MOLECULAR CHARACTERIZATION OF SHOOT AND ROOT PROTEIN CONTENTS OF SALT STRESSED BARLEY SEEDLINGS INOCULATED WITH *AZOSPIRILLUM BRASILENSE* NO40

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Abstract

The present work was performed to study the effect of inoculation with *Azospirillum brasilense* NO40 on the protein pattern of shoots and roots of seedlings of two barley cultivars (Giza 123 and Giza 2000) which are known by their different tolerance to salt stress. A green house experiment was conducted to evaluate the effect of inoculation on the molecular masses of protein contents of both studied barley cultivars cultivated under 350 mM NaCl through the determination of SDS-PAGE protein profile of shoots and roots. Results showed that the salt stress, bacterial inoculation and the interaction between them resulted in noticed changes in the protein patterns of shoot and root in all treatments.

INTRODUCTION

Salinity is an increasing problem in many irrigated, arid and semi-arid areas of the world where rainfall is insufficient to leach salts from the root zone, and it is a significant factor in reducing growth and crop productivity (Ghoulam *et al.*, 2002). Salt stress affects all the major physiological processes in plants such as growth, photosynthesis, protein synthesis, and energy as well as lipid metabolism (Parida and Das, 2005).

Plants develop a plethora of biochemical and molecular mechanisms to cope with salt stress such as accumulation of nitrogen-containing compounds like proline, activation of the antioxidant defense mechanism and expression of many genes leading to the formation of stress proteins (Sairam and Tyagi, 2004).

Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that can actively colonize plant roots and improve plant growth and yield by direct and indirect mechanisms (Noel *et al.*, 1996). They are of two general types: those that form a symbiotic relationship with the plant such as the nitrogen-fixing *Rhizobium* spp. and those that are free-living (Glick, 1995). They produce plant growth promoting compounds including phytohormones, auxins, cytokinins and gibberellins (Garcia de Salamone *et al.*, 2001).

Azospirillum strains are a free-living, plant-growth-promoting rhizobacteria (PGPR), capable of affecting growth and yield of numerous plant species, many of

agronomic and ecological significance. Initially it was believed that they were found only in the rhizosphere, but later on they were isolated from the soil. Certain endophytic strains are able to colonize internally the plant, supplying it more efficiently with nitrogen (Fischer *et al.*, 2003).

Omar *et al.* (2006) found that inoculation with *Azospirillum brasilense* NO40 significantly alleviated the deleterious effects of salinity stress on the photosynthetic apparatus of the two barley cultivars Giza 123 (salt sensitive) and Giza 2000 (salt tolerant), through increased pigment contents, reduced accumulation of the osmoregulator proline, and reduced activities of antioxidant enzymes. However, it was interesting to understand the effect of inoculation with *A. brasilense* NO40 on the formation of stress proteins in plants under salt stress. The aim of the present work was to study the effect of A. brasilense NO40 inoculant on protein pattern of the shoot and root of seedling of two barley cultivars, Giza 123 (salt sensitive) and Giza 2000 (salt tolerant) cultivated under salt stress.

The aim of the present work was to study the effect of *A. brasilense* NO40 inoculant on protein pattern of the shoot and root of seedling of two barley cultivars, Giza 123 (salt sensitive) and Giza 2000 (salt tolerant) cultivated under salt stress.

MATERIALS AND METHODS

The soil used in this study was consisted of 69.25%, 20.4% and 10.35% of sand, silt and clay, respectively. Soil pH was 7.53 and EC was 0.69 dS/m. Its chemical composition was: 0.9151, 0.511, 0.7, 0.131, 0.436, 0.331 and 0.005 g/100 g soil of N^{2+}, P^{3+}, K^{+}, Na^{+}, Ca^{2+}, Mg^{2+} and Fe^{2+}, respectively.

A mineral fertilizer (N, P and K) was applied according to the recommendations of the Egyptian Ministry of Agriculture.

Grains of the two barley cultivars (*Hordeum vulgare*) Giza 123 (salt sensitive) and Giza 2000 (salt tolerant) were obtained from Barley Department, Agricultural Research Center, Giza, Egypt.

1. Bacterial inoculation

Bacterial inoculation was performed by coating the grains of barley with the appreciate amount of bacterial strain *Azospirillum brasilense* (NO40). A single inoculated grain harbored 10^6 bacterial cells on its surface.

2. Plant growth

Grains were soaked in water for 24 hr (to accelerate the germination process) before coating with *Azospirillum brasilense* (NO40). Grains were sown in each plastic pot and irrigated every other day with 350 mM NaCl, or with tap water as control, the pots were drained with water once a week. Each treatment was represented by six

plastic pots. The seedlings were left to grow for 30 days at 25 ± 2 °C in a relative humidity of 65% and 16 hr photoperiod. From each treatment, seedlings were taken and kept frozen in liquid nitrogen till further analysis.

3. Qualitative characteristics of protein using SDS-PAGE

A. Extraction of protein

A sample of 0.5 g frozen root and leaf was homogenized with 1 ml of extraction buffer (25 mM Na-acetate, pH 4.5 and 1 mM phenyl methyl sulphonyl fluoride [PMSF]), vortexed and left for 2 hours at 4 °C. The extract was centrifuged at 10.000 rpm at zero °C for 15 min and the clear supernatant was taken as the total protein extract (Kalina and Evgueni, 2001).

B. Protein separation by SDS-PAGE

Characterization and molecular mass determination of proteins were carried out using one dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970).

RESULTS AND DISCUSSION

Protein pattern of barley shoots were illustrated in Figure1 and Table 1

Salt stress caused an induction or inhibition in the synthesis of some polypeptides in the shoots of 30-day old seedlings of both barley cultivars. SDS-PAGE showed the denovo synthesis of two polypeptide bands with molecular masses of 68 and 37 kDa in the shoots of cultivar Giza 123 under 350 mM NaCl which was undetected in the control plants whereas the synthesis of the polypeptide bands with molecular masses of 62 and 21 kDa were suppressed. In shoots of cultivar Giza 2000, the appearance of polypeptide bands with molecular masses of 73, 37,27,19,14 and 5 kDa and the disappearance of polypeptide bands with molecular masses of 70, 35, 17 and 11 kDa were recorded in the salt treated plants when compared with the control plants.

Inoculation with *A. brasilense* (NO 40) resulted in a remarkable change in the protein pattern in shoots of inoculated salt un-treated seedlings of both cultivars. The formation of three polypeptide bands with molecular masses of 73,62 and 5 kDa were completely inhibited in the inoculated salt un-treated shoots of cultivar Giza 123, while the formation of polypeptide bands with molecular masses of 70,35 and 14 kDa were recorded in same treatments as compared with un-inoculated plant. Shoots of inoculated Giza 2000 plants were characterized by the appearance of four polypeptide bands with molecular masses of 52, 27, 14 and 5 kDa and the synthesis of two polypeptide bands with molecular masses of 17 and 11 kDa when completely inhibited in the same treatments.

The response of expressed proteins to the interaction between the salt stress and the bacterial inoculation varied among the two cultivars. Shoots of inoculated salt stressed Giza 123 and Giza 2000 were characterized by the synthesis of 7 kDa polypeptide. The formation of the two polypeptide bands with molecular masses of 68 and 37 kDa was inhibited in shoots of inoculated salt stressed Giza 123 plants compared with un-inoculated treatments. In cultivar Giza 2000, inoculation with *A. brasilense* (NO 40) resulted in the disappearance of three polypeptide bands with molecular masses of 37, 21 and 19 kDa in shoots of salt stressed plants when compared with the un-inoculated treatment.

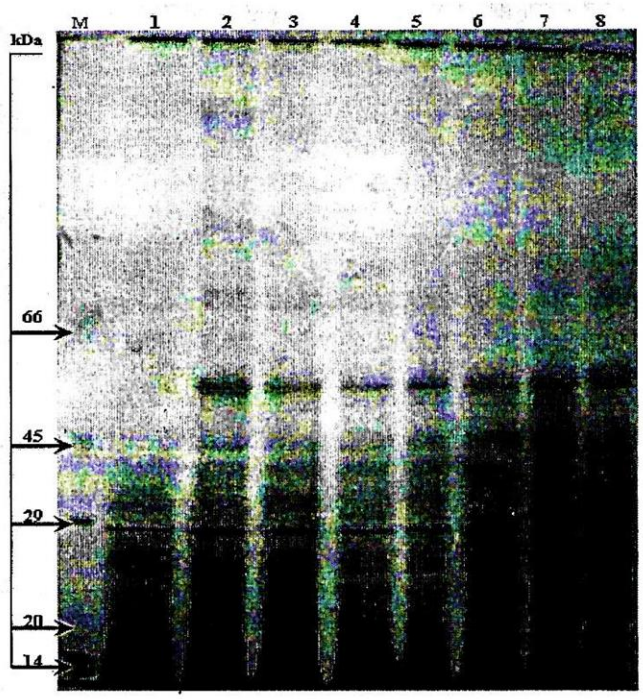

Figure 1. SDS-PAGE protein extracts of shoots of 30-day old barley seedlings of Giza 123 and Giza 2000 cultivars under 350 mM NaCl with and without *Azospirillum brasilense* (NO 40) inoculation

M- Marker protein 1- Giza 123, Control 2- Giza 123, 350 mM NaCl 3- Giza 123, Control+ Inoculation 4- Giza 123, 350 mM NaCl + Inoculation 5- Giza 2000, Control 6- Giza 2000, 350 mM NaCl 7- Giza 2000, Control+ Inoculation 8- Giza 2000, 350 mM NaCl + Inoculation

Table 1. SDS-PAGE protein extract of shoots of 30-day old barley seedlings of two cultivars (Giza 123 and Giza 2000) cultivated under 350 mM NaCl with and without *Azospirillum brasilense* (NO40) inoculation (M = Marker, MM = Molecular Mass, + = present, -- = absent)

M (kDa)	MM (kDa)	Giza 123				Giza 2000			
		Un- Inoculated		Inoculated		Un- Inoculated		Inoculated	
		Control	350 mM NaCl	Control	350 mM NaCl	Control	350 mM NaCl	Control	350 mM NaCl
--	73	+	+	--	+	--	+	--	+
--	70	--	--	+	--	+	--	+	--
66	68	--	+	--	--	--	--	--	--
	62	+	--	--	--	--	--	--	--
--	57	--	--	--	--	+	+	+	+
--	55	+	+	+	+	--	--	--	--
--	52	--	--	--	--	--	--	+	--
45	46	+	+	+	+	+	+	+	+
	40	+	+	+	+	+	+	+	+
--	37	--	+	--	--	--	+	--	--
--	35	--	--	+	--	+	--	+	--
--	32	+	+	+	+	--	--	--	--
29	29	+	+	+	+	+	+	+	+
--	27	+	+	+	+	--	+	+	+
--	25	+	+	+	+	+	+	+	+
--	23	+	+	+	+	+	+	+	+
20	21	+	--	+	--	+	+	+	--
	19	+	+	+	+	--	+	--	--
--	17	--	--	--	--	+	--	--	--
14	14	--	--	+	--	--	+	+	+
--	11	+	+	+	+	+	--	--	--
--	7	--	--	--	+	--	--	--	+
--	5	+	+	--	+	--	+	+	+

As shown in Figure 2 and Table 2 the electrophoretic separation of protein extract of 30-day old seedling roots of the two barley cultivars cultivated under 350 mM NaCl revealed that salt stress induced the synthesis of polypeptide bands with molecular masses of 67, 50, 37, 32, 22, 19 and 15 kDa in the roots of cultivar Giza 123. On the other hand, the disappearance of polypeptide bands with molecular masses of 86, 69,45,34,24 and 20 kDa were recorded in the same treatment compared with the control. In cultivar Giza 2000, the 350 mM NaCl stress resulted in the synthesis of several polypeptides in roots having bands with molecular masses of 76,71,63,55,43,40,37,24 and 15 kDa. Another set of polypeptide bands with molecular masses of 69, 59 and 17 kDa were disappeared in the roots of salt stressed Giza 2000 plant's compared with control.

A. brasilense inoculation resulted in a variation in the protein pattern in the roots of both cultivars. Five polypeptide bands with molecular masses of 40,37,22,15 and 13 kDa were detected in roots of the inoculated control treatment of Giza 123 plants, while another four polypeptide bands with molecular masses of 86, 71, 48 and 34 were suppressed in the same treatment compared with the un-inoculated treatments. In cultivar Giza 2000, bacterial inoculation caused the synthesis of four polypeptide bands with molecular masses of 55,43,37 and 24 kDa in roots and disappearance of another two polypeptide bands with molecular masses of 69,59 and 20 kDa which were detected in the roots of the un- inoculated plants.

Inoculated salt stressed seedling of cultivar Giza 123 was characterized by the presence of a polypeptide band with molecular mass of 81 kDa in the roots. Some new synthesized polypeptide bands with molecular masses of 69, 40, 24 and 13 kDa were formed in the roots of the inoculated salt stressed Giza 123 plants if compared with their corresponding un-inoculated counterparts. On the other hand, some of the polypeptides which were detected in the roots of salt stressed Giza 123 plants as polypeptide bands with molecular masses of 71, 67, 50, 32 and 15 kDa were never formed after the bacterial inoculation in the same treatment. In cultivar Giza 2000, the interaction between bacterial inoculation and salt stress resulted in the synthesis of two polypeptide bands with molecular masses of 67 and 45 kDa polypeptides in root and disappearance of another polypeptide bands with molecular masses of 71, 65, 63, 43 and 17 kDa in the roots compared with the un-inoculated salt stressed plants.

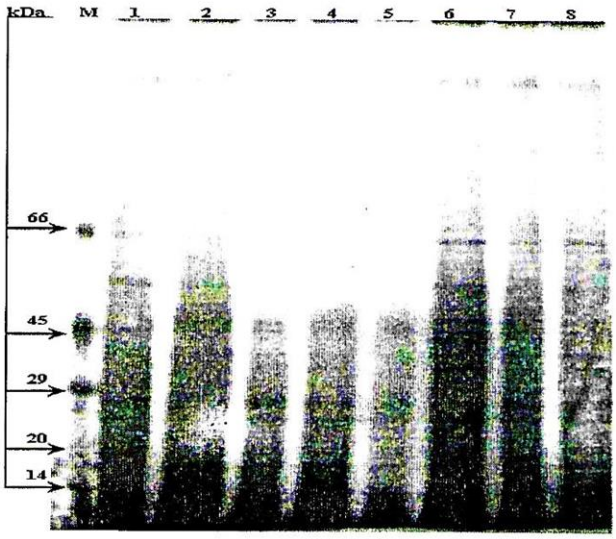

Figure 2. SDS-PAGE protein extract of roots of 30-day old barley seedlings of Giza 123 and Giza 2000 cultivars under 350 mM NaCl with and without *Azospirillum brasilense* (NO 40) inoculation

M- Marker protein 1- Giza 123, Control 2- Giza 123, 350 mM NaCl 3- Giza 123, Control+ Inoculation 4- Giza 123, 350 mM NaCl + Inoculation 5- Giza 2000, Control 6- Giza 2000, 350 mM NaCl 7- Giza 2000, Control+ Inoculation 8- Giza 2000, 350 mM NaCl + Inoculation

Table 2. SDS-PAGE protein extract of roots of 30-day old barley seedlings of two cultivars (Giza 123 and Giza 2000) cultivated under 350 mM NaCl with and without *Azospirillum brasilense* (NO 40) inoculation (M = Marker, MM = Molecular Mass, + = present, -- = absent)

M (kDa)	MM (kDa)	Giza 123				Giza 2000			
		Un- Inoculated		Inoculated		Un- Inoculated		Inoculated	
		Control	350 mM NaCl	Control	350 mM NaCl	Control	350 mM NaCl	Control	350 mM NaCl
--	101	+	+	+	+	+	+	+	+
--	86	+	--	--	--	+	+	+	+
--	81	--	--	--	+	--	--	--	--
--	76	--	--	--	--	--	+	--	+
--	71	+	+	--	--	--	+	--	--
--	69	+	--	+	+	+	--	--	--
66	67	--	+	--	--	--	--	--	+
	65	+	+	+	+	+	+	+	--
--	63	--	--	--	--	--	+	--	--
--	59	--	--	--	--	+	--	--	--
--	55	+	+	+	+	--	+	+	+
--	50	--	+	--	--	--	--	--	--
--	48	+	+	--	+	+	+	+	+
45	45	+	--	+	--	--	--	--	+
--	43	--	--	--	--	--	+	+	--
--	40	--	--	+	+	--	+	--	+
--	37	--	+	+	+	--	+	+	+
--	34	+	--	--	--	--	--	--	--
29	32	--	+	--	--	--	--	--	--
	28	+	+	+	+	+	+	+	+
--	26	+	+	+	+	+	+	+	+
--	24	+	--	+	+	--	+	+	+
--	22	--	+	+	+	+	+	+	+
20	20	+	--	+	--	+	+	--	+
--	19	--	+	--	+	--	--	--	--
--	17	--	--	--	--	+	--	+	--
14	15	--	+	+	--	--	+	--	--
	13	--	--	+	+	+	+	+	+
--	11	+	+	+	+	+	+	+	+

Expression of stress proteins is an important adaptive strategy of environmental stress tolerance. They are highly water soluble and heat stable, associate to cytoplasmic membranes and organelles and act as molecular chaperones. (Wahid and Close, 2006). In the present study, it could be demonstrated that stress-protein expression was induced in both shoots and roots in the seedlings of barley plants exposed to 350 mM NaCl. The expressed stress proteins were different among the two cultivars, but the molecular mass MM of the new formed polypeptide bands in the shoots of both cultivars were usually in the same ranges of MM which were from 27 kDa to 37 kDa and from 65 kDa to 75 kDa. Similar protein patterns were obtained in shoots of spring wheat by Ashraf and O'Leary (1996) they found that three new polypeptide bands with molecular mass of 29, 48 and 72 kDa were formed in shoots of spring wheat cultivated under 125 mM NaCl. Also, Kasim (2006) recorded the synthesis of polypeptide bands with molecular mass of 21.5 and 31 kDa in the *Vicia faba* seedlings cultivated under 200 mM NaCl.

The results of the present study also show that salt stress induced the formation of several polypeptides in roots of the two cultivars. The molecular mass of the formed polypeptides were found to be in the ranges of 30 to 43 kDa and 50 to 76 kDa. These results are in agreement with those reported by Goncalo *et al.* (2003) who found that several new proteins with molecular masses of 35 and 61 kDa were formed in roots of rice plant during its exposure to 170 mM NaCl. Formation of several polypeptides belonging to HSP family (60-100 kDa) in NaCl and/or heat stress-treated seedlings was confirmed in many crop plants such as wheat, rice and barley (Lee and Vierling, 2000). The HSPs perform several vital functions such as protein transport, folding, assembly and disassembly during growth and development of plants but their synthesis increased tremendously upon environmental stress (Kasim, 2006). Several polypeptides in the range between 15 kDa and 40 kDa act as molecular chaperones (Hettema *et al.,* 1998). Lee and Vierling (2000) and Heath *et al.* (2002) assigned four distinct functions to molecular chaperones; they can act as repair proteins, remove proteins that are irretrievably damaged, can facilitate the import of newly synthesized proteins into the interior of organelles such as the peroxisome and they act as antioxidant molecules in conjunction with protein. Molecular chaperones interact to protect against heat, water and salt stress through the repair of denatured proteins and these are evidences which were accumulated lead to an important role for heat shock proteins/molecular chaperones in stress resistance in plant systems (Harndahl *et al.,* 2001). It is thought that the molecular chaperones act to bind denatured proteins and to maintain them in a state that allows for ATP-dependent refolding by larger HSPs proteins (Lee and Vierling, 2000). Molecular chaperones can interact with

glutathione to protect against oxidative stress and stress resistance was dependent both on increases in reduced glutathione and on increases in expression of sHSPs, and was shown to decrease the levels of cellular ROS (Mehlen *et al.,* 1996). Grene (2002) demonstrated that sHSPs can protect against oxidative through increasing the antioxidant defense system in the cellular levels.

The present results indicated that bacterial inoculation was able to prevent the synthesis of most of the formed stress polypeptides band in shoots and roots of both cultivars under salt stress. It could be demonstrated that the formed polypeptides with molecular masses of 68 and 37 kDa in shoots of cultivar Giza 123 with 350 mM NaCl were inhibited after inoculation with *A. brasilense* (NO40) in the same treatment.

CONCLUSION

SDS-PAGE showed the denovo synthesis of two polypeptide bands with molecular mass of 68 and 37 kDa in the shoots of Giza 123 under 350 mM NaCl which were inhibited in their corresponding inoculated counterparts. In Giza 2000, salt stress resulted in the synthesis of polypeptide bands with molecular mass of 37, 21 and 19 kDa which were also disappeared in the inoculated treatments. Protein patterns of roots showed similar trend as that of shoots.

Generally it may be concluded that, salt stress resulted in the synthesis of some new polypeptides and in most cases inoculation with *A. brasilense* (NO40) inhibited the synthesis of some of these new polypeptides. It seems that with PGPR, the plants no longer need their innate defense mechanisms represented by the expression of stress proteins in the shoots and roots of barley plants to combat salinity stress. Application of PGPR seems to be a useful biological tool in agriculture to alleviate the negative effects of salinity and to improve salt tolerance of crop plants, although further studies are needed to assess the impact of this procedure on other micro-flora in the plants' rhizosphere.

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تأثير الملوحه على الخواص الجزيئيه للمحتوى البروتينى للمجموع الخضرى والجذرى لنباتات الشعير الملقحه بالازوسبيريلليم برازيلينز سلاله رقم NO40

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تمت دراسه تاثير التلقيح البكتيرى بالازوسبيريلليم برازيلينز سلاله رقم NO40 على الطرز البروتينيه لكل من المجموع الخضرى والجذرى لنباتات الشعير صنف جيزة ١٢٣ الغير مقاومه للملوحه و جيزة ٢٠٠٠ المقاومه للملوحه و لهذا الغرض اجريت تجربه فى صوبه معهد بحوث الاراضى والمياه والبيئه بالجيزة لتقيم تأثير التلقيح البكتيرى على الخواص الجزيئيه للمحتوى البروتينى فى السيقان والجذور لكل من اصناف الشعير تحت تأثير الملوحه بتركيز ٣٥٠ ملليمول كلوريد الصوديوم بطريقه SDS-PAGE. اوضحت النتائج ان تحت تأثير الملوحه والتلقيح البكتيرى منفردا او مختلطا حدث تغير فى الطرز البروتينيه للسيقان والجذور فى كل المعاملات .