



Sodium, potassium and nitrogenous osmolyte accumulation in relation to the adaptation to salinity of *Elytrigia pycnantha*, an invasive plant of the Mont Saint-Michel Bay

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Abstract: *Elytrigia pycnantha* (Godr.) Löve (also named *E. aethERICA* or *Elymus athericus*) is a monocot species native of salt marshes of the Mont Saint-Michel Bay. Usually found at the landward edge of the bay, this grass plant is now spraying in the middle of the marsh and even at the tidal flat boundaries. In this study, we have outlined some biochemical traits that may be linked to the spread of this invasive native clonal grass. Plants were collected at the west part of the bay (Le Vivier sur mer) along the salt marsh zonation, some of them were grown further in a glasshouse under different NaCl concentrations and nitrogen sources while others were analysed immediately without treatment. The plants collected from the bay and those treated under controlled conditions were sampled according to different vegetative organs, frozen dried, and water soluble compounds extracted. Potassium and sodium contents were measured by flame photometry and amino acids and betaine through HPLC procedure analysis. It is shown that sodium accumulation and amino acid profiles depend both on the location of the plant on the salt marsh and on the plant organ. We have also highlighted an effect of the nitrogen source (no N, N-NO₃⁻ or N-NH₄⁺) on plant response to salt treatment. It is suggested that recent ability of this species to spray is related to its ability to perform nitrogenous metabolic adjustment under new nitrogen source availability.

Résumé : Accumulation de sodium, de potassium et de produits azotés en relation avec l'adaptation à l'environnement salin chez *Elytrigia pycnantha*, une plante invasive des marais salés de la Baie du Mont Saint-Michel. *Elytrigia pycnantha* (*E. AethERICA* ou *Elymus athericus*) est une monocotylédone, se développant naturellement dans la zone du haut-schorre (baie du Mont Saint-Michel). Cependant, cette graminée a priori peu tolérante à de fortes salinités, colonise actuellement les zones médiane et basse du schorre. Nos résultats mettent en évidence quelques caractéristiques biochimiques de cette plante témoignant de cette invasion récente. Certaines plantes collectées dans le haut schorre (Le Vivier sur mer) ont été cultivées en serre en présence de différentes concentrations en NaCl et en Azote tandis que les autres plantes récoltées ont été analysées directement à l'issue du prélèvement sur le terrain. Les plantes collectées dans la baie et celles traitées en conditions contrôlées ont été fractionnées selon les différents organes végétatifs, lyophilisées et les solutés hydrosolubles extraits. Les teneurs en ions potassium et sodium ont été mesurées par photométrie de flamme et les acides aminés et

bétaïnes par analyses CLHP. Les résultats montrent que l'accumulation de sodium et les profils d'acides aminés sont dépendants de la zonation et de l'organe dans la plante. De plus, nous mettons en évidence de façon originale un effet de la source d'azote (pas de N, N-NO_3^- ou N-NH_4^+) sur la réponse de la plante au traitement salin.

Keywords: *Elytrigia pycnantha*; Glycine betaine; Nitrogen; Proline; Salinity; Sodium Potassium ratio

Introduction

Elytrigia pycnantha (Godr.) Löve (also named *E. aetherica* or *Elymus athericus*) is a monocot species native of salt marshes of the Mont Saint-Michel Bay, usually found at the landward edge of the bay so called the "high marsh" (Bouchard & Lefeuvre, 2000). In the last decade, this grass plant has been spraying in the middle of the marsh and even to the tidal hat boundaries (low marsh) (Valéry et al., 2004). It has been reported that the spread usually started from the banks of the drainage creeks (Valéry et al., 2004), which contain pore water enriched in $\text{NO}_3\text{-N}$ (Troccaz, 1996). In this area, *Spartina townsendii* (Groves) and *Salicornia europaea* (L.) are growing in the pioneer area, the low marsh is well represented by *Puccinellia maritima* (Huds.) Parl. and *Suaeda maritima* (Dum.), while the middle marsh is dominated by *Atriplex portulacoïdes* (L.) (Bouchard & Lefeuvre, 2000). More than 20 years ago, Briens & Larher (1982) made an overall study on the ability of the different halophytic species from the salt marsh to support the high salinity. They recognized three groups of species according to their capacity to store sodium ion and water in their tissues, (i) a first group including *Puccinellia*, and *Spartina* with an inorganic ion accumulation less than 20% of dry matter (DM) and a low water level (less than 300% of DM), (ii) a second group including *Atriplex* with high levels of inorganic ions (20 to 35 % of DM) and water (450 to 800% of DM), and (iii) a third group (*Suaeda* and *Salicornia*), with a tremendous capacity to store both inorganic ions (50% of DM) and water (1000% of DM). All these halophytic phanerogams are able to grow under high salinity environments by adjusting their osmotic pressure with inorganic and organic solute accumulation. In addition to carbohydrate, almost all species accumulate nitrogenous compounds such as proline or glycine betaine, with the quantitatively predominant solutes being carbohydrate (*Puccinellia*) or nitrogenous compounds (*Atriplex*, *Salicornia*, *Suaeda*) (Briens & Larher, 1982). What is expected with the species under study is that its recent invasion through the salt marsh may be linked to i) a new source of nitrogen from ground water and 2) a higher nitrogen use efficiency of this species (compare with native plant species of this area) allowing a better ability to adjust its nitrogenous metabolism under salt conditions. For instance,

it is expected that this species under higher nitrogen availability will be able to co-accumulate nitrogen compounds such as proline and glycine betaine, well described in plant response to salt adaptation in halophytic plants (Tipirdamaz et al., 2006). The aim of our study was to uncover the biochemical and physiological traits that are associated to the adaptation of *Elytrigia* to its new habitats and to propose mechanisms that permitted the changes and the expansion seaward, to lower levels of the salt marsh.

Material and Methods

Plants were collected at the west part of the Mont Saint-Michel bay (Le Vivier sur mer, F35) along the salt marsh zonation. We followed two experimental designs. First, we collected plants in the bay that we analyzed directly. In a second time, we transplanted plants from the bay to controlled environmental conditions in a glasshouse, where we grew them under different NaCl concentration and nitrogen sources.

For the experiment in the bay, plants were harvested on the 27 of April 2004 in 3 locations in the salt marshes, (i) a "pioneer location" at the low / middle marsh edge where *Elytrigia* was found as individual among *Puccinellia* or *Atriplex*, (ii) a "stabilized location", at the middle marsh, where *Elytrigia* was found as large spots (more than 1 m²) surrounded by *Atriplex*, and (iii) a "native location" at the high marsh where *Elytrigia* covered almost all the canopy. For each location, the youngest ("young leaf" selected because they appears green and not affected by salt) and second youngest ("old leaf" so called because they will start senescing as soon as a new leaf will growth) fully expanded leaf from 10 plants were collected (3 replicates, each replicate being the addition of 10 young or 10 old leaves).

For the controlled condition experiment, intact plants with their root systems were collected at the high marsh on the 27th of April 2004. Plants were separately cleaned and their root system washed. Plants were splitted in 6 groups of 40 individuals in pots containing 500 mL of full strength Hoagland's solution (Hoagland & Arnon, 1938). After 8 days (on the 5th of May 2004), the solution was replaced by modified Hoagland solution added (referred as salt) or not (referred as control) with 200 mM of NaCl and containing 0

meq.L⁻¹ of nitrogen (referred as control/no N and salt/no N), 3 meq.L⁻¹ of N-NO₃⁻ (referred as control/nitrate and salt/nitrate), 1.5 meq.L⁻¹ of N-NO₃⁻ plus 1.5 meq.L⁻¹ of N-NH₄⁺ (referred as control/ NH₄⁺ and salt/NH₄⁺). The solutions were renewed weekly. Plants were harvested on the 27th of May 2004 (3 weeks of treatment). Out of the 40 plants, the biggest and smallest were removed and 3 groups (3 replicates) of 6 to 7 plants were made per treatment. For each plant, we recognized the different leaves (from the youngest to the oldest). Within each plant, we separated the part of the leaf flattened (flag), the part of the leaf (rolled leaf) below the flag and enrolled in the internode above the leaf, and the internode just below the leaf fixation to the stem (Stem). All corresponding parts from 6 to 7 plants were pooled together. Some plants were harvested (as described above) at the start of treatment and were referred as T₀ (3 replicates of 6 plants).

The different vegetative organs sampled from the plants collected in the bay and the ones treated under controlled conditions were immediately frozen in liquid nitrogen, frozen dried, and grounded. Dried material (up to 50 mg) were transferred to microtube and suspended in 96% ethanol. Suspensions were heated at 85°C until complete evaporation of ethanol. The residues were resuspended with de-ionized water and shaken at +4°C for 1h. Crude extracts were clarified by centrifugation (15000 g, +4°C, 20 min) and stored at -20°C until analysis. These crude supernatants were used to quantify mineral and organic solutes without further purification step. Potassium and sodium contents were measured using flame photometer. Free proline was quantified according to Magné & Larher (1992) and total free amino acids according to Yemm & Cocking (1955), using a spectrophotometer. Glycine betaine was quantified through HPLC procedure analysis after derivatization with dibromoacetophenone, and individual amino acids after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) using the procedure optimized by Cohen & Michaud (1993) adapted to plant tissues by Bouchereau et al. (1999).

From the data collected (n = 3), means and standard errors calculation as well as T-test and regression fitted curves were performed using Microsoft Excel 2000 (©Microsoft Corp., 1985).

Results

Plants collected at the salt marshes

Inorganic compounds. The potassium content was stable and about 0.4 ± 0.04 meq.g⁻¹ DW, independently of location ("native" to "pioneer") and organ (young or old leaf).

In contrast, sodium was accumulated in old parts and to higher contents (0.4 ± 0.01 meq.g⁻¹ DW) in the "pioneer" and "stabilized" locations than in the "native" location (0.2 ± 0.02 meq.g⁻¹ DW) and differences between Na⁺ content in native and other locations was found significant ($P < 0.05$). In young leaves, sodium remained at low level (0.1 ± 0.02 meq.g⁻¹ DW) in all locations. In young leaves, Na⁺ content from stabilized and pioneer locations was significantly different from Na⁺ content of old leaf of the same location ($P < 0.01$) and native location ($P < 0.05$). Young leaf Na⁺ content in native location was significantly different ($P < 0.05$) from old leaf from other locations but no significant difference was found with old leaves from the same location.

Nitrogenous compounds. The main free amino acids detected by HPLC (Table 1) were proline, glutamate, glutamine, aspartate, asparagine, and γ -aminobutyric acid (GABA). Proline is accumulated similarly in old and young leaves with an increase from the "native" location (trace) to the "stabilized" location (about $10 \mu\text{mol.g}^{-1}$ DW) and the "pioneer" location (around $35 \mu\text{mol.g}^{-1}$ DW). The glycine betaine content was constant and around $40 \mu\text{mol.g}^{-1}$ DW. We did not find variation with location in GABA content, which was about $7 \mu\text{mol.g}^{-1}$ DW, nor in content of total free amino acids, other than proline, which was about $30 \mu\text{mol.g}^{-1}$ DW. Under such conditions, only PRO content showed significant differences among the different conditions (see Table 1).

Plants collected from glasshouse experiment

Inorganic compounds. Under free nitrogen growing conditions, potassium content (Fig. 1 a) was maintained at a high level after 3 weeks of treatments compared with T₀ while in the old leaf, we could notice a slight decrease. Under the other conditions, K⁺ was not affected with only a slight increase when ammonium was used as nitrogen source together with nitrate. In contrast, sodium (Fig. 1b), which was high at T₀ decreased drastically in all leaves. Under ammonium condition, almost all the sodium was remobilized from the young leaves. In all N-treatments, old leaves kept a significant amount of sodium, especially under no N or with nitrate as the only source of N. When salt treatment was applied, we could observe a dramatic decrease of K⁺ content in free N plants while K⁺ content was well maintained in plants fed with N, especially those with ammonium. Under NaCl conditions, all plants were able to maintain a lower sodium content in young leaves than in the others. Plants fed with nitrate showed the highest sodium content, particularly in oldest leaves where it reached 0.87 meq.g⁻¹ DW. It is noticeable that in all leaves, when NO₃⁻ was the only N source, sodium content

Table 1. Content ($\mu\text{mol.g}^{-1}$ DW) of glycine betaine (GB), proline (PRO) and total free amino acids minus PRO (AA-PRO), including glutamate (GLU), glutamine (GLN), Aspartate (ASP), asparagine (ASN), alanine (ALA) and γ -aminobutyric acid (GABA) in the young and old flattened leaves of *Elytrigia* harvested at different location (“native”, “stabilized” and “pioneer”) in the salt marshes (standard error in brackets, $n = 3$).

Tableau 1. Teneur ($\mu\text{moles.g}^{-1}$ DW) en glycine bétaine (GB), proline (PRO) et acides aminés libres totaux moins PRO (AA-PRO), incluant les teneurs en glutamate (GLU), glutamine (GLN), aspartate (ASP), asparagine (ASN), alanine (ALA) et γ -aminobutyric acid (GABA) dans les feuilles jeunes (young) et âgées (old) d'*Elytrigia* récoltée en différents points du shore, le haut-shore (“native”), le moyen-shore (“stabilized”) et le bas-shore (“pioneer”) (erreur standard entre parenthèses, $n = 3$).

Location	Leaf	GB	PRO*	AA-PRO	GLU	GLN	ASP	ASN	ALA	GABA
“native”	Young	35.91	3.16 cd	26.86	4.72	1.66	1.56	4.61	2.30	7.63
		(2.68)	(1.82)	(0.86)	(2.13)	(0.07)	(0.20)	(1.34)	(1.21)	(0.41)
	Old	31.87	0.82 d	41.91	9.27	4.26	2.88	7.81	3.87	5.69
		(3.56)	(0.47)	(3.14)	(3.67)	(1.34)	(0.18)	(3.12)	(0.41)	(0.38)
“stabilized”	Young	41.55	10.18 cd	24.14	1.57	1.28	3.23	3.30	4.04	5.83
		(5.50)	(1.94)	(7.19)	(0.39)	(0.41)	(1.25)	(0.50)	(2.04)	(0.64)
	Old	38.82	12.96 bc	43.46	5.99	1.65	11.84	5.94	4.68	6.40
		(6.54)	(3.36)	(5.27)	(2.51)	(0.31)	(6.51)	(2.79)	(0.90)	(0.60)
“pioneer”	Young	45.83	34.94 ab	14.92	0.38	2.98	2.45	0.64	0.27	8.69
		(7.86)	(5.34)	(7.59)		(2.36)	(2.07)			(2.06)
	Old	37.34	36.63 a	30.85	0.42	2.90	4.60	5.20	4.55	8.14
		(2.64)	(6.11)	(14.39)	(0.40)	(0.30)	(2.51)	(0.61)	(2.58)	(1.39)

*PRO: same letter after different mean values indicate no significant difference ($P < 0.05$)

was almost twice higher than under conditions when NO_3^- was partly replaced by NH_4^+ .

Organic compounds. At T_0 , plants contained already a high amount of proline (27 to 67 $\mu\text{mol.g}^{-1}$ DW) and glycine betaine (up to 90 $\mu\text{mol.g}^{-1}$ DW). Without salt treatment (Fig. 1c), proline content was close to 0 $\mu\text{mol.g}^{-1}$ DW under all N treatment except in plants fed with nitrate only where the amount of proline was maintained to a quarter for young leaves and to a half for other leaves compared to T_0 . When plants were submitted to saline conditions, proline content remained low under free N conditions but this amino acid was accumulated in young leaves under nitrate conditions and in all leaves with ammonium, compared to T_0 . For instance, proline content reached up to 140 $\mu\text{mol.g}^{-1}$ DW in young leaves. When data related to sodium and proline contents under NaCl conditions were plotted, a negative correlation between the two parameters was highlighted (Fig. 2). The GB content in control and stress plants was maintained to a high level as an overall in plants under N conditions (more than 75 $\mu\text{mol.g}^{-1}$ DW, data not shown).

Distribution of compounds within the leaf and the stem

Under conditions where nitrogen is supplied as an equimolar solution of ammonium and nitrate, we compared the

distribution of solutes in the different parts of the intermediate leaf and stem below (Fig. 3). Under control conditions, potassium was distributed equally in the different organs while sodium and proline were not accumulated. When salt treatment was applied, potassium slightly decreased in the leaves and stem except flag leaves where it was kept high. Sodium was kept low in the leaves, specially in the flag and was mainly stored in stem. The same observation was made for proline. In both conditions GB was mainly accumulated in the flag and in the stem. The lack of GB accumulation in the rolled part of the leaf seems to be a general feature as similar data were found at T_0 and in nitrate only fed plants and for other leaves (data not shown). However, under free nitrogen condition, this organ seems to accumulate large amounts of GB (about 120 $\mu\text{mol.g}^{-1}$ DW). In salt treated plants, the distribution of all solutes analyzed was similar to the one found at T_0 (data not shown).

Discussion

Data obtained in both experimental designs clearly showed that plants submitted to NaCl at high concentration (200 mM) are able to adjust their inorganic ion content, keeping potassium in the young and photosynthetic parts and using

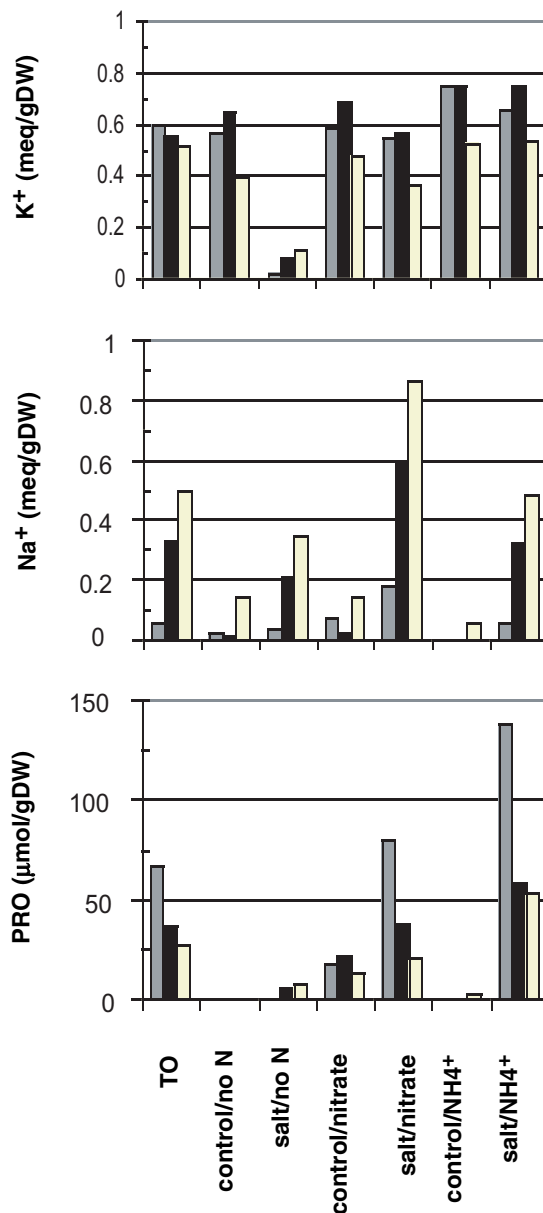


Figure 1. Content of inorganic ions (K^+ and Na^+) and proline (as PRO) in the different flattened leaf (flag) of *Elytrigia* (grey bar: youngest; black bar: intermediate; white bar: oldest) under different conditions (no N: no nitrogen; nitrate: 3 meq.L⁻¹ of $N-NO_3^-$; NH_4^+ : 3 meq.L⁻¹ of $N-NO_3^-$ and $N-NH_4^+$; control: no NaCl; salt: 200 mM NaCl. T_0 = start of treatment).

Figure 1. Teneur en ions inorganiques (K^+ et Na^+) et en proline (PRO) dans le limbe de feuilles de différents âges (bare grise: jeune; bare noire: intermédiaire; bare blanche: vieille) chez *Elytrigia* sous différentes conditions (no N: pas d'azote; nitrate: 3 meq.L⁻¹ de $N-NO_3^-$; NH_4^+ : 3 meq.L⁻¹ de $N-NO_3^-$ et $N-NH_4^+$ en mélange équimolaire; control: pas de NaCl; salt: 200 mM NaCl. T_0 = début du traitement).

other parts of the plant, stem and senescing leaves, as storage organs for sodium. This strategy has already been well described in other species and allows *Elytrigia* to maintain a high K^+/Na^+ ratio (above 1 in N fed plants) in its tissues where active metabolism occurs. Such ability is typical of glycophytic plants tolerant to salt such as tomato (Hernandez et al., 2000). In response to this distribution of inorganic ions, *Elytrigia* seems to accumulate mainly two compatible solutes, proline (PRO) and glycine betaine (GB). The ability of *Elytrigia* to adjust its nitrogen metabolism consequently to co-accumulate these two nitrogenous compounds has already been reported by Briens & Larher (1982) in this species growing on salt marshes (data on *Agropyron pungens* (Pers.) R.). While proline is remobilised quickly when stress is removed (Trotel-Aziz et al., 2000), GB is known to be stored in the plant without remobilisation (Murakeozy et al., 2003). Our data suggest such a metabolic remobilisation of proline in *Elytrigia* and a drastic spatial redistribution on GB depending of growth conditions. In this general strategy of *Elytrigia*, in order to cope with salt conditions, we can suggest that the plant is able to modify its metabolism and to distribute all its inorganic and organic solutes in the way to allow a fast growth of the young parts before the oldest one become senescent. However, growth rate parameters will have first to be recorded to confirm this point.

Under nitrogen deficiency, plants are unable to accumulate proline under salt conditions and are unable to synthesize more GB especially in old organs. The effect of nitrogen nutrition has already been reported in plants submitted to salt treatment such as in *Spartina* (Mulholland & Otte, 2000). In this species, it is shown that nitrogen supply decrease GB content. However, their data suggest that the nitrogen effect on GB content may be linked to a difference in dry matter production and probably in repartition of GB in the plant. In *Spartina*, like in our data, the differential response of GB and proline is also reported with a salt treatment increasing proline content and without drastic effects on GB (Mulholland & Otte, 2002). Under nitrogen deficiency, plants are also unable to maintain a proper K nutrition and under these conditions, K^+/Na^+ ratio decreased below 0.6. In addition, our data strongly suggest that when nitrogen is provided as ammonium, plants are able to handle the saline conditions better than when nitrogen is present only as nitrate. We can propose that ammonium uptake by roots may allow a better control of sodium uptake. It is interesting to notice that under ammonium feeding conditions in the glasshouse, sodium content in the plant was similar to the one in plants collected in the marsh, assuming that under such conditions, nitrogen was provided partly under ammonium uptake. Analysis of the soil water solution (data not shown) indicated an actual availability of ammonium for roots. This putative positive

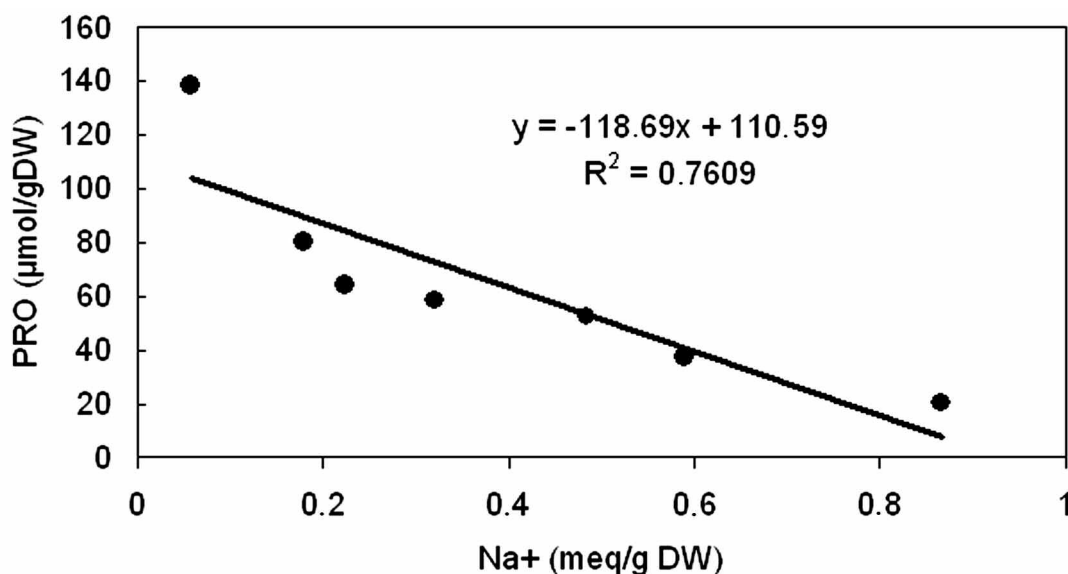


Figure 2. Content of proline (PRO) plotted against Na^+ content in the different flattened leaf of *Elytrigia* under different nitrogen condition (nitrate: 3 meq.L⁻¹ of N-NO_3^- ; NH_4^+ : 3 meq.L⁻¹ of N as NO_3^- and NH_4^+) in plants submitted to 200 mM NaCl. The line is the fitted linear regression.

Figure 2. Teneur en proline (PRO) en fonction de la teneur en Na^+ des limbes de feuilles de différents âges chez *Elytrigia* sous différentes conditions d'alimentation azotée (nitrate: 3 meq.L⁻¹ de N-NO_3^- ; NH_4^+ : 3 meq.L⁻¹ de N as NO_3^- and NH_4^+ en mélange équimolaire) chez des plantes soumises à un traitement à NaCl 200 mM NaCl. La courbe correspond à la droite de régression de ces valeurs.

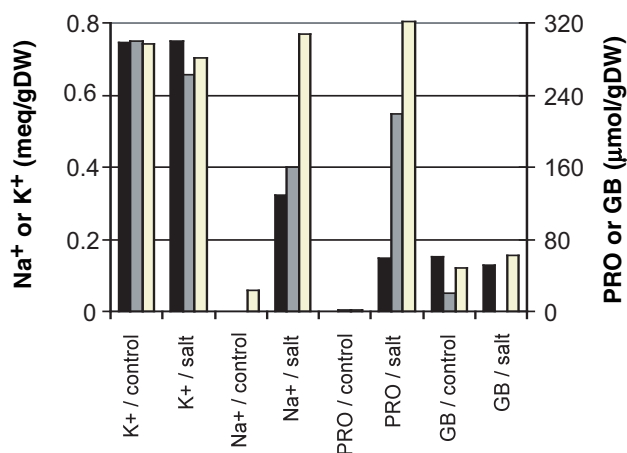


Figure 3. Content of inorganic ions (K^+ and Na^+) and nitrogenous organic solutes (proline PRO and glycine betaine GB) in the second youngest leaf and the stem below (black bar: flag; grey bar: rolled leaf; white bar: stem) in *Elytrigia* with 3 meq.L⁻¹ of N as NO_3^- and NH_4^+ (control : 0 mM NaCl; salt: 200 mM NaCl).

Figure 3. Teneur en ions inorganiques (K^+ and Na^+) et en composés azotés solubles (proline PRO et glycine bétaine GB) dans la feuille intermédiaire et la tige d'*Elytrigia* (barre noire: limbe; barre grise: partie inférieure de la feuille, barre blanche: tige) sur 3 meq.L⁻¹ de N-NO_3^- et N-NH_4^+ en mélange équimolaire (control: pas de NaCl; salt: 200 mM NaCl).

effect of ammonium nutrition on *Elytrigia* may also be found in plants not submitted to salinity. In fact, in such plants fed with ammonium, proline was not detectable, in opposite to what is shown in plants fed with nitrate only. Proline accumulation in control-nitrate plants may be explained by N toxicity as it has been reported in *Phaseolus* (Sanchez et al., 2002).

Conclusion

This investigation of the biochemical traits that could be related to the halotolerance of *Elytrigia pycnantha* enlightened the effect of nitrogen availability both quantitatively and qualitatively on the metabolic adaptation. *Elytrigia* tolerance to salt is associated to an accumulation of nitrogenous compounds, i.e. proline and glycine betaine. We suggest that the recent ability of this plant to colonize the middle and low salt marshes may be related to a recent increase of N availability especially from the pore-water of the drainage creeks as proposed by Valéry et al. (2004). However, we propose than in addition to N availability, the ratio of ammonium to nitrate available for roots is also important. Ammonium may provide a better control of sodium transport. The capacity of the plant to accumulate organic nitrogenous compounds such as proline and glycine betaine in the stem may also participate to the overall regu-

lation of sodium flux and distribution within the plant. Nitrogen availability could appear as a limiting factor for metabolic adjustment.

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