ksar Hdada mountain, showed the highest levels, alongside major declines in fresh weight and water content. This study casts doubt on the utility of Pro as a reliable marker of drought tolerance in barley genotypes, and the authors suggested that the reduction of chlorophylls or MDA contents, or the increase in total phenolic compounds could be used as more appropriate parameters for selecting drought-tolerant barley varieties [9]. This work supports previous studies with different barley genotypes, which also showed similar negative correlations, in this case between Pro contents and salt tolerance, suggesting that hyperaccumulation of Pro or other major compatible solutes in barley does not play a major role in salt-tolerance, but is rather a symptom of salt-susceptibility [45].

The responses to drought of one-year-old Norway spruce (Picea Abies) seedlings from seven different provenances in the Romanian Carpathian Mountains were analysed by growing them for 42 days without any irrigation. Determination of several markers, such as water and chlorophylls contents in the needles, allowed to identify the populations from Sudrigiu and Gioristea-Calimanut as the most drought-tolerant, in agreement with the climatic characteristics of their geographical locations. Needles of these two spruce populations did not show any significant increase in Pro contents in response to the water stress treatment. Conversely, the most sensitive population ("Valea Mare") exhibited the highest relative increase, about 8-fold, reaching concentrations of more than 60 µmol g^{-1} DW. Therefore, in P. abies, Pro does not seem to be directly involved in the mechanisms of drought tolerance but simply act as an indicator of the level of stress affecting the plants. [14].

In other study, drought responses were analysed in several commercial cultivars of three Tagetes (marigold) species, T. patula, T. tenuifolia and T. erecta, by subjecting the plants to three weeks without irrigation. The stress treatment induced a significant increase in Pro contents in all genotypes, with quantitative differences observed between species and between cultivars within each species. For most cultivars, Pro levels in stressed plants increased between 30 and 70-fold compared to the corresponding controls, reaching concentrations ranging from ca. 50 to 350 µmol g^{-1} DW. Determination of additional variables and Principal Component Analysis allowed to identify the most tolerant and sensitive cultivars within each species, and it could be concluded that Pro was negatively correlated with the relative degree of tolerance: higher levels of Pro were detected in the more sensitive genotypes of the same species—for example, T. erecta's "Cupid Golden Yellow", or T. tenuifolia's "Luna Orange" and "Luna Lemon" [10].

In the above examples, the participation of Pro in stress tolerance mechanisms cannot be ruled out, either by contributing with other osmolytes to osmoregulation, as ROS scavenger or signalling molecule. However, other stress responses, different from Pro accumulation, must be responsible for the observed differences in tolerance between closely related genotypes.

5. Conclusions

In conclusion, proline is widely used as a reliable biochemical marker of abiotic stress in plants, but its contribution to stress responses and plant survival is strongly context-dependent and cannot be generalised across all species. Pro accumulation may provide a useful indication of the intensity of the stress affecting the plants, although, obviously, only in those species that use it as a functional osmolyte. However, its accumulation in response to stress conditions, such as water deficit or salt treatments, does not necessarily imply a direct, relevant role in stress tolerance mechanisms. Because these responses are often shaped by species-specific genetics and adaptations to particular environments, broad generalisations are inappropriate, and the functional significance of Pro should be evaluated on a case-by-case basis.

To avoid a "Pro-centric" perspective, researchers should assess possible stress-induced changes in the concentration of a broader spectrum of osmolytes—including glycine betaine, polyalcohols, and soluble sugars—rather than focusing exclusively on Pro. Equally important, future studies should standardise osmolyte reporting (and other variables) to molar concentrations on a dry weight basis (e.g., μmol g^{-1} DW) to ensure physiological relevance and facilitate meaningful comparisons across experiments. Without dry weight standardisation, changes in tissue hydration under drought or salinity stress can artificially distort fresh weight–based metabolite measurements, thereby reinforcing the very misinterpretations highlighted in this review.

Furthermore, whenever feasible, studies should incorporate comparative analyses of related taxa that differ in stress resilience. Linking relative tolerance levels with absolute Pro concentrations is essential for determining whether Pro functions as an active adaptive defence mechanism or simply reflects physiological stress and damage.

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