Evidence of non-structural carbohydrates-mediated response to flooding and salinity in Limonium narbonense and Salicornia fruticosa

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Evidence of non-structural carbohydrates-mediated response to flooding and salinity in *Limonium narbonense* and *Salicornia fruticosa*

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Highlights

- Soluble NSCs content was determined primarily by flooding and secondly by salinity
- Soluble NSCs were more affected during seed ripening compared to flowering season
- Amino acids and starch contents were not affected by flooding, salinity or phenology
- Proline could be involved in stress tolerance regardless of the intensity of the stress

Abstract

In plant species, the effects of flooding and salinity are commonly studied under controlled conditions in order to understand their acclimation to environmental stresses. Nevertheless, each stress is usually considered separately and laboratory conditions cannot encompass the complexity of the natural ecosystem, often concealing the true plant response. Our work aimed therefore at studying plant responses to flooding and salinity in the field, focusing on two target halophytes and on their reserve organs, i.e. rhizomes of *Limonium narbonense* and woody stems of *Salicornia fruticosa*. The physiological response was investigated measuring non-structural carbohydrates (NSCs) and amino acids (AA), and considering the two growing stages of the species, i.e. the growing and the seed ripening seasons. *L. narbonense* rhizomes showed a larger amount of starch and all measured osmolytes, i.eNSCs, AA and proline, compared to *S. fruticosa* woody stem, where plant response to environmental stress seemed linked chiefly to soluble NSCs. The effects on soluble NSCs were mainly driven by flooding and were influenced by salinity only at low flooding stress. The two species showed a contrasting strategy against flooding and salinity based on soluble NSCs, and with a more intense response during the seed ripening season. Large amount of AA, proline in particular, suggested the involvement of these osmolytes in the salinity tolerance in *L. narbonense*, regardless to the intensity of the stress.

Keywords: Swamp sea-lavender, Shrubby swampfire, osmolytes, salinity, saltmarshes halophytes

1. Introduction

The interest on plant and vegetation responses to flooding is increasing among the scientific community (Pedersen et al., 2017; Noto and Shurin, 2017), as response to the forecasted sea level rise due to climate change (ICCP, 2019). Among natural environments, saltmarshes are particularly sensitive to the increase of flooding and frequency of storm surges. These events lead to the increase of soil salinity (Fagherazzi et al., 2019), forcing plants to face a changing environment.

Plant survival in a dynamic environment, such as saltmarshes, lays on plant phenotypic plasticity. Halophytes and glycophytes manifest their plasticity against flooding or salinity stresses on both morphological (Zhao et al., 2010) and physiological traits (Casolo et al., 2015), where hormonal response, antioxidants and secondary metabolites production are often involved (Rozentsvet et al., 2017; Arbelet-Bonnin et al., 2020).

Morphological acclimations against flooding include adventitious roots development, aerenchyma formation or shoot elongation and among physiological responses, underwater photosynthesis can sustain plant metabolism during submergence (Colmer and Flowers, 2008). Non-structural carbohydrates (NSCs) are of particular interest to overcame the ‘energy crisis’ induced by tissue anoxia (Colmer and Voesenek, 2009), even if the mechanism is still not clearly understood. In fact, NSCs in herbs are reported to increase in stems and leaves during flooding but the amount of soluble

NSCs is expected to be constant in underground organs (Chen et al., 2005), even if starch content changes (Qin et al, 2013).

Maintenance of a good water status, transpiration efficiency and antioxidants production are important physiological mechanisms involved in salinity tolerance. To avoid water withdrawal from the cell due to lower water potential in the apoplast, several species use strategies for osmotic compensation, i.e. the accumulation in the cytosol of soluble and compatible organic metabolites (Rhodes et al., 2002).

Halophytes are well-adapted species to flooding and salinity, showing many constitutive or inducible traits. Typical traits against flooding are root aerenchyma, shoot elongation and the formation of a root barrier to radial oxygen loss (Colmer and Flowers, 2008 and references therein), while salinity is controlled by different strategies: e.g. ion compartmentation, salt glands and a quicker leaf turnover (Flowers and Colmer, 2008 and references therein). In halophytes, the increase in specific NSCs and polyols have been associated to salt tolerance (Gil et al., 2013). In halophile monocots, osmotic adjustment is preferentially performed with NSCs and polyalcohols, whereas dicots respond with different types of osmolytes involving or not NSCs (Ye, 2010). Free amino acids (AA) pools may increase upon a stress-induced lowering of protein synthesis rate (Good and Zaplachinski, 1994) but such accumulation may contribute in maintaining a lower water potential inside the cell. In many cases, free proline was found to increase much more than the other AA, suggesting an active adaptive mechanism against environmental stresses (Trovato et al., 2019). Besides osmotic compensation, increased proline content may benefit the cell through a variety of possible mechanisms, ranging from enzyme and membrane stabilization to free radical scavenging and redox regulation (Forlani et al., 2019). Active osmotic adjustment provides osmotic balance between the cytosol and the vacuole, and the cytosol and the apoplast (Gagneul et al., 2007). Moreover, the AA-based adaptation is considered more important than NSCs in the salinity tolerance mechanism of halophytes (Hartzendorf and Rolletschek, 2001).

Plant acclimation to flooding or salinity has been largely investigated, but little attention has been paid to their interaction (see e.g. Colmer and Flowers, 2008). NSCs and AA in halophytes were mostly investigated in the context of salinity tolerance and the seasonal effect (Murakeözy et al., 2003; Mouri et al., 2012). Flooding is usually not considered, despite being the main driver of halophytes zonation. Moreover, the role of NSCs or AA in halophytes upon a combined effect of flooding and salinity has been only marginally investigated (Colmer and Pedersen, 2009). To our knowledge, the field research on flooding, salinity or their interaction in halophytes, compared to lab experiments, is limited (Gimeno et al., 2012) and therefore encouraged.

In this work, we focused on two commonhalophytes, namely *Limonium narbonense* Mill. and *Salicornia fruticosa* (L.) L. These two species show a similar distribution along the flooding gradient being both negatively affected by flooding but showing contrasting strategies to face against submergence (Pellegrini et al., 2017). The size of both species has been proved to be significantly reduced by flooding (Pellegrini et al., 2018). Flooding effects are particularly strong during maximum high tide, when plants are completely submerged in low sites, while leaves or stems remain emerged in more elevated sites (Supplementary material Fig. S1). Furthermore, *S. fruticosa* was used as a model plant to study the effect of salinity on photosynthesis (Redondo-Gómez et al., 2009), on oxidative stress (Gil et al., 2014) and solute partitioning (Marco et al., 2019). We investigated the combined effect of salinity and flooding on NSCs and AA in these two halophytes considering two different growing stages of plants, i.e. growing and seed ripening seasons. We focused on the rhizome of *L. narbonense* and the basal stem of *S. fruticosa* in which we analysed the content of: i) soluble NSCs, which are osmotically active; ii) starch, an osmotic non-active carbon reserve; iii) total AA; and iv) proline content.

We expected flooding to drive the decrease of NSCs, whereas salinity to increase the AA content, proline in particular. Considering the presence of a broad rhizome in *L. narbonense*, we hypothesized that soluble NSCs and starch are the main strategies of the species to counteract salinity and flooding, respectively, whereas AA may have larger importance for salinity tolerance in *S. fruticosa*. Eventually, we hypothesized that these relations may change during the season due to plant phenology (i.e. growing and seed ripening seasons).

2. Material and methods

2.1 Study area and selected halophytes

The Marano and Grado lagoon (centroid coordinates: 45°42’50’’N, 13°20’30’’E, Northern Adriatic Sea) is a Natura 2000 site (IT3320037) that covers an area of about 160 km^2. It is delimited by six barrier islands and most of the emerged surface is covered by saltmarshes fragmented by a complex channel network and tidal flats. The average tidal fluctuation and the spring tidal range were of 65 cm and 105 cm, respectively (Ret, 2006) and submerged soils are largely covered by seagrass meadows (Boscutti et al., 2015).

Four saltmarshes were considered in this study (Fig. 1): Belvedere and Gran Chiusa islands are saltmarshes with a loamy soil texture, located in the inside part of the lagoon, while Marina di Macia and Banco d’Orio are back barrier saltmarshes with predominantly sandy soils. *L. narbonense* and *S. fruticosa* were the most abundant species (about 41 and 16% of species cover on average), followed by *Juncus maritimus* (13%) and *Spartina maritima* (4%). Flooding is highly dependent to site elevation. The elevation above the mean sea level (a.m.s.l.) of sampling sites ranged from 0.28 up to 0.67 m. Soil salinity of the selected marshes ranges from 1.50 g L $^{-1}$ in Banco d'Orio to 3.56 g L $^{-1}$ in Gran Chiusa islands (Supplementary material Tab. S1).

L. narbonense is a rhizomatous geophyte with basal leaves (rosette) and bare flowering stems. Flowering occurs from the end of May to August and most of plants present fully ripened fruits at the end of September. *S. fruticosa* is a suffrutex, with green succulent articles supported by woody basal stems with branched surficial roots. Flowering ranges from the end of July to September, while seed ripening is postponed compared to *L. narbonense* and of about 1 month.

Being the two species affected by flooding, two sampling sites were selected in each saltmarsh (Fig. 1), according to species distribution and to LiDAR elevation model (1 m resolution data available online at http://irdat.regione.fvg.it/WebGIS/GISViewer.jsp). Following a belt transect, the "high" sites are characterised by a mean elevation of 56.3 cm above the m.s.l. (range 41-67 cm, depending on the saltmarsh), thus by a low and less frequent flooding, in average 1.[illegible]4 h day $^{-1}$. The "low" sites are characterised by a low elevation of about 30.8 cm above the m.s.l. (range 28-47 cm) and by a large and more frequent flooding of about 4.37 h day $^{-1}$.

2.2 Sampling and analysis

At each "high" and "low" site, plant and soil samples were collected. Sampling was carried out in June and in October and represented the growing and the seed ripening seasons for the two species considered, thus the period of larger rate of carbon assimilation and carbon storage, respectively.

In each sampling site, 3 square plots of 4 m ^{2} were selected. The 3 plots were homogenous in terms of plant community and site elevation.

Surface soil (10 cm depth) was collected and transported to the laboratory in plastic sealed bags (one sample for each plot). Aqueous extract (5:1) was prepared using 10 g of soil and shaken for 1h. Conductivity was measured in the filtered solution (Whatman n. 42 filter paper) at 25°C with the conductivity meter CM35 (Crison, Spain) and converted to salinity (g L $^{-1}$).

Three rhizomes of *L. narbonense* and three basal woody stems of *S. fruticosa*, were collected in each plot and transported to the laboratory in plastic sealed bags, in a portable fridge. Plants selected were representative of the mean plant size within the plot. Plants collected in June had not yet developed flowering stems or showed only sketched flowering stems. Plants collected in October showed already ripe seeds. We selected storage organs as they have been demonstrated to be main carbon reserves playing a pivotal role on plant survival (Martínez‐Vilalta et al., 2016). Within the day, plant samples were microwaved (600 Watt, 3 min) to prevent further enzymatic activity, then dried at 60°C for at least 24 h and stored in silica gel until further analysis.

2.2.1 Non-structural carbohydrates extraction and quantification

The method of Quentin et al. (2015) was applied for NSCs extraction with slight modifications, as described below.

About 15 mg of dried tissue (hereafter dry weight - DW) were ground (diameter less than 150 µm) and placed in a 1.5 mL Eppendorf vial with 300 µL of 80% (v/v) ethanol and vortexed. The Eppendorf was placed in a water-bath at 80°C for 30 min. After centrifugation for 3 min (12,000 rpm) in Eppendorf centrifuge, the supernatant was transferred into another Eppendorf vial. The same operation was repeated as above and the supernatants were pooled together, then dried overnight at 55°C. Finally, 500 µL of 50 mM TRIS-HCl pH 7.5 was added at room temperature to the pellet and, after a new centrifugation, used to re-suspend the dried carbohydrates resulting from ethanol extraction. For starch extraction, 1 mL of acetate buffer (0.4 M $NaCH_3COO$, pH 4.6) was added to the pellet residue and treated at 100°C for 1 h to allow starch gelification. After, the sample was incubated overnight at 55°C with 20 µL of amylase (100 units per sample) and amyloglucosidase (20 units per sample) and the day after boiled for [illegible] min to denature the enzymes.

Soluble sugars and starch were determined colorimetrically using Anthrone (0.1 % w/v in 98 % sulfuric acid). The dye was read at 620 nm. Standard curves were obtained with glucose and amylose for soluble sugars and starch, respectively. The absorbance was determined at 620 nm with a Wallac Victor 1420 multiplate counter (PerkinElmer, Inc., USA).

2.2.2 Amino acid extraction and quantitation

Finely ground dried material was resuspended in 20 mL g^{-1} of an ice-cold 3% solution (w/v) of 5-sulfosalicylic acid. Samples were equilibrated on ice and extraction was allowed to proceed for 30 min with vortexing every 5 min. Following centrifugation for 3 min at 12,000 rpm, the sedimented material was extracted as above, and the two supernatants were combined. In preliminary trials this treatment was found to extract at least 90% of free AA. Each sample was carried out in triplicate (biological replicates).

AA content was determined by the acid ninhydrin method as described by Williams and Frank (1975), with minor modification. Proper extract dilutions in a final volume of 15 µL were sequentially mixed in a 96-microwell plate with the same volume of a 3% (w/v) Na acetate solution and 200 µL of a 0.15% (w/v) freshly-prepared solution of ninhydrin in acetic acid, and immediately read with a Ledetect plate reader (Labexim, Lengau, Austria) equipped with LED plugins at 352 and 540 nm. After heating at 50°C for 12.5 min, samples were cooled to room temperature and read as above. The difference of absorbance between final reading and time-zero value was considered. For each sample, at least 8 dilutions were tested (technical replications). Proline and total AA concentrations were calculated based on calibration curves obtained with different dilutions of reference solutions containing 1 mM L-proline (read at 352 nm) or a 25 mM mixture of AA similar to their abundance in plant extracts [all proteinogenic AA at 1 mM but Gln (4 mM), Asn, Glu and Asp (2 mM)] (read at 540 nm).

2.3 Statistical analysis

Statistical analyses were performed in R 3.6.0 software (R Core Team, 2019).

Linear mixed-effects models (LMM) were applied to test the effects of salinity (as continuous variable), flooding (factor with two levels, i.e. “low” or “high”), plant growth stage (i.e. flowering and seed ripening) and their interactions on NSCs (i.e. soluble sugars or starch) and osmolytes (i.e. AA or proline), in the two species selected. The saltmarsh site was considered as random factor. LMM were applied using the “nlme” package. Model assumptions were verified using diagnostic plots of model residuals.

The “dredge” function of the MuMIn package was applied for model selection, which ranked models on AICc (Akaike's Information Criterion corrected for small samples). The best fit refers to the model with the lowest AICc. Results from the best models were tested using the Analysis Of Variance (ANOVA) and plotted using the “visreg” function.

3. Results

3.1 Soluble non-structural carbohydrates

The content of soluble NSCs recorded in rhizome and basal stem of *L. narbonense* and *S. fruticosa* differed between the two species and was on average 2.17 ± 0.50 and 0.70 ± 0.48 mmol g $^{-1}$ DW for *L. narbonense* and *S. fruticosa*, respectively.

The best model selected by the multi-model inference analysis was the only one plausible (Δ AICc<2) and explained the 72.6 % of the total variation in soluble NSCs.

Soil salinity ranged from 1.4 to 4.3 g L $^{-1}$ and was slightly higher in high sites compared to low sites (Supplementary material Tab. S1). Despite the small variability, the two species responded significantly to salinity and differently according to flooding stress (low or high) and plant growth stage (flowering or seed ripening, Tab. 1). In particular, in both species the content of soluble sugars was affected by salinity but mainly under occasional flooding (low flooding stress, Fig. 2A, B). Soil salinity negatively affected the content of soluble NSCs in *L. narbonense* rhizomes, while improved the content of soluble NSCs in *S. fruticosa* basal woody stems. Salinity affected soluble NSCs stronger during seed ripening compared to the growing season, in both species. Finally, frequent flooding (high flooding stress) entailed a constant content in soluble NSCs in both halophytes and during both growth stages (growing or seed ripening), despite the increase in soil salinity.

3.2 Starch

The starch content measured in rhizome or woody basal stem of *L. narbonense* and *S. fruticosa*, respectively, differed significantly only between species (one plausible model Δ AICc<2, Fig. 3) (Tab. 2) explaining ca. 62.7% of the total variation in starch content. No significant relationship between starch content and salinity or differences due to flooding stress or plant growth stages (flowering or seed ripening) were recorded.

Starch content was higher in the rhizome of *L. narbonense* compared to the woody stem of *S. fruticosa*. *L. narbonense* recorded in average 26.4 ± 4.8 mg g^{-1} of starch whereas *S. fruticosa* recorded in average 17.3 ± 3.7 mg g^{-1}.

3.3 Amino acids and proline

AA and proline contents recorded in rhizome or woody basal stem of *L. narbonense* or *S. fruticosa*, respectively, differed only between species (Fig. 4A, B). The difference was highly statistically-significant (one plausible model Δ AICc<2, Tab. 3) and the model explained the 74.0% and 96.6% of the total variance for AA and proline contents, respectively. No differences were recorded due to flooding stress or salinity, as well between plant growth stages (flowering or seed ripening).

AA and proline content were higher in the rhizome of *L. narbonense* compared to the woody stem of *S. fruticosa*. *L. narbonense* recorded in average 119.9 ± 76.4 µmol g^{1} of AA, where proline represented 33.9 % of total AA content. *S. fruticosa* recorded on average 16.0 ± 16.2 µmol g^{-1} of AA and proline represented only the 11.6 % of total AA content (average). AA and proline contents were highly variable, in both *L. narbonense* rhizome and *S. fruticosa* stem.

4. Discussion

The present work provided interesting insights on the physiological response to flooding and salinity in two halophytes well adapted to high-stress conditions, and on interaction between these two environmental stresses. Among the considered osmolytes, only soluble NSCs seemed involved in the osmotic adjustment in both species, whereas AA and proline contents were not affected by salinity or flooding, suggesting that both species could support a soluble NSC-based strategy to face flooding and salinity. Nevertheless, this strategy is not common in dicots, which usually exploit other osmolytes to drive the osmotic adjustment (Gil et al., 2013). Furthermore, since starch is not affected by salinity or flooding, soluble NSCs could be used for sprouting and vegetative propagation. Halophytes can tolerate tissue anoxia thanks to carbohydrate supply, enabling ATP consuming processes (Colmer and Flowers, 2008). Reserve organs represent the main source for carbohydrates and respond to long-term effects of environmental stresses, differently to leaves or roots that are highly variable even within the same season (Duque and Setter, 2016).

4.1 NSCs and osmolytes content in *L. narbonense* and *S. fruticosa*

Patterns of soluble NSCs observed in *S. fruticosa* are consistent with literature and the low values of free proline content are highly comparable with those reported by García-Caparrós et al. (2017). Soluble NSCs and AA contents in *S. fruticosa* poorly explain the salinity tolerance of the species. Osmolytes concentration after a stress is expected to be in the range between 40 and 400 mmol g $^{-1}$ (Subbarao et al., 2001) but, in our samples, soluble NSCs were only about 0.70 mmol g $^{-1}$ and AA were in average 16 µmol g $^{-1}$. It is plausible that other compounds, such as glycine betaine, could be used for osmotic adjustments, being the latter already reported in large concentration in the succulent stems of *S. fruticosa* (Gil et al., 2014).

L. narbonese rhizomes showed a large content of all osmolytes (i.e. soluble NSCs, AA and proline) and starch. Soluble NSCs content was larger than what measured in other species of the same genus (Murakeözy et al., 2002; Liu and Grieve, 2009), whereas starch content in *L. narbonense* (on average 26.34 mg g $^{-1}$) was consistent with literature data (Liu and Grieve, 2009). Rhizomes are expected to accumulate more sugars compared to other plant tissues and the large content in osmolytes could be probably a response to the high soil salinity in our sampling sites. In our work, soluble NSCs were on average 2166 µmol g ^{1}, 9- fold than in non-salty conditions (Liu and Grieve, 2009) and 5-fold than in soil with low salinity (< 2 g L $^{-1}$, Murakeözy et al., 2002). Several osmolytes could be involved in osmotic stress tolerance and the effective acclimation response of *L. narbonense* to environmental stresses could be referred, in addition to morphological traits, to this large amount of NSCs and AA recorded. Osmolytes could probably affect plant plasticity and enhance plant stress tolerance.

The high content of soluble NSCs in *L. narbonense* could be also attributed to a fraction of soluble amorphous starch or fructans, but no data on fructan content are available for the Plumbaginacee, a family where the biochemical diversity of osmolytes seems quite peculiar among halophytes (Gagneul et al., 2007).

4.2 Soluble NSCs response to salinity and flooding

The response mediated by soluble NSCs differed between species, seasons and with the interaction between salinity and flooding.

Despite the growth of both *L. narbonense* and *S. fruticosa* is limited by flooding (Pellegrini et al., 2018), plants from high stress sites did not show an accumulation of carbon reserves, contrary to what suggested for altitude stress (growth limitation hypothesis) in trees (Fajardo et al., 2012) or shrubs (Casolo et al., 2020). Furthermore, at high flooding stress, accumulation/consumption of soluble NSCs did not differ markedly with the increase of salinity, in both species.

Carbohydrates are expected to be rapidly depleted upon anoxia because of the energy demand to satisfy plant physiological activity and reserve consumption is slower in flood-tolerant plants than in non-tolerant ones (Dubey and Singh, 1999; Marcílio et al., 2019).

Literature reports contrasting results on the relationship between NSCs and flooding. Some authors recorded a decrease of total soluble NSCs in belowground tissues with the increase of flooding (Gimeno et al., 2012), due to sugar depletion, while others observed a lack of relationship between soluble NSCs and flooding (García-Sanchez et al., 2007).

The ability to use NSCs effectively is an inherent characteristic of flood-tolerant species (Ye, 2010), in order to face the upcoming flooding or salinity stress regardless to its strength, and the high soluble NSCs reported for halophytes could be a constitutive trait (Gong et al., 2005).

In our study, salinity had a significant effect on soluble NSCs only at low flooding stress. Salinity tolerance is expected to increase osmolyte content more consistently than flooding, regardless to the species (Sanchez et al., 2008). However, the two considered species behaved oppositely at increasing soil salinity. Soluble NSCs in *L. narbonense* decreased with the increase of salinity, whereas in *S. fruticosa* soluble NSCs increase with the increase of salinity.

The decrease of soluble NSCs in *L. narbonense* may be due to the mobilisation of soluble NSCs from the rhizome to other tissue sinks. In mature plants, many functions are performed by sugars, e.g. signalling, turgor maintenance, phloem transport (Gibson, 2005), and only some of them are related to environmental stresses (Savage et al., 2016). NSCs could be accumulated during plant rest and used all of the year for plant growth and seed production. Therefore, despite responding to salinity, we expect that other osmolytes are involved in the osmotic adjustment in *L. narbonense*, e.g. *chiro*-inositol (Liu and Grieve, 2009).

On the contrary, the increase of soluble NSCs in *S. fruticosa* highlighted the role of this sugar fraction in driving salinity tolerance in this species. At low flooding stress, less energy is depleted for stem elongation (Pellegrini et al., 2017) and a larger amount of sugars could be used to contrast the salinity stress. Soluble NSCs are commonly reported to increase with salinity in many species (Dubey and Singh, 1999), included some of the Chenopodiaceae family (Murakeözy et al., 2003).

Plant response to salinity was more pronounced upon the seed ripening season, in both species. Plants are likely to prevent acute depletions of the NSC pool at all times, but seasonal variations in soluble NSCs were reported in literature (Martínez-Villalta et al., 2016). Nevertheless, the relationship between seasonal changes and salt or flood stress is not clear, and literature showed contrasting results (Murakeözy et al., 2002).

The decrease of soluble NSCs in *L. narbonense* is probably due to a decrease in photosynthesis activity during seed ripening, with a subsequent allocation of sucrose in seeds, during the seed ripening season. Indeed, a decrease of sucrose was observed in underground organs during fruit ripening (Petrussa et al., 2018). On the contrary, a lower soluble NSCs production is expected in stems of *S. fruticosa* during summer (i.e. growing season), because photosynthesis is limited in this halophytic C_3 shrub during the warmer hours of the day (Redondo-Gómez et al., 2007).

4.3 Species-specificity of starch content

As expected, starch in *L. narbonense* rhizome was higher compared to *S. fruticosa* stems but, even if the analysed tissues are both reserve organs, starch did not depend on the season, contrary to what reported for other species with underground reserve organs (Petrussa et al., 2018). In storage organs, starch can be stored over the seasons or over many years, sustaining plant growth at the start of the growing season. In our work, starch did not depend on flooding or salinity. Flooding is reported to reduce starch amount in roots and rhizomes of halophile species (Peña-Fronteras et al., 2009) but this trend is inconsistent especially in flood-tolerant plants (García-Sánchez et al., 2007). Salinity could reduce starch content but the independency of starch to salinity stress was demonstrated for some halophytes as well (Liu and Grieve, 2009). Halophytes are extremely adapted to stresses and variation in the plant responses are probably restricted, being physiological acclimation a constitutive trait in these species.

4.4 Lack of AA and proline response to salinity and flooding

AA and proline contents were higher in *L. narbonense* than in *S. fruticosa*, and did not differ between season, despite seasonal fluctuations were expected (Mouri et al., 2012). In *S. fruticosa*, low levels of AA and proline were found and the contents were far below the required for osmotic compensation (Gagneul et al., 2007), while the high AA and proline contents in *L. narbonense* may have a role in flooding and salinity tolerance of this species. Nevertheless, no effect of flooding or salinity on AA and proline levels was found in these two halophytes and such results strengthen the significance of soluble NSCs variations as response to salinity and flooding.

Changes in AA content related to flooding are poorly studied and uncertain (García-Sánchez et al., 2007) but the occurrence of significant variations in AA homeostasis as a consequence of osmotic stress, such as salinity (Zhonghua et al., 2011), is frequently reported. Proline usually increases more than other AA under the salinity stress (Sanchez et al., 2008) possibly contributing to osmotic adjustment, stabilizing subcellular structures and scavenging free radicals (Forlani et al., 2019). Hartzendorf and Rolletschek (2001) reported a 4-fold increase of total AA in *Phragmites australis* rhizomes exposed to 10‰ salinity, which was mainly due to a 200-fold increase of free proline. However, some halophytes showed species-specific patterns with different trends regarding salinity (Nasir et al., 2010). Previous reports showed the lack of osmo-induced accumulation and a strong reduction of proline concentration in roots of *S. fruticosa* following salt treatments (García-Caparrós et al., 2017). Another possible compatible osmolyte, glycine betaine, was found in *S. fruticosa* in larger amount compared to proline (Gil et al., 2014). Considering that plants do not use both compounds at the same time for the osmotic adjustment (Slama et al., 2015), salinity tolerance in *S. fruticosa* could potentially be attributed to glycine betaine but further investigations are required to support this hypothesis.

Large amount of AA and proline in *L. narbonense* could be interpreted as an evolutionary adaptation to face flooding and salinity fluctuations of tidal saltmarshes. Accordingly, proline has been found to increase with salinity in roots of *L. latifolium*, but only in relatively low amounts at which it might behave as cytoprotectant (Gagneul et al., 2007). However, contrasting responses were recorded in the *Limonium* genus. In *L. linifolium*, proline accumulation was not induced by salinity and high intracellular concentrations were consistently interpreted as a constitutive adaptation of this halophyte to the osmotic stress (Tabot and Adams, 2014).

5. Conclusions

In our work, some possible physiological responses to flooding and salinity were studied in the field, highlighting the role of the combining effect of these two environmental stresses on two target halophytes.

Stress tolerance seems to be driven by a soluble NSCs-based strategy in both *L. narbonense* and *S. fruticosa*. These species underlined opposite strategies against stresses, but plant response was similarly stronger during seed ripening. Flooding appeared the major stress affecting the osmotic response, whereas salinity tolerance was modulated only at low intensity of flooding. The osmotic adjustment seems therefore a result of the combination of flooding and salinity, playing NSCs a central role in plant stress tolerance.

The high AA and proline contents in *L. narbonense* suggested, in addition, a constitutively expressed, osmolyte-based mechanism to preserve the tissue water potential, independently from flooding or salinity intensity. The large amount of soluble NSCs recorded in this species could be probably due to soluble starch and/or fructans, included in the soluble sugar fraction.

Despite halophyte responses to flooding and salinity are complex and still unclear, our results focus the role of soluble NSCs in stress tolerance. Moreover, the combining effect of flooding and salinity highlighted that stresses cannot be individually examined and that their interaction could be pivotal in plant response to environmental stresses.

Author statement

Elisa Pellegrini: Conceptualization, Investigation, Formal analysis, Writing

Giuseppe Forlani: Investigation, Writing, Resources

Boscutti Francesco: Conceptualization, Methodology, Formal analysis

Valentino Casolo: Conceptualization, Writing, Resources, Project administration

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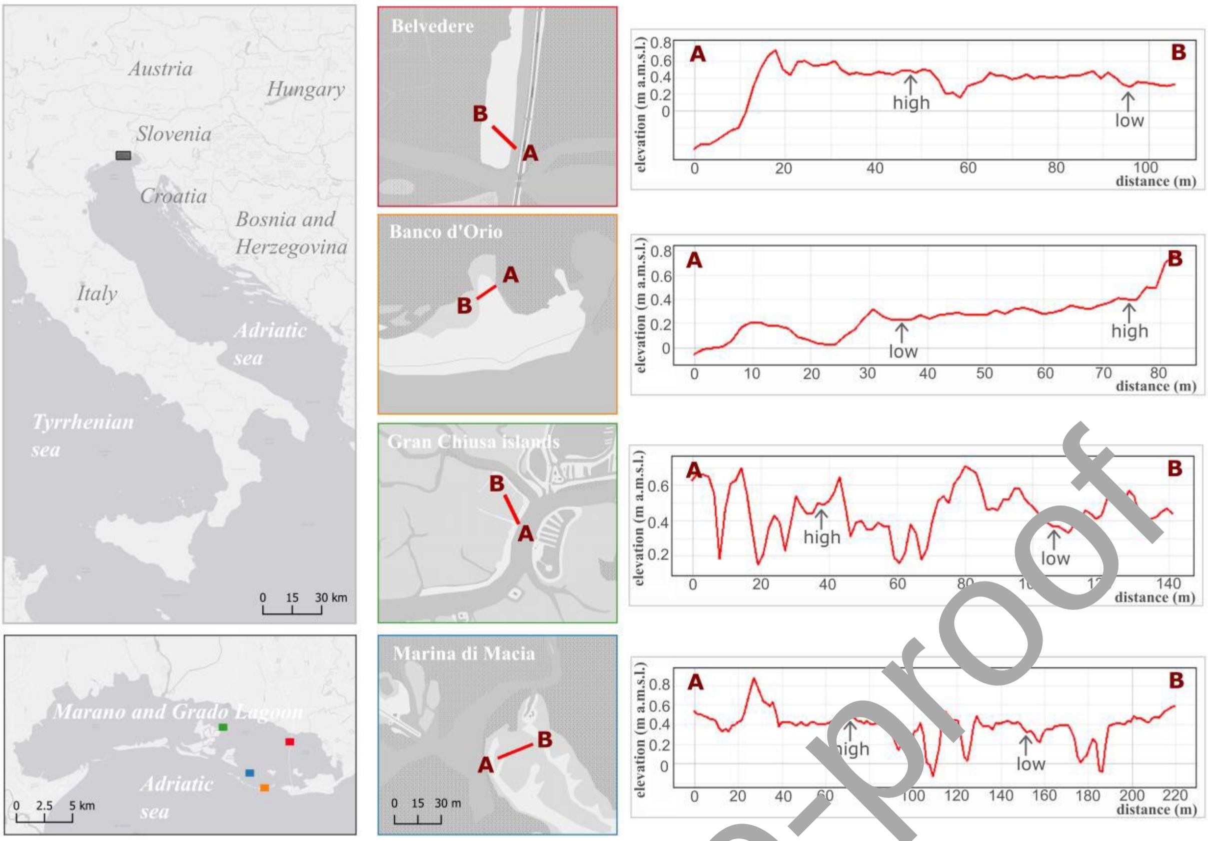
Figure captions

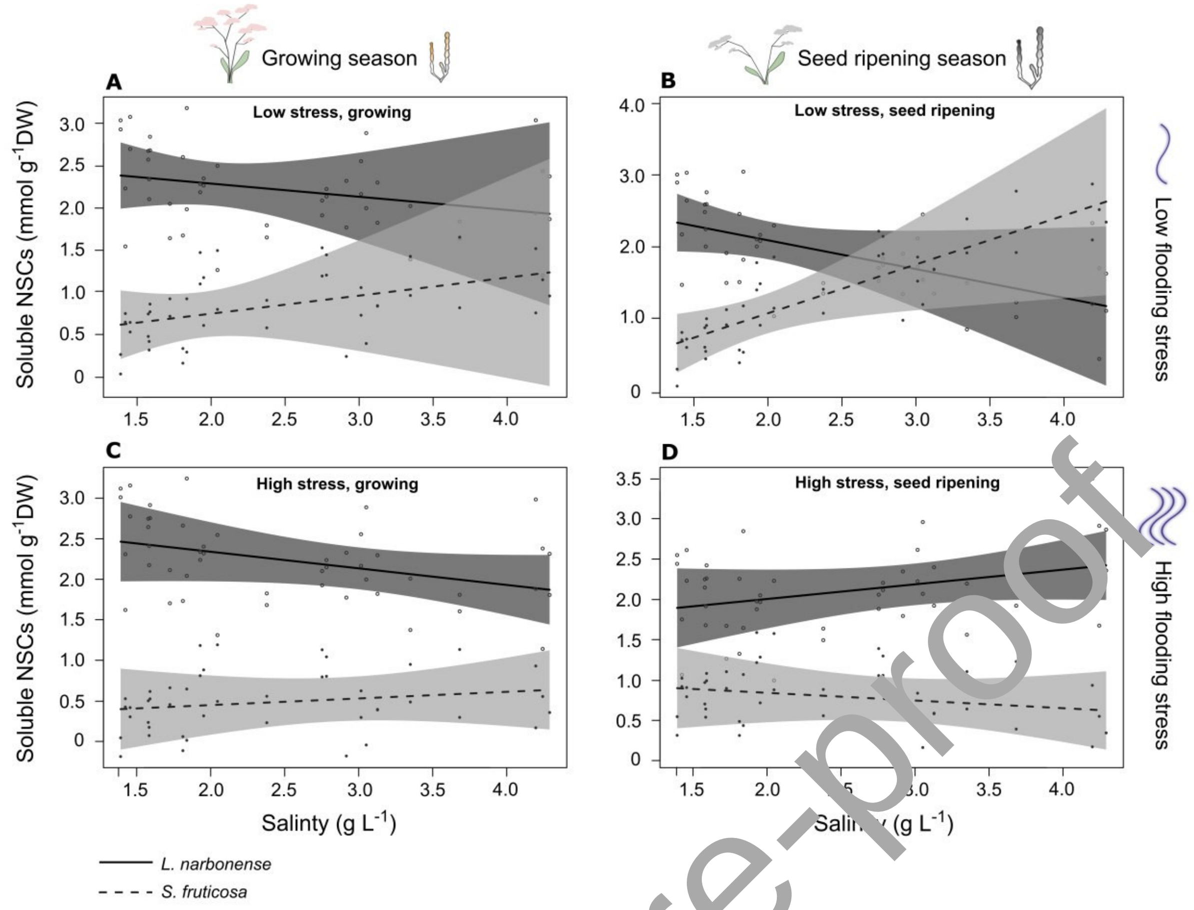
Fig. 1. Marano and Grado Lagoon in the Northern Adriatic Sea and the four saltmarshes selected. In each saltmarsh, the direction and the length of the belt transect is shown. Along the transect, two sampling sites (“low” and “high”) were selected according to species distribution and LiDAR elevation model. Terrain elevation profiles with location of “high” and “low” sites are reported on the right for each saltmarsh.

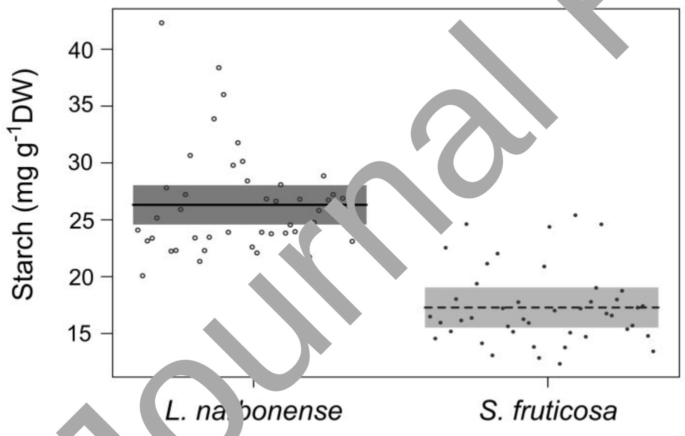
Fig. 2. Effects of salinity on soluble NSCs content in rhizome and woody stem of *L. narbonense* (solid line) and *S. fruticosa* (dashed line), respectively. The effects of the best model selected are shown. Flooding stress (low or high) and season (flowering or seed ripening) are factor-type variables.

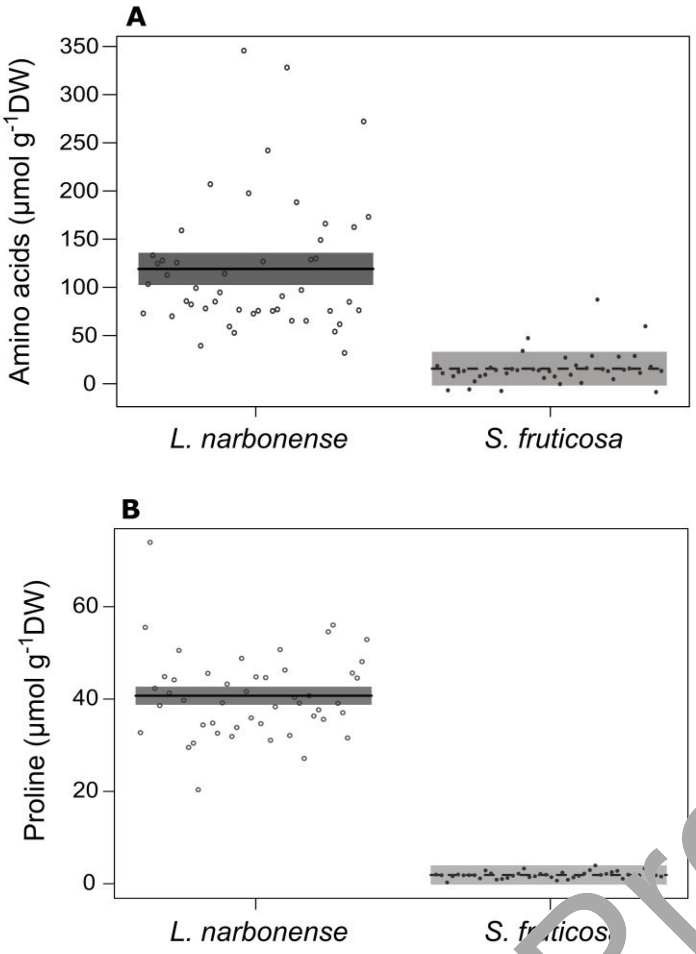
Fig. 3. Starch content in rhizome of *L. narbonense* (solid line) and woody stem and *S. fruticosa* (dashed line), respectively. The effects of the best model selected are shown.

Fig. 4. AA and proline concentration in rhizome of *L. narbonense* (solid line) and woody stem and *S. fruticosa* (dashed line), respectively. Effects of the best models selected are shown for AA and proline contents, respectively.


Austria
Hungary
Slovenia
Croatia
Bosnia and Herzegovina
Italy
Adriatic sea
Tyrrhenian sea
0 15 30 km
Marano and Grado Lagoon
Adriatic sea
0 2.5 5 km
Belvedere
Banco d'Orio
Gran Chiusa islands
Marina di Macia
0 15 30 m
A
B
elevation (m a.m.s.l.)
distance (m)
high
low


Growing season
Seed ripening season
A
Low stress, growing
B
Low stress, seed ripening
Low flooding stress
C
High stress, growing
D
High stress, seed ripening
High flooding stress
Soluble NSCs (mmol g-1DW)
Salinty (g L-1)
1.5
2.0
2.5
3.0
3.5
4.0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
L. narbonense
S. fruticosa


Starch (mg g-1DW)
15
20
25
30
35
40
L. narbonense
S. fruticosa


A
Amino acids (µmol g-1DW)
350
300
250
200
150
100
50
0
L. narbonense
S. fruticosa
B
Proline (µmol g-1DW)
60
40
20
0
L. narbonense
S. fruticosa

Tables

Table 1: Results of ANOVA applied to the best linear mixed-effects model (lowest Δ AICc) describing soluble NSCs content in rhizome and woody stem of *L. narbonense* and *S. fruticosa*, respectively. Explanatory variables are salinity, season (flowering or seed ripening), flooding (high or low flooding stress) and their interactions. Season and site are factors. DF = degrees of freedom, F = results of the Fisher's test, *P* = level of significance. Significant relationships are in bold.

Explanatory variables	**DF**	**F**	***P***
(Intercept)	1, 56	995.40	<.0001
Salinity	1, 17	0.01	0.94
Season	1, 56	0.70	0.41
Species	1, 56	218.07	**<.0001**
Flooding	1, 17	1.62	0.22
Salinity:season	1, 56	1.05	0.31
Salinity:species	1, 56	0.27	0.60
Season:species	1, 56	3.81	0.06
Salinity:flooding	1, 17	0.03	0.86
Season:flooding	1, 56	0.001	0.98
Species:flooding	1, 56	3.08	0.08
Salinity:season:species	1, 56	1.96	0.17
Salinity:season:flooding	1, 56	0.06	0.80
Salinity:species:flooding	1, 56	6.08	**0.02**
Season:species:flooding	1, 56	0.01	0.91
Salinity:season:species:flooding	1, 56	4.52	**0.04**

Table 2: Results of ANOVA applied to the best linear mixed-effects model (lowest Δ AICc) describing starch in rhizome and woody stem of *L. narbonense* and *S. fruticosa*, respectively. DF = degrees of freedom, F = results of the Fisher's test, *P* = level of significance.

Explanatory variables	**DF**	**F**	***P***
Starch			
(Intercept)	1, 67	5695.4	<.0001
Species	1, 67	134.6	<.0001

Tab. 3. Results of ANOVA applied to the best linear mixed-effects model (lowest Δ AICc) describing AA and proline content in rhizome and woody stem of *L. narbonense* and *S. fruticosa*, respectively. DF = degrees of freedom, F = results of the Fisher's test, *P* = level of significance.

Explanatory variables	**DF**	**F**	***P***
AA			
(Intercept)	1, 66	771.39	<.0001
Species	1, 66	243.66	<.0001
Proline			
(Intercept)	1, 65	5286.57	<.0001
Species	1, 65	2537.75	<.0001