Acta Physiol Plant (2011) 33:2289–2299
DOI 10.1007/s11738-011-0769-x

ORIGINAL PAPER

Case study of a biological control: *Geobacillus caldoxylosilyticus* (IRD) contributes to alleviate salt stress in maize (*Zea mays* L.) plants

Amal Fadl Abdelkader · Mona Abdeltawab Esawy

Received: 2 April 2010 / Revised: 7 April 2011 / Accepted: 11 April 2011 / Published online: 25 May 2011

Abstract The inevitable exposure of crop plants to salt stress is a major environmental problem emerged from the presence of excess NaCl radicals in the soil. Handling the problem in maize plants using a biological agent was the main interest of the present study. The non-pathogenic, halophytic, facultative aerobic bacterium *Geobacillus caldoxylosilyticus* IRD that was isolated from Marakopara pond in the Atoll Tikehau (French Polynesian, 2005) and found tolerant to salt stress until 3.5% NaCl (w/v). An artificial symbiosis was achieved by inoculating *Geobacillus* sp. into 5-day-old maize cultivars of triple hybrids (321 and 310) and singlet hybrids (10 and 162). Thereafter, maize seedlings were exposed to 350 mmol NaCl for 10 days. The data revealed that *Geobacillus* sp. had interacted with salinized maize and improved maize overall growth, dry weight and relative water content. Na^+ accumulation was six times less and Cl^- accumulation was 13 times less in the tips of salinized maize seedlings upon *Geobacillus* sp. inoculation. Salinized maize without *Geobacillus* viewed decayed cortical cells of seedlings. In addition, proline content was two times higher in salinized seedlings lacking *Geobacillus*. Photosynthetic pigments and antioxidant enzymes were significantly regulated upon inoculation. Beyond this study, we presented a novel insight into a possible role of *Geobacillus caldoxylosilyticus* bacteria in controlling/protecting maize plants against high salt stress.

Keywords Chloride · *Geobacillus caldoxylosilyticus* · Maize · Potassium · Proline · Relative water content · Salt stress · Sodium

Communicated by B. Barna.

A. F. Abdelkader
Department of Botany, Faculty of Science,
Ain Shams University, Abbassia, Cairo 11566, Egypt

M. A. Esawy
Department of Chemistry of Natural and Microbial Products,
National Research Center, Dokki, Giza 12622, Egypt

A. F. Abdelkader (✉)
Department of Plant Science, Faculty of Science,
Ain Shams University, Abbassia, Cairo 11566, Egypt
e-mail: amal.abdelkader@yahoo.com

Introduction

Soil contamination with high salts radicals is a fundamental threat to agriculture (Kijne 2006). Maize (*Zea mays* L.) is the principal food for several nations and recently, maize is been industrialized to become green source and strategic commodity of biofuel in number of countries worldwide. Maize breeders categorized maize as a sensitive crop to salinity and other stressors (Katerji et al. 1996). Thus, maize production and propagation should be highly maintained for its high economical values.

Salinity-mediated suppression of plants growth was related basically to ionic disturbance. Therefore, salt tolerance is strongly connected to the ability of plants to re-establish ionic homeostasis (Hasegawa et al. 2000; Zhu 2000, 2001). Plants could acclimate to varying degrees the salt stress by various mechanisms. Salinity also alters numerous physiological and molecular pathways in plants (Munns 2002). Resent studies highlighted the particular sensitivity of maize to Na^+ and the mechanisms exploited by tolerant maize plants to avoid salt stress. That was mostly based on the exclusion of excess Na^+ of from the photosynthetic apparatus of young leaves (Fortmeier and Schubert 2006) via the Na^+/H^+ antiport activity which is

extremely low in salt-sensitive crops (Mahajan and Tuteja 2005).

The halophytes possess a defined physiological and structural appearance that facilitates their adaptation to the extreme salty environment; they also implicated number of tolerance strategies. For example, they are capable for reducing the osmotic stress through fluxing out the inorganic ions from the cell (Hasegawa et al. 2000).

Varied impacts of salt stress on plants could be detected using an indicator such as proline, which was yet been discovered in plants tissues under the unfavorable conditions, such as drought, salinity, extreme temperatures, and even infeasible light intensity (Aspinall and Paleg 1981; Mansour 2000). Proline has, therefore, been identified as global stress indicator (Chen et al. 2001; Claussen 2005; Gadallah 1993; Gzik 1996; Monreal et al. 2007; Rai et al. 2004). Proline stabilizes macromolecules and organelles, such as membranes and also maintains proteins and protein complexes (Bohnert and Shen 1999; Bray et al. 2000). In addition, proline monitors the cytosolic pH and detoxifies the excess NH_4^+ (Gilbert et al. 1998).

Symbiosis is a dual beneficial plant–microbial relationship on the biosphere within which a microorganism is associated with specific plant parts. Some 65 years, scientists practiced artificial inoculation of microbial cells to control plants different problems. For instance, growing bacteria in the rhizosphere of some plants had manipulated soil pathogen (Weller 1988). Furthermore, plant growth was enhanced when soil bacteria were introduced to roots (Chanway 1997).

Metabolic substances, such as photosynthetic pigments (chlorophyll $a + b$ and carotenoids) are indicators for the biomass and the capacity of photosynthesis, their concentrations also reflect the physiological status of plants. Photosynthetic pigments are known to degrade by hyperosmotic stress (Meloni et al. 2003; Sairam et al. 2002; Sudhakar et al. 2002). As a consequence to unfavorable environmental conditions, a group of very reactive, short-lived chemicals termed as reactive oxygen species (ROS) are induced. They encompass superoxide anion, hydrogen peroxide and hydroxyl radical. They could also appear during normal metabolism or upon an oxidative stress (Sun 1990). The activities of antioxidant enzymes play role in scavenging ROS. Enzymes, such as superoxide dismutase (SOD) and catalase (CAT) catalyze the dismutation of superoxide to hydrogen peroxide and oxygen (Foyer et al. 1994).

The aim of the present study was *first*, isolate and characterize *Geobacillus* sp. Second, study the potential role played by *Geobacillus* sp. on maize protection from salinity by injecting *Geobacillus* sp. into plant young seedlings prior seedlings exposure to salt stress. Our steps to achieve these aims were (1) isolation of salt tolerant microorganism. (2) Inoculation of the microorganism into maize plants prior they were sown with 350 mmol NaCl. (3) Study the physiological status of maize within the biological control.

Materials and methods

Isolation of *Geobacillus caldoxylosilyticus* IRD (*Geobacillus*)

The bacterial strain was isolated according to Esawy et al. (2007).

Growth conditions of *Geobacilluss caldoxylosilyticus* IRD

The pH, temperature, and NaCl growth experiment performed in duplicates, using Hungate tubes (Hungate and Macy 1973) containing BM and glucose (20 mmol) as energy source. Prior to inoculation for growth experiments, the cultivar sub cultured at least once under the same experimental conditions. For all experiments, the bacterial growth was monitored by measuring the increase in turbidity at 600 nm in aerobic tubes inserted directly into a model UV-160A spectrophotometer (Shimadzu). The presence of spores was sought by microscopic examination of the culture at different phases of growth.

Substrates test

Substrates injected before tested, from sterile stock solutions, to a final concentration of 20 mmol into Hungate tubes containing BM. The use of elemental sulfur (2% w/v), thiosulfate (20 mmol), sulfite (20 mmol), nitrate (10 mmol), nitrite (10 mmol) and fumarate (20 mmol) as terminal electron acceptors was tested using BM supplemented with glucose (20 mmol) as energy source.

Plant material and growth conditions

Grains from four maize cultivars, *triple hybrids* (TH 321 and TH 310) and *simple hybrids* (SH 10 and SH 162) were purchased from Agricultural Research Center, Dokki, Egypt. Grains were pre-soaked overnight in distilled water and grown in distilled water for 5 days. The inoculation procedure was performed using syringe (10 cm) needle. The germinated seedlings were planted in Petri dishes for additional 10 days. Based on the growth conditions of maize, seedlings were labeled as follows: 'Control' for seedlings grown without salt, 'Control+' for seedlings grown with 350 mmol NaCl for 10 days, 'Geobacillus' for seedlings pre-inoculated with 0.2 ml of *Geobacillus*

suspension and grown for 10 days without salt; Geobacillus+, for seedlings pre-inoculated with 0.2 ml *Geobacillus* suspension before sown for 10 days with 350 mM NaCl. Seedling heights and dry weights were determined on the end of the incubation period.

Seedling dry mass

One of the most commonly used measure is the dry mass of plants. Oven dry seedlings of the studied cultivars were weighed and expressed as seedling dry weight of 1 g fresh weight (Chapman 1976).

Relative water content

Relative water content was measured by the method of Weatherley (1950) and its modification by Weatherley and Barrs (1962) was adopted to correct mainly for continued water uptake by leaf tissue after attaining full turgidity due to growth. Leaf cuts (1–2 cm in length) were immediately floated on distilled water. It was found that keeping the floating cuts at low temperature in the refrigerator (4°C) and in dark or under low light intensity reduced the error due to continued growth because it can be greatly slowed down. Saturation of tissue cuts was attained in 24–36 h. Leaf cuts were then rapidly and thoroughly blotted dry, weighed immediately (turgid weight), oven-dried at 80°C for 24 h then reweighed (dry mass) and fresh weight determine from water content. RWC of leaves was expressed as a percentage and evaluated according to the equation:

$$\text{RWC} = \frac{\text{Fresh weight of leaf cuts} - \text{oven dry weight of leaf cuts}}{\text{Saturation weight of leaf cuts} - \text{oven dry weight of leaf cuts}} \times 100$$

Anatomical analysis

Sections from fresh root and leaf of maize seedlings were dehydrated and then placed in formalin–acetic acid–alcohol (FAA; 5:5:95) for 24 h. Small portions of the leaves were cut and then treated according to the glycol methacrylate (GMA) method of Feder and O'Brien (1968). This involves dehydrating the material through a graded alcohol series before infiltrating with GMA and embedding in capsules containing GMA. The capsules were placed in an oven at 60°C for 24 h to polymerize. Sections, 3–5-μm thick, were made using an ultramicrotome. Staining was done with Schiff's reagent and toluidine blue. The microscopic slides been observed under a light microscope equipped with a digital camera and a computerized data capturing system. On day 15 from maize growth, the anatomy of leaf and root were examined using light microscopy.

Determination of mineral content

The minerals (Na^+, Cl^- and K^+) in the acid digested samples were determined photometrically using flame photometer (Perkin Elmer model-149) as described by Brown and Lilleland (1946).

Determination of proline content

Proline in dry biomass seedlings was investigated according to Bates et al. (1973) as follows:

The acidic ninhydrin prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml of 6 M phosphoric acid with agitation until dissolved. The mixture was kept to cool and stored at 4°C. The reagent remains stable for 24 h. Approximately, 0.1 g of ground dried tissue was homogenized in 10 ml of 3% aqueous sulfosalicylic acid, and then filtered through filter paper Whatman No. 2. 2 ml of the filtrate was mixed with equal volume of glacial acetic acid and 2 ml of acidic ninhydrin in a test tube and heated for 1 h at 100°C. The reaction mixture was extracted with 4 ml toluene, mixed vigorously in a test tube for 15–20 s. The chromophore containing toluene was aspired from the aqueous phase and warmed to room temperature. The absorbance was read at 520 nm using toluene as a blank. Referring to proline standard curve, the proline concentration was determined and calculated on dry matter basis as microgram proline g^{-1} dry weight.

Determination of photosynthetic pigments

Photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and carotenoids) were determined in the leaves of the investigated plants using the spectrophotometric method recommended by Metzner et al. (1965). Fresh leaf samples (0.2 g) were ground in 8 ml of 80% acetone. The homogenate was centrifuged at 5,000 rpm for 5 min. The absorbance of the supernatant was measured at 452.2, 644 and 663 nm against the solvent blank. Contents of chlorophyll *a* and *b* as well as carotenoids were expressed as μg/0.2 g tissue and calculated using the following equations:

$$\text{Chl}a = 10.3\,A_{663} - 0.918\,A_{644}$$

$$\text{Chl}b = 19.7A_{644} - 3.87A_{663}$$

$$\text{Carotenoids} = 4.2\,A452.5 - (0.0264\ \text{Chl}a + 0.426\ \text{Chl}b).$$

Antioxidant enzyme activities

Catalase

Catalase (CAT) activity (EC 1.11.1.6) was assayed in a reaction mixture (3 ml) composed of phosphate buffer

(50 mmol, pH 7.0), 30% (w/v) H_2O_2 and 0.5 ml of enzyme extract (Aebi 1983). The activity of catalase enzyme was estimated by the decrease of absorbance at 240 nm as a consequence of H_2O_2 consumption and was expressed according to Havir and Mellate (1987).

Superoxide dismutase

Superoxide dismutase (SOD) activity (EC 1.15.1.1) was measured using the method described by Marklund and Marklund (1974). The solution (10 ml) consisted of 3.6 ml of distilled water, 0.1 ml of the enzyme extract from Chinese brake, 5.5 ml of 50 mmol phosphate buffer pH 7.8 and 0.8 ml of 3 mmol pyrogallol (dissolved in 10 mmol HCl). The rate of pyrogallol reduction was measured at 325 nm with a UV–Vis spectrophotometer (UV 160 U UV–visible recording spectrophotometer, Shimadzu, Japan). 1 U of enzyme activity was defined as the amount of the enzyme that resulted in 50% inhibition of the auto-oxidation rate of pyrogallol at 25°C (Kong et al. 1999).

Statistical analysis

Statistical analyses were pursued using SPPS 11.5 for windows; one-way ANOVA test at $p \leq 0.05$ evaluated the present data according to Shapiro–Wilk test. Standard deviation (SD) was used for evaluating data of figures.

Results and discussion

Characterization of *Geobacillus caldoxylosilyticus*

The bacteria isolate (*Geobacillus*) was isolated from Marakopara pond in the Atoll Tikehau (French polynesian). The analyses of the most recent 16S rRNA gene sequences available from the RDP and Gene Bank revealed that our isolate belonged to the genus *Geobacillus*; *Geobacillus caldoxylosilyticus* being its closest phylogenetic relative similarity of 95%. The level of DNA–DNA relatedness between our isolate and *Geobacillus* was 26.6%; this revealed that (*Geobacillus*) isolate should be assigned to a novel species of the genus *Geobacillus* (Esawy et al. 2007). The halophilic obligate aerobic isolate was characterized by gram positive cocci, central to terminal endospore. Generation time under optimum conditions was 1–2 h. Growth was observed in a pH range 4.5–9.5, with an optimum at 7.5. The isolate was moderately thermopile growing at temperature ranging form 37–52°C, the optimum growth obtained at 45°C and no growth was observed at 55°C. *Geobacillus* was capable of hydrolyze sucrose, starch, glucose, maltose, casein, lactose. No growth was observed with ribose, fructose, xylose, mannose and fumarate

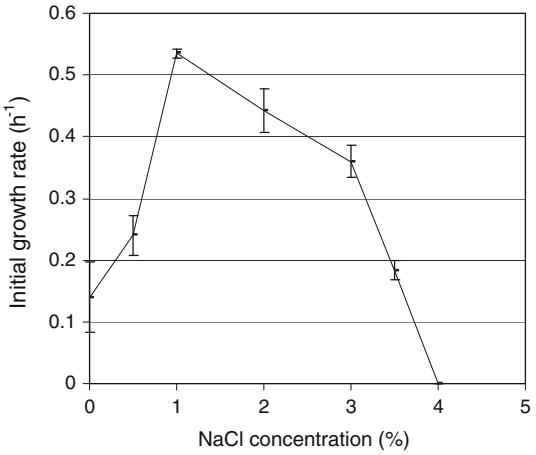

Fig. 1 Growth rates of *Geobacillus caldoxylosilyticus* exposed to different NaCl concentrations

(data not shown). Elemental sulfur, sulfate, thiosulfate, sulfite, nitrate and nitrite are not used as electron acceptors. It grew in a wide range of carbon sources, including glucose, lactose, starch, sucrose, raffinose and xylose (Ahmed et al. 2000). The NaCl not obligatory required for growth of the isolate. The NaCl concentration optimal for growth was 1% (w/v). The isolate was tolerant to 3.5% NaCl (w/v, Fig. 1). At the end of the experiment (10 days), the isolate was taken and propagated from maize plants tissue, and showed normal successful growth.

Impacts of *Geobacillus* inoculation on growth of salt-stressed maize

The NaCl was frequently used in high doses in laboratory experiments to achieve various purposes. For example, tobacco cells exposed to 428 mmol NaCl manifested proline levels of induction and accumulation in the intracellular and cytoplasm (Binzel et al. 1987). In the present investigation, using a relatively high NaCl concentration (i.e. 350 mmol) which equals to 2% 'w/v' in treating maize seedlings after germination was suggested to fulfill two goals: *first*, to study the shock effect, which known to induce abrupt endogenous and continuous physiological and structural variations inside plants (Abdelkader et al. 2007). Second, *Geobacillus*-salt-adapted bacteria-grew efficiently under high osmotic stress (from 1 to 2% NaCl, Fig. 1). The NaCl concentration used here was still within the optimal concentration required for *Geobacillus* growth.

Salt stress lead to growth suppression was extensively recorded (Ozturk et al. 2004; Sari and Ceylan 2002). The maximum growth obtained in maize seedlings grown normally or grown with salt stress was illustrated in Fig. 2. In the four used maize cultivars, the height of 'Control' plants mimics the heights of 'Geobacillus' plants. The maximum seedlings heights were 14, 11, 15.4 and 17.7 cm in

cultivars, TH 321, TH 310, SH 10 and SH 162, respectively. Owing to the short inoculation period of maize which was 10 days only, the heights of 'Geobacillus+' seedlings although were up regulated slightly were not significantly better than the heights of 'Control+' seedlings. The height values were 4.3, 1.76, 5.48 and 4.2 cm in 'Control+' and 4.47, 3.1, 5.37 and 5.3 cm in 'Geobacillus+' for TH 321, TH 310, SH 10 and SH 162 cultivars, respectively (Fig. 2). Maize cultivars responded to salt stress heterogeneously, the best growth was detected in SH 10 and SH 162 cultivars. This implied to the bacterial–maize varied levels of interaction during maize plant exposure to salt stress. However, *Geobacillus* inoculation into maize must has governed an increase in cell elongation and cell division in a direction lead to maize growth enhancements, this effect must be described as significant in terms of the high salt dose within short period (i.e. 10 days only following maize inoculation).

Impacts of *Geobacillus* inoculation on the dry mass

Maize grain was detached before maize seedlings dry mass (DM) was calculated at the end of the experiment time course. Salt stress lead to significant increase in DM in maize seedlings (Fig. 3). Several authors argued that the reduction in shoot DM upon plant desiccation is a sign of stress adaptation, taking this symptom as a good morpho-physiological indicator of tolerance in these plants (Alberico and Cramer 1993; Azevedo Neto and Tabosa 2000).

It was further reported that plants exposure to salt stress has governed DM reduction in tolerant plants (Azevedo Neto et al. 2004). In the present study; based on height measurements of maize, we presumed that both SH 10 and SH 162 might be tolerant cultivars- as seen from their DM values compared to the other two cultivars (Fig. 2). Generally, the combined effects of salt stress and *Geobacillus* in maize have caused a significant increase in DM in the inoculated seedlings. Here, we concluded that *Geobacillus* inoculation in maize before exposure to salt stress has modified seedlings dry mass; these modifications were towards growth advances.

Anatomical structure of root and leaf

Salt stress influences the anatomy of plants tissues. For example, lipid peroxidation was the main reason for membranes damage as seen in ultrastructure of plastids under salt stress (Abdelkader et al. 2007). Our micrographs taken after a microscopic examination of leaf and root of maize plants exposed to salt stress, viewed seedlings inoculated or

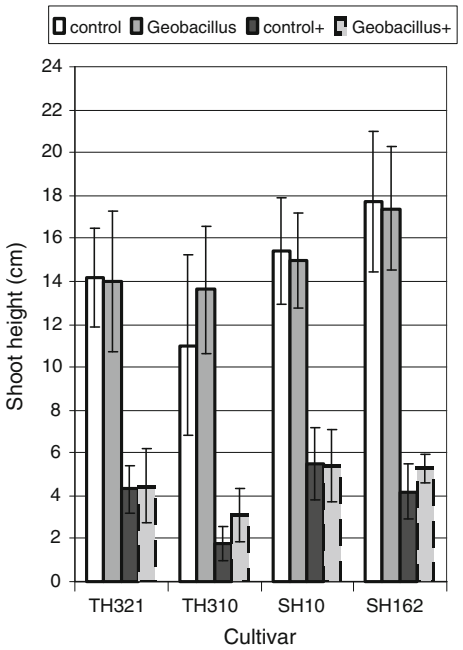

Fig. 2 Shoot heights of 15-day-old maize seedlings after exposed to salt stress. Seedlings of maize were germinated for 5 days in distilled water, inoculated or non-inoculated with *Geobacillus*, thereafter left to grow in 350 mmol NaCl for 10 days. Unstressed maize lacking *Geobacillus*, Control; unstressed maize inoculated with *Geobacillus*, Geobacillus; stressed maize lacking *Geobacillus*, Control+; stressed maize inoculated with *Geobacillus*, Geobacillus+. *Error bars* represent SD of the mean

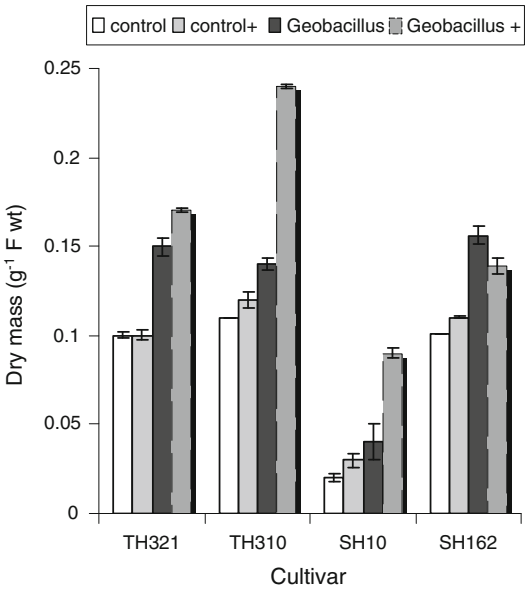

Fig. 3 Dry mass of 15-day-old maize seedlings exposed to salt stress. Seedlings of maize were germinated for 5 days in distilled water, inoculated or non-inoculated with *Geobacillus*, thereafter left to grow in 350 mmol NaCl for 10 days. Unstressed maize lacking *Geobacillus*, Control; unstressed maize inoculated with *Geobacillus*, Geobacillus; stressed maize lacking *Geobacillus*, Control+; stressed maize inoculated with *Geobacillus*, Geobacillus+. *Error bars* represent SD of the mean

un-inoculated with *Geobacillus*. An impaired root cortex in 'Control+' seedlings was a striking consequence of salt stress effects on unprotected plant (Fig. 4g). On the other hand, the stressed root was intact only in seedlings inoculated with *Geobacillus* for 10 days (Fig. 4h).

In concomitant to other kinds of a biotic stresses, salt stress causes leaf injuries and lead to decrease in leaf size through decreasing the machinery of both cell expansion and cell division (Curtis and Lauchli 1987; Fricke and Peters 2002; Hasegawa et al. 2000). In stressed plants, the leaf thickness was decrease at the midrib region in ST10 and ST162 cultivars (data not shown), whereas an increase in the midrib thickness was recorded in TH321 and TH310 cultivars. Other authors (Azevedo Neto et al. 2004) had, also reached these findings suggested differential responses to salt stress by different plants genotypes. Upon *Geobacillus* inoculation to stressed plants, the midrib thickness has increased significantly in all cultivars (data not shown).

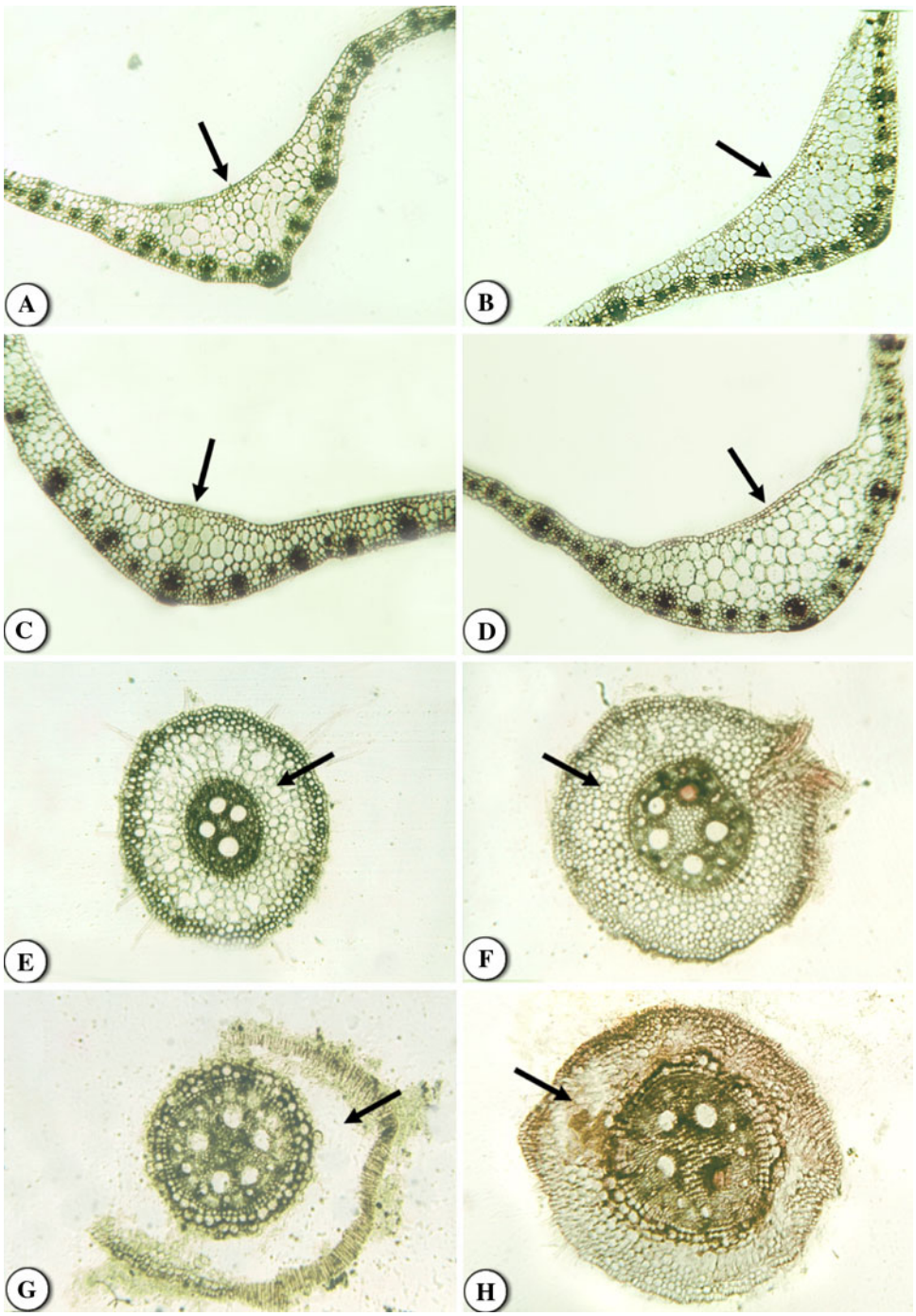

Fig. 4 *Light microscopic photographs* show the appearance of leaf and root in maize plants after been exposed to salt stress. Seedlings of maize were germinated for 5 days in distilled water, inoculated or non-inoculated with *Geobacillus*, thereafter left to grow in 350 mmol NaCl for 10 days. Unstressed leaf, **a** stressed leaf, **b** unstressed root, **e** stressed root, **f** unstressed leaf inoculated with *Geobacillus*, **c** stressed leaf inoculated with *Geobacillus*, **d** unstressed root inoculated with *Geobacillus*, **g** stressed root inoculated with *Geobacillus*, **h** *thick arrows* pointed to leaf midrib and to root cortex. Magnification used (×32)

Herein, the role of halophytic bacteria in minimizing the adverse symptoms of salt stress was aggravated.

In this section, we proposed that *Geobacillus* sp. inoculation into stressed seedlings conferred protection to the root cortical structures and increased the number of leaf vascular bundles, for example, number of vascular bundles increased from 25 in 'Control+' seedlings to 73 in 'Geobacillus+' seedlings and from 84 in 'Control+' seedlings to 99 in 'Geobacillus+' seedlings, for TH321 and SH162 cultivars, respectively. (Fig. 4h). Moreover, the inoculation has increased the number of leaf vascular bundles even in unstressed seedlings (data not shown).

Geobacillus sp. found to have manipulated the general root area under all treatments, with two exceptions: 'Geobacillus+' seedlings of TH 321 cultivar and 'Geobacillus' seedlings of TH 310 cultivar.

Mineral content (Na^+, Cl^- and K^+)

Based on the previous reports, tolerance is dependent on the ability of plant cells to re-establish ion homeostasis and since salt tolerance is strongly dependent on the net selection of K^+ (*less toxic*) over Na^+ (*high toxic*), tolerant plants must potentially exclude Na^+ from photosynthesizing young leaf (Moradi et al. 2003).

Tolerance in maize was not merely dependent on Na^+ content in shoots but on cells efficiency to compartmentalize ions in the vacuole (Alberico and Cramer 1993; Rai and Takabe 2006). Researchers presumed that compartmentalization delayed the effect of the ionic stress, which could take days, weeks or even months before the physical damage appeared in the plant (Munns 2002). In the present investigation, we proposed that *Geobacillus* sp. must had consumed NaCl to run some cellular activities necessary for its growth; on the same level, it shielded salts deleterious effects from plants tissue (Table 1). The data in hand showed that Na^+ content has decreased markedly in TH321, TH310 and ST10 seedlings after been inoculated with *Geobacillus* sp. before exposed to salt stress (Fig. 5a), whereas 'Control+' seedlings accumulated much more Na^+.

The accumulation of Cl^- per 1 g dry weight ranged between 4 and 21 mg in 'Control+' seedlings. On the other hand, 'Geobacillus+' seedlings accumulated less Cl^- content as Cl^- had ranged between 1.0 and 2.5 mg in seedlings after 10 days exposure to salt stress (Table 1; Fig. 5b).

Geobacillus is presumed potential in reducing the levels of Cl^- accumulation in maize plants under salt stress.

The accumulation of K^+ in salt-stressed seedlings followed opposite strategy compared to strategies of Na^+ and Cl^- accumulation. Generally, K^+ has *decreased* in level in salt-stressed seedlings than in unstressed seedlings. Recently, it was discovered (Yilmaz et al. 2004) that NaCl lead to Na^+ increase, K^+ and K^+/Na^+ decrease in tomato plant seedlings. In the four investigated maize cultivars, K^+ had increased in control seedlings whether inoculated with *Geobacillus* or not. Except for ST 10 cultivar, the K^+ level was generally higher in 'Geobacillus' seedlings (Fig. 5c). In salt-stressed seedlings, the decrease in K^+ level was pronounced in 'Geobacillus+' seedlings as compared to 'Control+' seedlings (Fig. 5c). These results suggested that *Geobacillus* sp. had provoked maize plants to pursue all possible mechanisms that are necessarily initiated by plants when their survivals were threatened by undesirable environmental conditions.

Proline content

The osmotic adjustment using net solutes (e.g. carbohydrates, proline and other amino acids) is a crucial mechanism encounters salt and drought effects in plants (Hasegawa et al. 2000). Proline is a stress indicator (Liu and Zhu 1997) increased heavily in plants under salt stress. Based on the literature and our data, proline has intensively accumulated in salt-sensitive plants (Jain et al. 1991). In such case, TH321 and TH310 cultivars could be more sensitive than ST10 and ST162 cultivars (Fig. 5d).

Table 1 The percentage of minerals (Na^+, Cl^- and K^+) accumulated in leaf tips of 15-day-old maize plants grown for 10 days in 350 mmol NaCl

Mineral (%)	Cultivar							
	TH 321		TH 310		ST 10		ST 162	
	+	−	+	−	+	−	+	−
Na^+	187	941	279	1,675	193.4	302.5	270	186
Cl^-	111	254	150	240	120	792	532	984
K^+	0.14	0.40	0.03	0.4	0.93	0.47	0.20	0.50

On day 5, germinated maize seedlings were inoculated with *Geabacillus caldoxylosilyticus*. Non-inoculated seedlings were labeled with 'minus'; inoculated seedlings were labeled with 'plus'

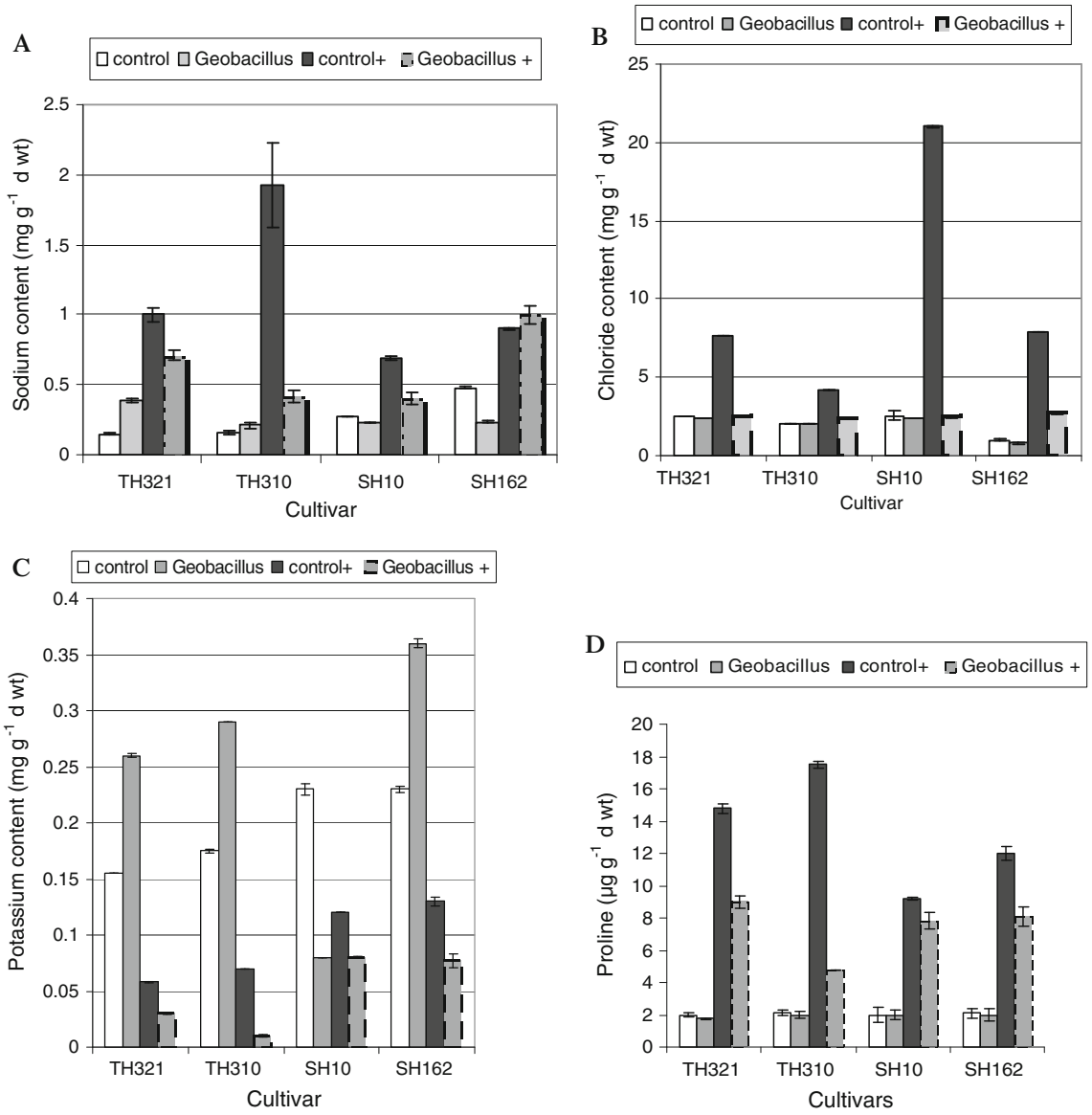

Fig. 5 **a** Sodium content in 15-day-old maize plants. Seedlings of maize were germinated for 5 days in distilled water, inoculated or non-inoculated with *Geobacillus*, thereafter left to grow in 350 mmol NaCl for 10 days. Unstressed maize lacking *Geobacillus*, Control; unstressed maize inoculated with *Geobacillus*, Geobacillus; stressed maize lacking *Geobacillus*, Control+; stressed maize inoculated with *Geobacillus*, Geobacillus+. *Error bars* represent SD of the mean. **b** Chloride content in 15-day-old maize plants. Seedlings of maize were germinated for 5 days in distilled water, inoculated or non-inoculated with *Geobacillus*, thereafter left to grow in 350 mmol NaCl for 10 days. Unstressed maize lacking *Geobacillus*, Control; unstressed maize with *Geobacillus*, Geobacillus; stressed maize lacking *Geobacillus*, Control+; stressed maize inoculated with *Geobacillus*, Geobacillus+. *Error bars* represent SD of the mean. **c** Potassium content in 15-day-old maize plants. Seedlings of maize were germinated for 5 days in distilled water, inoculated or non-inoculated with *Geobacillus*, thereafter left to grow in 350 mmol NaCl for 10 days. Unstressed maize lacking *Geobacillus*, Control; unstressed maize with *Geobacillus*, Geobacillus; stressed maize lacking *Geobacillus*, Control+; stressed maize inoculated with *Geobacillus*, Geobacillus+. *Error bars* represent SD of the mean. **d** Proline content in 15-day-old maize plants. Seedlings of maize were germinated for 5 days in distilled water, inoculated or non-inoculated with *Geobacillus*, thereafter left to grow in 350 mmol NaCl for 10 days. Unstressed maize lacking *Geobacillus*, Control; unstressed maize inoculated with *Geobacillus*, Geobacillus; stressed maize lacking *Geobacillus*, Control+; stressed maize inoculated with *Geobacillus*, Geobacillus+. *Error bars* represent SD of the mean

The proline level was high in this order: TH310, TH321, SH162 and SH10 suggesting that SH10 cultivar is probably the most tolerant and TH310 is the most sensitive cultivar to salt stress. Upon *Geobacillus* introduction, the properties of maize cultivars for tolerance have changed slightly, as proline level became high in this order: TH321, SH162, SH10 and TH310 (Fig. 5d). The new changes were obtained due to the interactions occurred between maize

and *Geobacillus* sp. these interactions are certainly not homologous among cultivars.

Proline-retrieved plants membranes and protein structures under stress; proline accumulation was a sign that plants experienced no physiological stress (Binzel et al. 1987). Studying the level of maize plant proline in the presence of *Geobacillus* has asserted the biological control of *Geobacillus* in salt stress alleviation, confirmed by a decreased proline level in 'Geobacillus+' seedlings than 'Control+' plants (Fig. 5d).

Impacts of *Geobacillus* inoculation on the relative water content

Relative water content (RWC) was determined in four maize cultivars grown under conditions of high salt stress combined to inoculation with *Geobacillus* sp. Our data clearly showed that the RWC of 'Geobacillus+' seedlings was generally higher than 'Control+' seedlings. RWC of 'Geobacillus+' seedlings was even higher compared to 'Geobacillus' seedlings (Table 2). This proposed that these plants upon inoculation possess sufficient water and suffer no potential stress. Despite this, RWC is not always correlated to the physiological status of some cereal plants or their level of sterility. For example, in a previous study conducted on rice plants (Lafitte 2002) a weak correlation was discovered between both RWC and yield production and between RWC and the percentage of spike sterility.

Photosynthetic pigment content

Photosynthetic pigments mainly constitute chlorophyll $a + b$ and carotenoids are of vital importance in photosynthesis. Reduction in these pigments often occurs in response to prolonged plant exposure to different stresses. Regulation of chlorophyll *a*, chlorophyll *b* and carotenoids under stress combined to microbial inoculation was presented in Table 2.

On one hand, the overall photosynthetic pigments were increased in 'Geobacillus' seedlings as compared to 'Control' seedlings. On the other hand, by comparing 'Control+' and 'Geobacillus+' seedlings together, chlorophyll *a* and carotenoids were found significantly higher in 'Geobacillus+' seedlings of TH321 and SH162 cultivars, whereas chlorophyll *b* was significantly higher in 'Geobacillus+' seedlings of TH321 cultivar (Table 2). The variation among the tested cultivars was statistically analyzed using one-way ANOVA ($p < 0.05$) test. There was chlorophyll *a* significant difference between treated cultivars except 'Control' plants. Furthermore, chlorophyll *b* and carotenoids values exhibited significant difference for 'Control+' cultivars. The present data are in concomitant to previous studies

Table 2 Relative water content expressed as (%), Chlorophyll (*a* and *b*) and carotenoids content expressed as μg/0.2 g fresh weight, antioxidant enzymes; catalase (CAT) and superoxide dismutase (SOD) measured in unit per gram fresh weight in maize plants leaf

Cultivar	Treatment	RWC (%)	Chl*a* (μg)	Chl*b* (μg)	Carotenoids (μg)	CAT (unit/g)	SOD (unit/g)
TH 321	C	89.6 ± 0.5*	10.6 ± 0.2	5.2 ± 1.1	6.4 ± 0.5	2.74 ± 0.06	0.134 ± 0.01
TH 310	C	96.0 ± 1.7*	5.7 ± 0.2	3.2 ± 0.2	3.8 ± 0.3	4.32 ± 0.02	2.4 ± 0.4
SH 10	C	92.0 ± 2.0*	7.5 ± 0.03	4.6 ± 0.2	4.6 ± 0.1	1.97 ± 0.1	0.09 ± 0.001
SH 162	C	95.1 ± 1.0*	4.5 ± 0.2	2.3 ± 0.1	3.3 ± 0.2	0.41 ± 0.04	3.28 ± 0.2
TH 321	G	73.0 ± 1.8	8.35 ± 0.2*	2.4 ± 0.1	5.2 ± 0.2	2.21 ± 0.04	0.27 ± 0.01
TH 310	G	68.7 ± 2.0	8.0 ± 0.2*	5.4 ± 0.3	4.5 ± 0.3	2.43 ± 0.1	0.2 ± 0.01
SH 10	G	95.6 ± 1.6	11.6 ± 0.1*	4.7 ± 0.2	7.0 ± 0.02	1.00 ± 0.02	1.53 ± 0.1
SH 162	G	87.1 ± 2.2	7.7 ± 0.2*	4.6 ± 0.1	5.0 ± 0.1	3.60 ± 0.1	0.3 ± 0.01
TH 321	C+	70.1 ± 3.2*	5.0 ± 0.5*	2.9 ± 0.2*	2.6 ± 0.4*	7.00 ± 0.2	0.33 ± 0.01
TH 310	C+	68.2 ± 1.2*	5.0 ± 0.1*	3.0 ± 0.2*	3.5 ± 0.3*	6.33 ± 0.1	0.11 ± 0.001
SH 10	C+	43.2 ± 1.5*	10.3 ± 0.2*	6.4 ± 0.21*	6.3 ± 0.2*	2.86 ± 0.1	7.1 ± 0.5
SH 162	C+	88.6 ± 2.4*	6.0 ± 0.1*	6.6 ± 0.2*	3.7 ± 0.2*	2.33 ± 0.01	11.33 ± 2.1
TH 321	G+	94.1 ± 2.1	12.7 ± 1.2*	4.6 ± 0.02	8.6 ± 0.2	14.60 ± 0.3*	0.31 ± 0.01*
TH 310	G+	87.0 ± 2.5	3.5 ± 0.2*	2.2 ± 0.1	2.4 ± 0.1	4.16 ± 0.2*	4.82 ± 0.2*
SH 10	G+	89.0 ± 1.6	4.2 ± 0.1*	2.6 ± 0.03	3.2 ± 0.3	5.66 ± 0.1*	0.73 ± 0.01*
SH 162	G+	92.3 ± 2.5	8.9 ± 0.2*	5.3 ± 0.1	5.7 ± 0.01	5.30 ± 0.2*	14.1 ± 1.0*

Seedlings of maize were germinated for 5 days in distilled water, inoculated or non-inoculated with *Geobacillus*, thereafter left to grow in 350 mmol NaCl for 10 days

Unstressed maize lacking *Geobacillus*, *C* unstressed maize inoculated with *Geobacillus*, *G* stressed maize-lacking *Geobacillus*, *C+*, stressed maize inoculated with *Geobacillus*, *G+*. Data presented as mean ± SD

Each value is a mean of three independent determinations. Asterisks indicate values that differ significantly between cultivars at $p \leq 0.05$ according to Shapiro–Wilk test

which revealed that pigments of photosynthetic apparatus often destroyed by external factors such as salt stress and UV radiation-mediated loss of photosynthetic capacity (Jordan et al. 1994). The effect of these factors was targeting PSII complex (Turcsányi and Vass 2000).

Activity level of catalase and superoxide dismutase

Regardless to whether seedlings were *Geobacillus* inoculated or not, it was indicated (Table 2) that both CAT and SOD level of activities had generally increased with the effect of salt stress in most of the cultivars. It was early documented that abiotic stress induces the production of reactive oxygen species (ROS) and results in significant damage to cellular constituents (Halliwell and Gutteridge 1993). Thereafter, it was discovered that plants ability to resist oxidative stress is dependent on plant species as well as the type of stress (Parmar et al. 2002). Plants could overcome oxidative stress either enzymatically, nonenzymatically or in presence of both mechanisms (Israr and Sahi 2006). One-way ANOVA test at ($p < 0.05$) was consulted to analyze the level of activity of CAT and SOD. One-way ANOVA showed a significant difference between 'Geobacillus+' seedlings (Table 2).

Conclusion

The present study is a case of a biological control, aimed for protecting maize growth/survival under salt stress by examining physiological and structural impacts of maize upon halophytic bacterial cells introduction into its seedlings. The in vivo measurements of Na^+, Cl^-, K^+ and proline have evaluated concisely the potent effect of high salt stress. *Geobacillus caldoxylosilyticus* played a significant role with ions exclusion from maize plant seedlings. We conclude that *Geobacillus* impacts in protecting maize was potential particularly that the NaCl concentration used here was high and the experimental span was short. Accordingly, *Geobacillus* sp. is recommended for protecting plants more efficiently against moderate salt stress.

Acknowledgments The authors thank Yannick Combet for offering facilities in 'Laboratoire de microbiologie, IRD, IFR-BAIM, Universités de Provence et de la Méditerranée, ESIL case 925, 13288 Marseille Cedex 9, France' during isolation and characterization of *Geobacillus caldoxylosilyticus*. The authors would like to thank the technician Medhat Zareef for his assistance during the microscopic examinations.

References

Abdelkader AF, Henrik A, Katalin S, Bela B, Christer S (2007) High salt stress induces swollen prothylakoids in dark grown wheat and alters both prolamellar body transformation and alters both prolamellar body transformation and reformation after irradiation. J Exp Bot 58:2553–2564

Aebi HE (1983) Catalase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. 3:273–286

Ahmed S, Scopes RK, Rees GN, Patel KC (2000) *Saccharococcus caldoxylosilyicus* sp.nov., an obligatory thermophilic, xylose-utilizing, endospore-forming bacterium. Int J Syst Evol Microbiol 50:517–523

Alberico GL, Cramer GR (1993) Is the salt tolerance of maize related to sodium exclusion? I. Preliminary screening of seven cultivars. J Plant Nutr 16:2289–2303

Aspinall D, Paleg LG (1981) Proline accumulation: physiological aspects. In: Paleg LG, Aspinall D (eds) The physiology and biochemistry of drought resistance in plants. Academic Press, Sydney, pp 205–241

Azevedo Neto AD, Tabosa JN (2000) Salt stress in maize seedlings: I. Growth analysis. Rev Bras Eng Agric Amb 4:159–164

Azevedo Neto AD, Prisco JT, Enéas-Filho J, De Lacerda CF, Silva JV, Da Costa PHA, Gomes-Filho E (2004) Effects of salt stress on plant growth, stomatal response and solute accumulation of different maize genotypes. Braz J Plant Physiol 16:31–38

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207

Binzel ML, Hasegawa PM, Rhodes D, Handa S, Handa AK, Bressan RA (1987) Solute accumulation in tobacco cells adapted to NaCl. Plant Physiol 84:1408–1415

Bohnert HJ, Shen B (1999) Transformation and compatible solutes. Sci Hortic 78:237–260

Bray EA, Bailey-Serres J, Weretilnyk E (2000) Responses to abiotic stresses. In: Buchanan BB, Gruissem W, Jones RL (eds) Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville, pp 1158–1203

Brown JD, Lilleland O (1946) determination of potassium and sodium in plant material and soil extracts by flame photometry. Proc Am Soc Hort Sci 12:341–364

Chanway CP (1997) Inoculation of tree roots with plant growth promoting soil bacteria (an emerging technique for reforestation. For Sci 43:99–112

Chapman SB (1976) Methods in plant ecology. Blackwell, Oxford, pp 96–97

Chen CT, Chen LM, Lin CC, Kao CH (2001) Regulation of proline accumulation in detected rice leaves exposed to excess copper. Plant Sci 160:283–290

Claussen W (2005) Proline as a measure of stress in tomato plants. Plant Sci 168:241–248

Curtis PS, Lauchli A (1987) The effect of moderate salt stress on leaf anatomy in *Hibiscus cannabinus* (kenaf) and its relation to leaf area. Am J Bot 74:538–542

Esawy MA, Wafaa A, Samia H, Ahmed A, Combet Y (2007) Natural material role in production, activation and stabilization of alkaline protease produced from a new isolated *Geobacillus caldoxylosilyticus* IRD. J Appl Sci Res 10:1062–1068

Feder N, O'Brien TP (1968) Plant microtechnique: some principles and new methods. Am J Bot 55:123–142

Fortmeier R, Schubert S (2006) Salt tolerance of maize (*Zea mays* L.): the role of sodium exclusion. Plant Cell Environ 11:1041–1047

Foyer CH, Lelandais M, Kunert KJ (1994) Photooxidative stress in plants. Physiol Plant 92:696–717

Fricke W, Peters WS (2002) The biophysics of leaf growth in salt-stressed barley. A study at the cell level. Plant Physiol 129:374–388

Gadallah MAA (1993) Effect of water stress, abscisic acid and proline in cotton plants. J Arid Environ 30:315–325

Gilbert GA, Gadush MV, Wilson C, Madore MA (1998) Amino acid accumulation in sink and source tissues of *Coleus blumei* Benth during salinity stress. J Exp Bot 49:107–114

Gzik A (1996) Accumulation of proline and α-amino acids in sugar beet plants in response to osmotic, water and salt stress. Environ Exp Bot 36:29–38

Halliwell B, Gutteridge JMC (1993) Free radicals in biology and medicine, 2nd edn. Oxford University Press, New York

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol 51:463–499

Havir EA, Mellate NA (1987) Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. Plant Physiol 84:450–455

Hungate RE, Macy J (1973) The roll-tube method for cultivation of strict anaerobes. In: Norris JR, Ribbons DW (eds) Method in microbiology, 3B. Academic Press Inc., New York, p 132

Israr M, Sahi SV (2006) Antioxidative responses to mercury in the cell cultures of *Sebania drummondii*. Plant Physiol Biochem 44:590–595

Jain S, Nainawatee HS, Jain RK, Chowdhury JB (1991) Proline status of genetically stable salt-tolerant *Brassica juncea* L. somaclones and their parent cv. Prakash. Plant Cell Rep 9:684–687

Jordan BR, James PE, Strid A, Anthony RG (1994) The effect of ultraviolet-B radiation on gene expression and pigment composition in etiolated and green pea leaf tissue UV-B induced changes are gene-specific and dependent upon the developmental stage. Plant Cell Environ 17:45–54

Katerji N, Van Hoorn JW, Hamdy A, Karam F, Mastrorilli A (1996) Effect of salinity on water stress, growth, and yield of maize and sunflower. Agric Water Manage 30:237–249

Kijne JW (2006) Abiotic stress and water scarcity: identifying and resolving conflicts from plant level to global level. Field Crop Res 97:3–18

Kong FX, Hu W, Chso SY, Sang WL, Wang LS (1999) Physiology responses of the lichen *Xanthoparmelia mexicana* to oxidative stress of SO_2. Environ Exp Bot 42:201–209

Lafitte R (2002) Relationship between leaf relative water content during reproductive stage water deficit and grain formation in rice. Field Crop Res 76:165–174

Liu J, Zhu JK (1997) Proline accumulation and salt-stress-induced gene expression in a salt-hypersensitive mutant of Arabidopsis. Plant Physiol 2:591–596

Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: an overview. Arch Biochem Biophys 444:139–158

Mansour MMF (2000) Nitrogen containing compounds and adaptation of plants to salinity stress. Biol Plant 43:491–500

Marklund S, Marklund G (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47:469–474

Meloni DA, Oliva MA, Martinez CA, Cambraia J (2003) Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environ Exp Bot 49:69–76

Metzner H, Ranum H, Senger H (1965) Unterschungen zur sychnonisier-Barkiet einze-Iner Pigmenmangel- Mutanten-von Chloprella. Planta 65:186

Monreal JA, Jiménez ET, Remesal E, Morillo-Velarde R, García-Mauriño S, Echevarría C (2007) Proline content of sugar beet storage roots: response to water deficit and nitrogen fertilization at field conditions. Environ Exp Bot 60:257–267

Moradi F, Ismail AM, Gregoria GB, Egdane JA (2003) Salinity tolerance of rice during reproductive development and association with tolerance at the seedling level. Ind J Plant Physiol 8:276–278

Munns R (2002) Comparative physiology of salt and water stress. Plant Cell Environ 25:239–250

Ozturk A, Unlukara A, Ipek A, Gurbuz B (2004) Effects of salt stress and water deficit on plant growth and essential oil content of lemon balm (*Melisa officinalis* L.). Pak J Bot 36:787–792

Parmar NG, Vithalani SD, Chanda SV (2002) Alteration in growth and peroxidase activity by heavy metals in *Phaseolus vulgaris*. Acta Physiol Plant 24:89–95

Rai AK, Takabe T (2006) A biotic stress tolerance in plants toward the improvement of global environment and food. Springer, Berlin, pp 1–267

Rai V, Vajpayee P, Singh SN, Mehrotra S (2004) Effect of Chromium accumulation in photosynthetic pigments, oxidative stress defense system, nitrate reduction, proline level and eugenol content of *Ocimum tenuiflorum* L. Plant Sci 167:1159–1169

Sairam RK, Rao KV, Srivastava GC (2002) Differential response of wheat genotypes to long term salinity stress in relation to oxidative stress, antioxidant activity and osmolyte concentration. Plant Sci 163:1037–1046

Sari AO, Ceylan A (2002) Yield characteristics and essential oil composition of lemon balm (*Melissa officinalis* L.) grown in the Aegean region in Turkey. Turk J Agric For 26:217–224

Sudhakar C, Lakshmi A, Giridarakumar S (2002) Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. Plant Sci 161:613–619

Sun Y (1990) Free radicals, antioxidant enzymes, and carcinogenesis. Free Radic Biol Med 8:583–599

Turcsányi E, Vass I (2000) Inhibition of photosynthetic electron transport by UV-A radiation targets the photosystem II complex. Photochem Photobiol 72:513–520

Weatherley PE (1950) Studies in the water relations of the cotton plant 10 the field measurements of water deficits in leaves. New Phytol 49:81–87

Weatherley PE, Barrs C (1962) A re-examination of the relative turgidity technique for estimating water deficit in leaves. Aust J Biol Sci 15:413–428

Weller DM (1988) Biological control of soil borne plant pathogens in the rhizosphere with bacteria. Annu Rev Phytopathol 26:379–407

Yilmaz K, Akinci IE, Akinci S (2004) Response of tomato (*Lycopersicon esculentum* Mill.) to salinity in the early growth stages for agricultural cultivation in saline environments. J Environ Biol 25:351–357

Zhu JK (2000) Genetic analysis of plant salt tolerance using Arabidopsis. Plant Physiol 112:152–166

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