IJBPAS, April, 2019, 8(4): 722-738

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

THE EFFECT OF NITRATE AND SALT LEVELS ON THE GROWTH OF ARABIDOPSIS MUTANTS WITH INSERTION IN *ASN2* GENE

HOUDA MAAROUFI DGUIMI[1, 2] AND ALI KHALAF AHMED ALBAGGAR[3]

[1] Unité de recherche: Nutrition et Métabolisme Azotés et Protéines de Stress (99 UR /09-20), Campus Universitaire, Faculté des Sciences de Tunis. Département de Biologie, Université Tunis EL MANAR, Tunis 1060, Tunisie

[2] Adaptation des Plantes à l'Environnement, IJPB, UMR1318 AgroParisTec, INRA Route de Saint-Cyr, 78026 Versailles cedex, France.

[3] Department of Biology, Faculty of sciences and arts in Baljurashi, Albaha University, Saudi Arabia

***Corresponding author: Houda Maaroufi Dguimi: E Mail address: houda_maaroufi@yahoo.fr; Tel: (216) 23860252; Fax: (216) 71885480**

Received 19th Oct. 2018; Revised 17th Nov. 2018; Accepted 24th Nov. 2018; Available online 1st April 2019

https://doi.org/10.31032/IJBPAS/2019/8.4.4683

ABSTRACT

Wild-type (WT) *Arabidopsis thaliana* (L.) Heynh. Col0, (*ASN2*)-deficient mutant (*asn2* mutant) and knockout T-DNA mutant inserted in the *ASN2* gene (*asn2-1* mutant) were grown for one month by hydroponic culture at high nitrate level (HN=12mM). Then, plants were divided into HN and low nitrate (LN=0.2 mM) medium. Effects of short term (6h) salt stress (100mM NaCl) were assessed in each kind of medium.

On HN-WT leaves, NaCl stress decreased chlorophyll, soluble protein (SP) contents and glutamine synthetase (GS, EC 6.3.1.2) activity. Despite the lack of ammonium accumulation, salt treatment induced aminatrice activity of glutamate dehydrogenase (NADH-GDH, EC 1.4.1.2). In (*Asn2*)-deficient mutant grown on HN medium, salt treatment reduced chlorophyll content and induced the SP and ammonium contents. In *asn2-1* leaves, salt treatment induced NADH-GDH activity. GS activity and SP content were reduced under salinity. The transfer of plant to low nitrate (LN) medium for 6h had a stimulator effect on chlorophyll, SP

contents, GS and NADH-GDH activities in WT leaves. This stimulator effect was lesser important in leaves of (*Asn2*)-deficient mutant. In *asn2-1* mutant, the transfer to LN medium had no significant effect on different studied parameters. In WT and (*ASN2*)-deficient mutant grown on LN medium, changes were related to low nitrate supply rather than salt stress effects. In *asn2-1* mutant, NaCl effects were accentuated by LN treatment in term of ammonium accumulation, NADH-GDH activity stimulation and GS activity inhibition. Salinity reduced GS1 protein level under LN medium, however it was more stable on HN one. The *asn2-1* mutant appeared to be more sensitive to salt treatment under LN medium compared to WT and (*Asn2*)-deficient mutant. The *asn2-1* mutant was the most resistant to nitrate level reduction in the culture medium relative to WT and (*ASN2*)-deficient mutant.

Keywords: Salt, *ASN*, nitrate, *Arabidopsis thaliana,* ammonium assimilation

INTRODUCTION

Salinity has become major constraint for plant growth and productivity of crop plants[1]. Among consequences, high salinity produces osmotic stress by decreasing the water chemical activity and affecting the cell turgor[2]. Salinity can inhibit enzymes of nitrogen assimilation especially glutamine synthetase (GS) and glutamate synthase (GOGAT)[3-4]. As well, salinity reduces photosynthetic capacity, resulting in a decrease of carbon assimilation[5]and an enhanced protein degradation, which leads to a rapid and excessive accumulation of ammonium[6-7], and amino acids remobilization[8]. Ammonium is generally re-assimilated into amides *via* the GS/GOGAT cycle. Alternatively, aminating activity of glutamate dehydrogenase (GDH) may be involved in ammonium reassimilation under stress conditions[9], while *in vivo* function remained to be elucidated. Ammonium can be also assimilated into asparagine by asparagines synthetase activity (AS, EC 6.3.5.4), in response to environmental stresses [10-12].

Asparagine synthetase is encoded by a small number of genes in plants. Three genes encoding asparagine synthetase have been identified in *Arabidopsis thaliana* (*ASN1*, *ASN2* and *ASN3*). Asparagine plays a key role in nitrogen storage and transport of nitrogen from sources to sinks organs because of its high nitrogen to carbon ratio and its stability. Asparagine is a predominant amino acid transported in phloem of *Arabidopsis thaliana*[13]. Instability of asparagine synthetase [14] and rapid turnover of asparagine by asparaginase[15] make it difficult to carry out biochemical approach by determining asparagine synthetase activity. By using molecular approaches, Wong et al.[16]

reported an importance of *ASN2* function in the stress-induced ammonium reassimilation. However, few data are available in distinguishing *ASN* functions in response to salt stress.

In the present study, *ASN2* knockout mutant was used to assess a role of *ASN2* in the regulation of ammonium metabolism under NaCl stress.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana wild-type Col0 and two T-DNA mutants disrupted in *ASN2* were used for the experiments. *Arabidopsis thaliana* (Col0) with T-DNA insertion into intron 3 (SALK_43167) was obtained from the Not-tingham Arabidopsis stock centre (Nottingham, UK), homozygous knockout line (*asn2-1* mutant) and (*asn2-d*)-deficient mutant, a T-DNA mutant disrupted partially in *ASN2* function were characterized[17]. Seeds were sterilized and stratified at 4°C for 4 days. Plants were grown under hydroponic culture in a growth chamber with an 8-h-light/16-h-dark cycle and 80% relative humidity. Plants were supplied with basic nutrient medium containing 8 mM KNO_3, 1 mM $MgSO_4$, 1mM KH_2PO_4, 2mM $CaNO_3$, 5µM $MnSO_4$, 30µM H_3BO_3, 1µM $ZnSO_4$, 1µM $CuSO_4$, and 30µM K- iron-EDTA. Plants were cultivated on control medium during four weeks (12mM). A group of plants were maintained in high nitrate medium with +/- 100mM NaCl (HN). Another group was transferred on low nitrate medium (0,2mM nitrate) with +/- 100mM NaCl (LN) during 6 hours.

Ammonium contents

Ammonium was extracted from plant material at 4°C with 0.3 mM H_2SO_4 and 0.5 % (w/v) polyclar AT. Ammonium content was quantified according to the reaction of Berthelot modified by Weatherburn[18].

Protein content

Soluble protein content was quantified using Coomassie Brilliant Blue[19] with bovine serum albumin as a protein standard.

Chlorophyll determination

Chlorophyll was determined by the method of Arnon[20]. The absorbance of each sample was read at 460, 645 and 663 nm, after centrifugation.

Enzyme assays

Glutamine synthetase

Frozen samples were homogenized in a cold mortar and pestle with grinding medium containing 25mM Tris-HCl buffer (pH 7.6), 1mM $MgCl_2$, 1mM EDTA, 14mM 2-mercaptoethanol and 1% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 25,000 g for 30 min at 4°C. GS activity was determined using hydroxylamine as substrate, and the formation of γ-glutamylhydroxamate (γ-GHM) was quantified with acidified ferric chloride[21].

Glutamate dehydrogenase

GDH extraction was performed according to the method described by Magalhaes and Huber[22]. Frozen samples were homogenized in a cold mortar and pestle with 100mM Tris-HCl (pH 7.5), 14mM 2-mercaptoethanol, and 1% (w/v) PVP. The extract was centrifuged at 12,000 g for 15 min at 4°C. GDH activity was determined by following the absorbance changes at 340 nm.

Western blot analysis

Proteins were extracted from frozen leaf material in cold extraction buffer containing 50mM Tris-HCl (pH 7.5), 1mM EDTA, 1mM MgCl2, 0.5% (w/v) PVP, 0.1% (v/v) 2-mercaptoethanol and 4mM leupeptine and separated by SDS-polyacrylamide gel electrophoresis (PAGE)[23]. Equal amounts of protein (40µg) were loaded in each track. The percentage of polyacrylamide in the running gels was 12%. Proteins were electrophoretically transferred to nitrocellulose membranes for western blot analysis. Polypeptide detection was done using polyclonal antiserum raised against a synthetic polypeptide common to GS1 and GS2 of *Arabidopsis thaliana*.

Detection of GDH activity on gel

Leaf and root soluble proteins were extracted from frozen material in cold extraction buffer containing 100mM Tricine, 1mM EDTA, 40mM $CaCl_2$, 0,5% (w/v) PVP, 0.1% (v/v) 2-mercaptoethanol and 1mM AEBSF ((4-(2-AminoEthyl)-Benzenesulfonylfluoride). The protein separation was carried out in 1 mm-thick non-denaturing gel as above. Equal amount of protein (40µg for root and 60µg for leaf) was loaded in each track. Native PAGE of the partially purified GDH extracts was performed by the method of Davis[24]on 5% running gel with 4% stacking gel. The buffer system was 100mM Tris-Glycine adjusted to pH 8 with HCl. Running was at 4°C, 120V for about 2 h and bands containing GDH activity were visualized with the tetrazolium system. The staining solution contained 150mM Tris-HCl (pH 8.8), 50mM glutamate, 0.5mM NAD^+, 0.5mM NBT and phenazine.

Statistical analysis

The data are presented in the figures and in the tables as the average of at least six replicates per treatment and means ± confidence limits at P = 0.05 level.

RESULTS

Chlorophyll contents

In order to determine whether salt-stress results in a phenotype of *Arabidopsis thaliana* lines, we first determined chlorophyll contents. In the different line and under high nitrate concentration, 6h of salt treatment reduced+/- slightly total chlorophyll (Chl) content mainly in (*ASN2)-deficient* mutant. The plant transfer

on low nitrate medium (LN) upon 6 hours, induced Chl content increase in WT. In *asn2-d* mutant, Chl content decreased strongly at low nitrate medium (36%). In asn2-1 leaves, the reduction of nitrate level in culture medium had no significant effect on Chl content.

In LN medium, salt addition had same effects of nitrate level reduction in WT and *asn2d.* In *asn2-1* mutant, salt addition on LN medium decreased strongly Chl content (44%).

Soluble protein and ammonium contents

After 6h of salt treatment on control medium (HL), soluble protein (SP) content was found to decrease in wild-type leaves and in *asn2-1* leaves (Fig. 1A). In (*ASN2)-deficient* mutant, salt treatment on HL medium induced SP content (14%). The plant transfer on LN medium had no significant effect on SP content in WT and two *ASN2* mutants. The salt stress application on LN medium induced SP accumulation in WT (15%).Under salt stress on LN medium, SP content decreased in (*ASN2*)-deficient mutant (12%) and mostly in *ans2-1* mutant leaves (19%).

Salt stress application on HL medium had no effect onNH4+ content in WT leaves (Fig. 1B). While, in (*ASN2)*-deficient mutant and *asn2-1*mutant, NH4+ content increased respectively with 19% and 15%. The plant transfer on LN medium upon 6 hours had no significant effect in WT leaves but slightly induced NH4+ content in (*ASN2*)-deficient mutant (7%) . In *asn2-1* mutant, NH4+ content decreased (12%) (Fig. 1B). Salt treatment addition on LN medium upon 6 hours had no significant effect in WT leaves. The salt treatment on LN medium induced NH4+ accumulation in (*ASN2*)-deficient mutant (9%) and mostly in asn2-1 mutant (30%).

Salt effects on ammonium-assimilating enzymes

Glutamine synthetase activity and enzyme protein level

The lack and the deficiency of *ASN2* mRNA had no significant effect on glutamine synthetase (GS) activity in the leaves under the control condition without NaCl (Fig.2A). In HL medium, the inhibitory effect of salt on GS activity in the leaves appeared 6h after a stress application to both the wild-type and *asn2-1* (Fig. 2A). In (*ASN2*) deficient mutant, salt addition on HL medium induced GS activity with 12% with refer to control. The GS activity induction was due to the GS1 protein accumulation detected by western blot in *asn2-d* mutant (Fig.2B).

The plant transfer on LN medium induced GS activity with 15% and 8% respectively in WT and asn2-d with refer to control. In *asn2-1* mutant, the reduction of nitrate concentration in culture medium

upon 6 hours had no significant effect on GS activity although the GS2 protein accumulation detected in leaves (Fig.2B). The salt addition on LN medium induced GS activity in WT and *asn2-d* mutant leaves with 15% referring to controls. Western blot analysis showed that GS activity induction was due primarily to plastidic GS2 accumulation (Fig. 2B). In *asn2-1* mutant, salt addition on LN medium reduced GS activity with 27% referring to control. In *asn2-1* mutant, The GS1 protein decrease induced the GS activity reduction under salt treatment on LN medium.

Glutamate dehydrogenase activity

NaCl stress on HN medium induced aminating GDH activity (NADH-GDH) with 9%, 14% and 16% respectively in WT, *asn2-d* and *asn2-1* with refer to control (Fig. 3A). The reduction of nitrate concentration in the culture medium stimulated slightly NADH-GDH activity in WT (14%) and in *asn2-d* mutant (6%). The plant transfer on LN medium upon 6 hours had no significant effect on NADH-GDH activity in *asn2-1* mutant (Fig. 3A). The salt addition on LN medium upon 6 hours stimulated NADH-GDH activity in WT (16%) and mostly in *asn2-1* mutant (27%). The salt treatment on LN medium had no effect on NADH-GDH activity in *asn2-d* mutant.

Glutamate dehydrogenase subunit patterns

The revelation of GDH activity on polyacrylamide gel showed the presence of 7 GDH subunit patterns in the leaves formed by the association of six of two different subunits α and β (Fig. 3B). The basal GDH band was formed by six subunits α, while the apical GDH band was formed by only six subunit β.

The detection of the GDH activity on gel revealed a slight decrease of GDH subunits by salt stress in the wild-type and *asn2-1* mutant leaves. In *asn2-d* mutant, 6 hours of salt treatment on HL medium had no effect on GDH subunits. The plant transfer on LN medium had no effect on GDH pattern in WT and *asn2-d* mutant but a slight decrease was detected in *asn2-1* mutant leaves.

The salt addition on LN medium upon 6 hours reduced all GDH subunits intensities in WT and mostly in *asn2-1* mutant. In *asn2-d* mutant, salt treatment on LN medium had no apparent effect on GDH subunits (Fig. 3B).

Table 1: NaCl effects on total chlorophyll level (mg/g FW) in wild-type (WT), (ASN2)-deficient mutant *asn2-d* and knock-out *asn2-1* mutants. Plants were cultivated on control medium during four weeks with high nitrate level (12mM). A group of plants were maintained in high nitrate medium with +/- 100mM NaCl (C and C+S). Another group was transferred on low nitrate medium (0,2mM nitrate) with +/- 100mM NaCl (C-N and C-N+S). Data are means of six replicates ± CL at 0.05

		C	C+S	C-N	C-N+S
Chl (mg/g FW)	WT	1.55± 0,03	1.39± 0,005	1.86± 0,03	1.81± 0,07
	Asn2-d	1.73± 0,045	1.18± 0,064	1.28± 0,108	1.29± 0,002
	Asn2-1	1.85± 0,05	1.82± 0,11	1.85± 0,045	1.23± 0,04

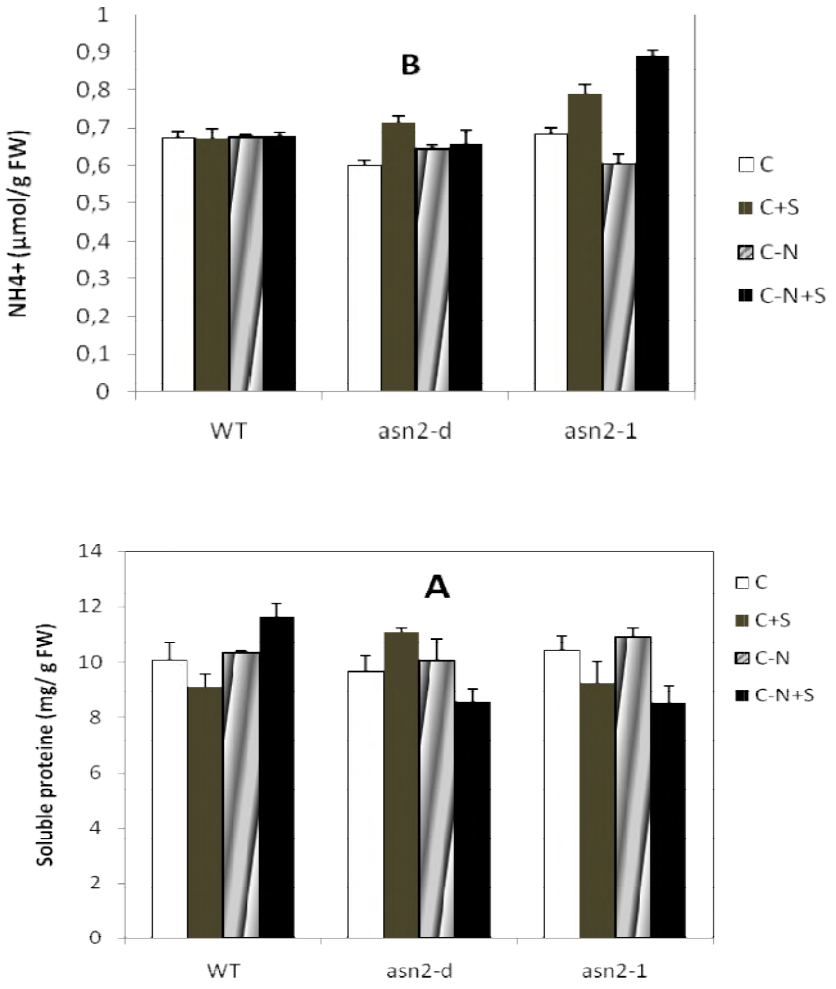
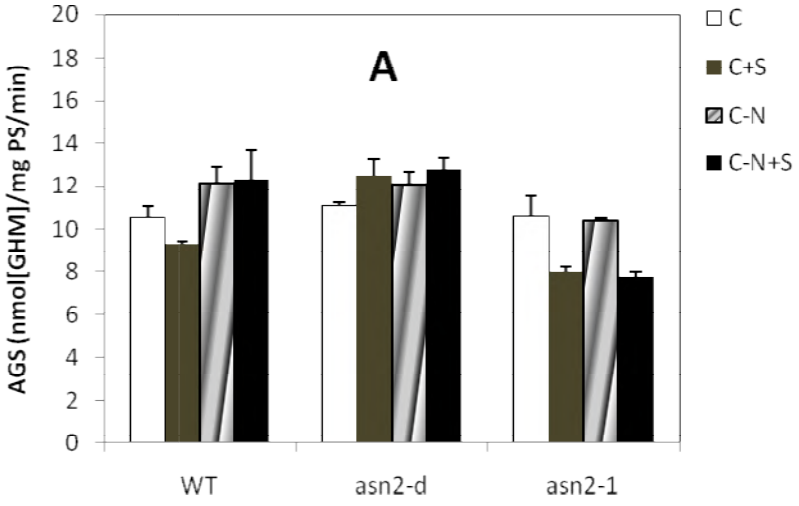


Fig. 1: NaCl effects on soluble protein levels in leaves (A) (mg/FW) and endogenous free ammonium contents (B) (µmol/g FW) in the wild-type (WT), *asn2-d* mutant and asn2-*1* mutant (*asn2-1*). Plants were cultivated on control medium during four weeks with high nitrate level (12mM). A group of plants were maintained in high nitrate medium with +/- 100mM NaCl (C and C+S). Another group was transferred on low nitrate medium (0,2mM nitrate) with +/- 100mM NaCl (C-N and C-N+S). Data are means of six replicates ± CL at 0.05 levels



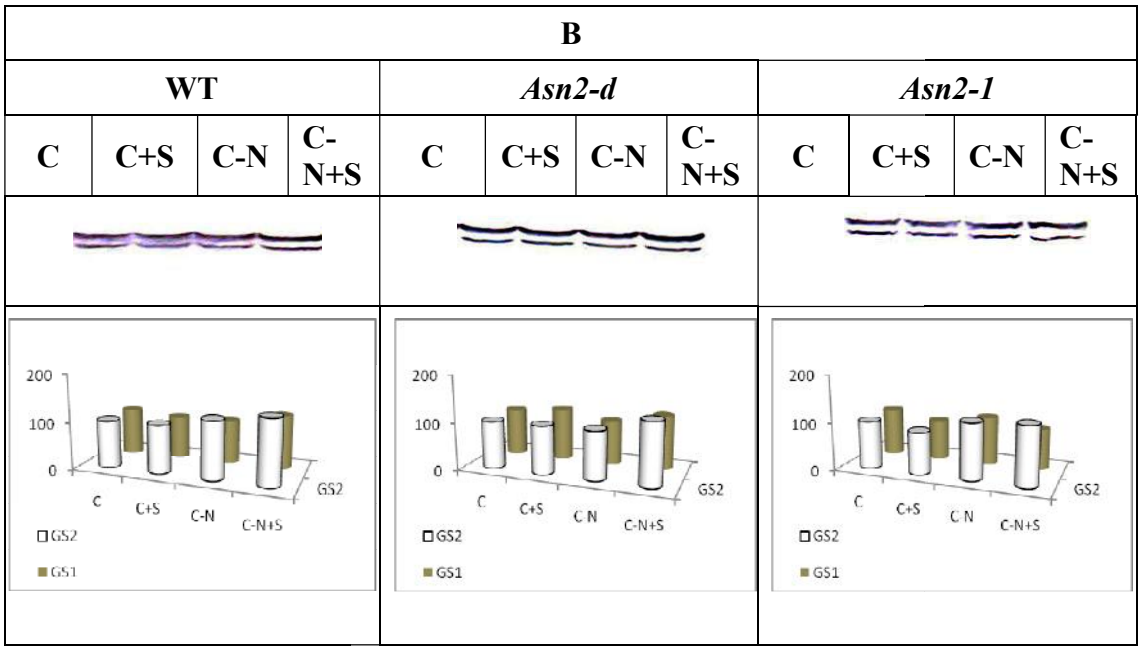
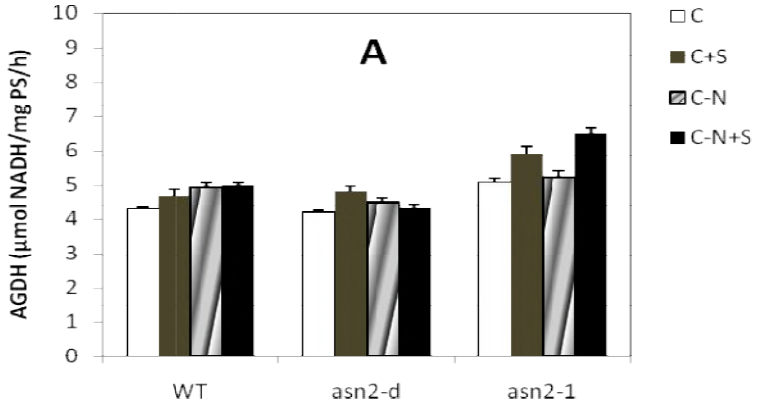


Figure 2: NaCl effects (100mM) on GS activity (nmol *γ*-glutamylhydroxamate (GHM) mg^{-1} soluble protein min^{-1}) (A) and GS protein (B), in the wild-type (WT), *asn2-d* and *asn2-1* mutants in response to 6h of salt addition. Plants were cultivated on control medium during four weeks with high nitrate level (12mM). A group of plants were maintained in high nitrate medium with +/- 100mM NaCl (C and C+S). Another group was transferred on low nitrate medium (0,2mM nitrate) with +/- 100mM NaCl (C-N and C-N+S). Data are means of six replicates ± CL at 0.05 levels. Western blot analysis was carried out from the wild-type (WT), *asn2-d* and *asn2-1* mutant (*asn2-1*) in response to 6h salt treatment. An aliquot of 40 µg of total protein from leaves of each line was loaded. Total proteins were extracted as described in "Materials and Methods". Bands were quantified with "Quantity one" program from BioRad. Histograms of band quantification were expressed as an arbitrary unit.



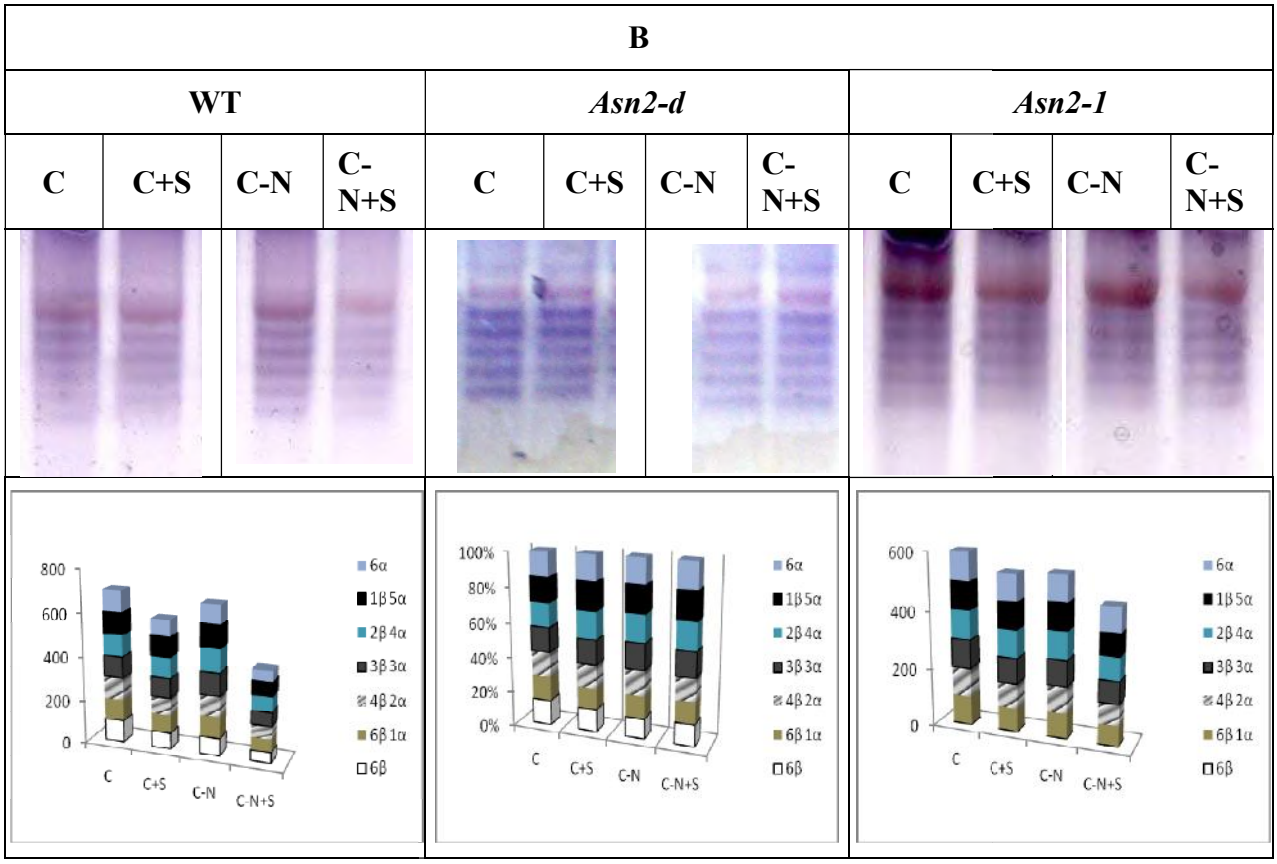


Figure 3: Effects of NaCl treatment (100mM) on NADH-GDH activity (µmol NADH oxidized mg^{-1} soluble protein h^{-1}) (A) and NADH-GDH activity on native polyacrylamide gel (zymogram) (B) in leaves of the wild-type (WT), *asn2-d* and *asn2-1* mutants. Data are means of six replicates ± CL at 0.05 levels. Electrophoresis analysis was carried out from the wild-type (WT) and *asn2* mutants. An aliquot of 60 µg of total protein from leaves of each line was loaded. Plants were cultivated on control medium during four weeks with high nitrate level (12mM). A group of plants were maintained in high nitrate medium with +/- 100mM NaCl (C and C+S). Another group was transferred on low nitrate medium (0,2mM nitrate) with +/- 100mM NaCl (C-N and C-N+S). Total proteins were extracted as described in "Materials and Methods". Bands were quantified with "Quantity one" program from BioRad. Histograms of band quantification were expressed as an arbitrary unit.

DISCUSSION

Salinity up to several days results in an inhibited growth phenotype of glycophytes such as *Arabidopsis thaliana*[25-26], tomato seedlings[4], soya bean[5]and sweet sorghum[27]. When the wild-type *Arabidopsis thaliana* was subjected to a salt treatment for 24h, the soluble protein contents inleaves were reduced, while root soluble protein contents remained constant (Fig. 1). We did not observe a reduction in the contents of chlorophyll a and b in the wild-type leaves (Table1). By contrast, the *asn2-1* leaves displayed a phenotype of highly reduced soluble protein contents and chlorophyll contents after 24h of salt stress (Table 1). Decrease in soluble protein contents by salt treatment as observed in *asn2-1* leaves is reported for many plant species[28-30]. Glycophytes such as chickpea and tomato seedlings develop a chlorosis and a fall in chlorophyll contents following a prolonged salt stress[4, 28, 29, 31-32].Diminution in the soluble protein and chlorophyll contents may reflect low nitrogen assimilation and/or proteolytic activity, which is induced by salinity[4].It is assumed that the salt stressed *asn2-1* leaves were affected in the nitrogen assimilation and translocation.

Asparagine rapidly accumulates in response to salt treatment[33-35]and it serves as ammonium detoxification amino acid in plants[36]. *ASN2* is the most highly expressed *ASN* gene in the wild-type leaves (Fig. 2). This suggests that *ASN2* expression controls ammonium assimilation in response to salt stress. *ASN2* mRNA levels were slightly increased under salinity in the wild-type leaves, where there was no ammonium accumulation (Figs. 1C, 2A) as described previously in the salt-treated *Arabidopsis thaliana*[16]. By contrast, *in silico* data showed that salt treatment down-regulated *ASN2* mRNA level within 6h in the wild-type seedlings (Arabidopsis eFP browser: http://bbc.botany.utoronto.ca/efp). As inorganic nitrogen (nitrate/ammonium) down-regulates *ASN2* expression[37], a slight induction of *ASN2* mRNA may be mediated by other metabolites such as amino acids (asparagine, glutamine and glutamate) [38].

We examined whether ammonium assimilation was modified under the salt stress in the absence of *A SN2* expression in the *asn2-1* mutant. *ASN1* mRNA levels were induced by salinity in the *asn2-1* leaves as well as in the wild-type leaves (Fig. 2). More pronounced increase of *ASN1* mRNA level in the *asn2-1* leaves than in the wild-type leaves (Fig. 2) indicates that *ASN1* compensates *ASN2* for its function in the *asn2-1* line. The increase of *ASN* mRNA level was also observed under abiotic and biotic stress[39-41]. While, several results from databases showed that salt stress for 6h represses *ASN1* mRNA

expression together with *ASN3* mRNA levels (Arabidopsis eFP browser: http://bbc.botany.utoronto.ca/efp). Consistently, *ASN1* mRNA levels in the roots of wild-type and *asn2-1* lines were repressed by the salt treatment (Fig. 2).

Ammonium is rapidly assimilated by the reaction of glutamine synthetase. The salt stress repressed *GLN2* expression in the leaves of the *asn2-1* mutant as well as the wild-type line (Fig. 3). This is in agreement with databases showing that *GLN2* expression is inhibited after 6h of salt treatment (eFP Browser, http://bbc.botany.utoronto.ca/efp). Low *GLN2* mRNA levels were correlated with low levels of chloroplastic GS2 protein in the leaves of the *asn2-1* and wild-type lines (Fig. 3). However, salinity did not affect the GS2 protein levels in the roots where cytosolic GS1 proteins are the major isoforms (Fig. 4). Among five *GLN1* genes for cytosolic GS1, we measured two *GLN1* genes and higher expression of *GLN1:2* was detected in the leaves and roots (Fig. 3). The salt treatment did not reduce *GLN1:2* mRNA levels in the leaves but down-regulated in the roots of wild-type and *asn2-1* lines (Fig. 3). This repression pattern confirms the results of databases from 18-days old *Arabidopsis thaliana* seedlings (Arabidopsis eFP browser: http://bbc.botany.utoronto.ca/efp). In contrast, it is reported that *GLN1* mRNA levels in soybean roots were induced by salt stress together with the GS1 protein[42].

Activity of GS to assimilate ammonium was evaluated in the *asn2-1* mutant under salt stress. Total GS activity was found to be inhibited by the salt treatment in the leaves of wild-type and *asn2-1* lines. This likely reflects a down-regulation of *GLN2* mRNA and GS2 protein, which is the major GS isoform in leaves (Figs. 2, 3, 4). Also, the decrease in GS protein may result from an enhancement of proteolytic activity, which affects GS protein stability and half-life[4,7]. Moreover, salt stress is accompanied by oxidative stress, which may also have an effect on GS2 activity *via* amino acid redox change[43]. Total GS activity in the roots under salinity was also found to be inhibited in both *asn2-1* wild-type and lines (Fig. 4).

It has been suggested that aminating GDH activity catalyzes an alternative ammonium assimilating pathway when GS activity is impaired by abiotic stress[3, 44]. Expression of two genes encoding the two subunits α and β of NADH-GDH is regulated by metabolites and environmental stress[45-47]. NADH-GDH is formed by seven bands following PAGE[48]. The 7 NADH-GDH bands were detected in grapevine shoot callus[49], while in triticale seedlings NAD-GDH activity revealed on gel showed

only one band[50]. In the wild-type leaves and roots, α-enriched NADH-GDH bands were accumulated under salinity (Fig. 5). This may be associated with an increase in aminating GDH activity in the wild-type roots under salinity (Fig. 5), while enhancement of GDH activity in the leaves was not detected (Fig. 5). The stimulation of aminating GDH activity is thought to improve salt resistance[3]. However, α-enriched GDH bands from the *asn2-1* leaves and roots under salinity remained low, indicating that there was no significant contribution of GDH aminating activity in the *asn2-1* mutant.

Ultimately, we concluded that *asn2-1* mutant was more sensitive to salt stress than the wild-type line. Despite a salt-induced *ASN1* mRNA levels in the *asn2-1* leaves, the *asn2-1* mutant showed a phenotype related to inhibited use of nitrogen. It remains to elucidate whether inhibition of asparagine synthesis by *ASN2*-encoded asparagine synthetase affected assimilation and transport for use of nitrogen in the *asn2-1* mutant.

ACKNOWLEDGEMENTS

H. Maaroufi Dguimi thanks the UTIQUE (CMCU) bilateral program for a doctoral fellowship. The authors thank Dr C. Masclaux-Daubresse for providing GS antibody.

REFERENCES

[1] Khan MH, Panda SK (2008) Alterations in root lipid peroxidation and antioxidative responses in two rice cultivars under NaCl-salinity stress. Acta Physiol Plant 30: 91-89.doi: 10.1007/s11738-007-0093-7

[2] Zhu JK (2001) Plant salt tolerance. Trends Plant Sci 6: 66–71.

[3] Gulati A, Jawail PK (1996) Effect of NaCl on nitrate reductase, glutamate dehydrogenase and glutamate synthase in *Vigna radiate calli.* Biol Plant 38: 177-183. doi: 10.1007/BF02873842

[4] Debouba M, Maâroufi-Dghimi H,Suzuki A,Ghorbel MH,Gouia H(2007) Changes in growth and activity of enzymes involved in nitrate reduction and ammonium assimilation in tomato seedlings in response to NaCl stress. Annals of Botany 99: 1143-1151.doi: 10.1093/aob/mcm050

[5] Çiçek N and Çakirlar H (2008) effect of salt stress on some physiological and photosynthetic parameters at three different temperatures in six Soya Bean (*Glycine max* L. Merr.) cultivars. J Agr Crop Sci 194: 34-46.doi: 10.1111/j.1439-037X.2007.00288.x

[6] James F, Brouquisse R, Pradet A,

Raymond P (1993) Changes in proteolytic activities in glucose-starved maize root tips. Regulation by sugars. Plant Physiol Biochem 31: 845-856.

[7] Silveira JAG, Viégas R, Rocha IMA, Moreira A, Moreira R, Oliveira J (2003) Proline accumulation and glutamine synthetase activity are increased by salt-induced proteolysis in cashew leaves. J. Plant Physiol 160: 115–123.doi:10.1078/0176-1617-00890

[8] Hartzendorf T, Rolletschek H (2001) Effects of NaCl-salinity on amino acid and carbohydrate contents of *Phragmites australis*. Aquatic Bot 69 (264): 195-208. doi:10.1016/S0304-3770(01)00138-3

[9] Papadakis AK, Stephanou EG, Roubelakis-Angelakis KA (2006) Abiotic Stress Generates ROS That Signal Expression of Anionic Glutamate Dehydrogenases to Form Glutamate for Proline Synthesis in Tobacco and Grapevine. The Plant Cell 18: 2767-2781.

[10] Stewart GR, Larher F (1980) Accumulation of amino acids and related compounds in relation to environmental stress, in: P.K. Stumpf, E.E. Conn (Eds.). The Biochemistry of Plants, Academic Press, New York, USA, 609-635.

[11] Rabe E (1990) Stress physiology: the functional significance of the accumulation of nitrogen-containing compounds. J Hortic Sci 65: 231-243.

[12] Chaffei C, Pageau K, Suzuki A, Gouia H, Ghorbel MH, Masclaux-Daubresse C (2004) Cadmium toxicity induced changes in nitrogen management in *Lycopersicon esculentum* leading to a metabolic safeguard through an amino acid storage strategy. Plant Cell Physiol 45:1681-93.doi: 10.1093/pcp/pch192

[13] Lam HM, Coschigano K, Schultz C, Melo-Oliveira R, Tjaden G, Oliveira I, Ngai N, Hsieh MH and Coruzzi G (1995) Use of *Arabidopsis* mutants and genes to study amino acid biosynthesis. Plant Cell 7: 887-898.

[14] Huber TA and Streeter JG (1985) Purification and properties of asparagines synthetase from soya bean root nodules. Plant Sci 42: 9-17.

[15] Joy KW, Ireland RJ, Lea PJ (1983) Asparagine synthesis in pea leaves, and the occurrence of an asparagine synthetase inhibitor. Plant Physiol 73: 165–168.

[16] Wong HK, Chan HK, Coruzzi GM, Lam HM (2004) Correlation of *ASN2* gene expression with ammonium metabolism in *Arabidopsis*. Plant Physiol134: 332-338.

[17] Gaufichon L (2009) Functional characterization of the genes encoding asparagine synthetase in *Arabidopsis thaliana*: role in ammonium assimilation and nitrogen metabolism. Thesis, University of Paris XI.

[18] Weatherburn MW (1967) Phenol hypochlorite reaction for the determination of ammonia. Anal Chem 39: 971-974.

[19] Bradford MM (1976) A rapid and sensitive method for the quantitative determination of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

[20] Arnon DI (1949) Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1-15.

[21] Wallsgrove RM, Lea PJ, Miflin BJ (1979) Distribution of the enzymes of nitrogen assimilation within the pea leaf cell. Plant Physiol 63: 232–236.

[22] Magalhaes JR, Huber DM (1991) Free ammonia, free amino acid and enzyme activity in maize tissue treated with Methionine sulfoximine. J Plant Nutr 14: 883-895.doi: 10.1080/01904169109364249

[23] Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature227: 680-685.

[24] Davis BJ (1964) Disc electrophoresis II. Method and application to human serum proteins. An n N Y Acad Sci 121: 404-427.

[25] Attia H, Karray N, Rabhi M, Lachaâl M (2008) Salt-imposed restrictions on the uptake of macroelements by roots of *Arabidopsis thaliana*. Acta Physiol Plant 30: 723-727.doi: 10.1007/s11738-008-0172-4

[26] Szekely G, Abraham E, Cseplo A, Rigo G, Zsigmond L, Csiszar J, Ayaydin F, Strizhov N, Jasik J, Schmelzer E, Koncz C, Szabados L (2008) Duplicated P5CS genes of *Arabidopsis* play distinct roles in stress regulation and developmental control of proline

biosynthesis. Plant J 53: 11-28.doi: 10.1111/j.1365-313X.2007.03318.x

[27] Almodares A, Hadi MR, Dosti B (2008) the effects of salt stress on growth parameters and carbohydrates contents in sweet sorghum. Res J Environ Sci 2 (4): 298-304.doi: 10.3923/rjes.2008.298.304

[28] Gadallah MAA (1999) Effects of proline and g lycine betaine on *Vica faba* responses to salt stress. Biol Plant 42: 249-257.doi: 10.1023/A:1002164719609

[29] Agastian P, Kingsley SJ, Vivekanandan M (2000) Effect of salinity on photosynthesis and biochemical characteristics in mulberry genotypes. Photosynthetica 38: 287–290.doi: 10.1023/A:1007266932623

[30] Parida A, Das AB, Das P (2002) NaCl stress causes changes in photosynthetic pigments, proteins and other metabolic components in the leaves of a true mangrove, *Bruguiera parviflora*, in hydroponic cultures. J Plant Biol 45: 28–36.doi: 10.1007/BF03030429

[31] Hernandez JA , Olmos E, Corpas FJ, Sevilla F, del Rio LA (1995) Salt-induced oxidative stress in chloroplasts of pea plants. Plant Sci 105: 151–167.doi:10.1016/0168-9452(94)04047-8

[32] Soussi M, Ocana A, Linch C (1998) Effect of salt stress on growth, photosynthesis and nitrogen fixation in chickpea (*Cicer arietinum* L.). J of Exp Bot 49: 1329-1337.doi: 10.1093/jxb/49.325.1329

[33] Moller MG, Taylor C, Rasmussen SK, Holm PB (2003) Molecular cloning and characterisation of two genes encoding asparagine synthetase in barley (*Hordeum vulgare* L.). Biochem Biophys Acta 1628: 123–32.

[34] Igarashi D, Ishizaki T, Totsuka K, Ohsumi C (2009) *ASN2* is a key enzyme in asparagine biosynthesis under ammonium sufficient conditions.Plant Biotechnol26: 153–159.

[35] Beato VM, Rexach J, Navarro-Gochicoa MT, Camacho-Cristobal JJ, Herrera-Rodriguez MB, Maldonado JM, Gonzalez-Fontes A (2010) A tobacco asparagine synthetase gene responds to carbon and nitrogen status and its root expression is affected under boron stress. Plant Sci 178(3): 289-298. doi:10.1016/j.plantsci.2009.12.008

[36] Lea PJ, Sodek L, Parry M, Shewry PR, Halford NG (2007) Asparagine in plants. Anal Apply Biol 150: 1–26. doi: 10.1111/j.1744-7348.2006.00104.x

[37] Lam HM, Hsieh MH, Coruzzi G (1998) Reciprocal regulation of distinct asparagine synthetase genes by light and metabolites in *Arabidopsis thaliana*. Plant J 16: 345–353.doi: 10.1046/j.1365-313x.1998.00302.x

[38] Gaufichon L, Reisdorf M, Rothstein SJ, Chardon F, Suzuki A (2010) Biological functions of asparagine synthetase in plants. Plant Sci 129: 141-153. doi:10.1016/j.plantsci.2010.04.010

[39] Olea F, Pérez-García A, Cantón FR, Rivera ME, Cañas R, Ávila C, Cazorla FM, Cánovas FM, de Vicente A (2004) Up-regulation and localization of asparagine synthetase in tomato leaves infected by the bacterial pathogen *Pseudomonas syringae*. Plant Cell Physiol 4: 770-780.doi: 10.1093/pcp/pch092

[40] Wong H, Liu D, Sun J, Zhang A (2005) Asparagine synthetase gene *ASN1* from wheat is up-regulated by salt stress, osmotic stress and ABA. J Plant Physiol 162: 81-89. doi:10.1016/j.jplph.2004.07.006

[41] Herrera-Rodriguez MB, Vicente RP, Maldonado JM (2007) Expression of asparagine synthetase genes in sunflower (*Helianthus annuus*) under various environmental stresses. Plant Physiol Biochem 45: 33-38. doi:10.1016/j.plaphy.2006.12.002

[42] Zilli CG, Balestrasse KB, Yannarelli GG, Polizio AH, Santa-Cruz DM, Tomaro ML (2008). Heme oxygenase up-regulation under salt stress protects nitrogen metabolism in nodules of soybean plants.Envir Exp Bot. 64: 83–89. doi:10.1016/j.envexpbot.2008.03.005

[43] Choi YA, Kim SG, Kxon YM (1999) the plastidic glutamine synthetase activity is directly modulated by means of redox changes at two unique cysteine residues. Plant Sci 149: 175-182. doi:10.1016/S0168-9452(99)00163-6

[44] Mâaroufi H, Debouba M, Ghorbel MH, Gouia H (2009) Tissue-specific cadmium accumulation and its effects on nitrogen metabolism in tobacco (*Nicotiana tabaccum*, Bureley v. Fb9). C R Biologies 332: 58-68.doi :

10.1016/j.crvi.2008.08.021

[45] Masclaux C, Valadier MH, Brugière N, Morot-Gaudry JF, Hirel B (2000) Charaterisation of sink/source transition tobacco (*Nicotiana tobaccum* L.) shoots in relation to nitrogen management and leaf senescence. Planta 211: 510-518.

[46] Britto DT, Kronzucker HJ (2002) NH_4^+ toxicity in higher plants: a critical review. J Plant Physiol 159: 567-584.doi:10.1078/0176-1617-0774

[47] Chafei C, Gouia H, Ghorbel MH (2003) Nitrogen metabolism of tomato under cadmium stress conditions. J.Plant Nutri 26: 1617-1634.doi: 10.1081/PLN-120022372

[48] Fontaine J, Saladino F, Agrimonti C, Bedu M, Laforgue T, Tétu T, Hirel B, Restivo FM, Dubois F (2006) Control of the synthesis and subcellular targeting of the two GDH genes products in leaves and stems of *Nicotiana plumbaginifolia* and *Arabidopsis thaliana*. Plant Cell Physiol 47: 410-418. doi: 10.1093/pcp/pcj008

[49] Loulakakis AK, Roubelakis-Angelakis KA (1991) Plant NAD (H)-glutamate dehydrogenase consists to two subunits polypeptides and their participation in the seven isoenzymes occurs in an ordered ratio. Plant Physiol 97: 104-111.

[50] Kwinta J, Cal K (2005). Effects of salinity stress on the activity of glutamine synthetase and glutamate dehydrogenase in triticale seedlings. Pol J Environ Studies 14: 125-130.