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In vitro Growth of Wheat (*Triticum aestivum* L.) Seedlings, Inoculated with *Azospirillum* sp., Under Drought Stress

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Abstract: This research was conducted to determine: (1) the effects of drought on wheat seedlings growth underi *in vitro* and dark conditions and (2) if inoculation of wheat seedlings with *Azospirillum* sp. can alleviate the unfavorable effects of drought on the growth of wheat seedlings. *In vitro* planted seedlings were subjected to different drought intensities using poly ethylene glycol and were inoculated with 25 *Azospirillum* strains including the isolated ones and the standard strains of *A. halopreaferanse*, *A. brasilense*, *A. irakense* and *A. lipoferum*. Different strains of *Azospirillum* sp. enhanced seedlings growth and adjusted their water behavior under drought. Such results in combination with the previously related results indicate that *Azospirillum* sp. are able to enhance plant growth and production under different physiological and ecological conditions.

Key words: Drought stress, *Azospirillum* population, plant growth promoting bacteria, turgor weight, coleoptile length

INTRODUCTION

The rate of seedlings growth under stress determine plant growth and hence corp production. Wheat seeds are able to germinate and grow under relatively dry conditions. However, under such conditions seedlings growth are very much influenced by drought stress affecting plant growth and production (Palta, 1990).

Wheat is planted in different arid and semi arid parts of the world and hence its germination rate and growth is very much under the influence of soil water potential. The successful establishment of seedlings is a function of appropriate coleoptile development, affected by soil osmotic potential. Hence, using any method to alleviate drought stress on coleoptile growth and development can enhance plant growth and its subsequent production (Creus *et al*., 1998).

Leaf water conditions affect plant growth and production. Leaf water potentials in the range of 10 to 15 atm influence most plant physiologiacl processes including leaf development, stomatal activities, photosynthesis rate and nitrogen (N) metabolism. In addition, water potential of stressed leaf at the flowering stage affects leaf development and growth resulting in smaller leaf size (Aggarwal and Sinha, 1984). As cellular turgesance is a necessary step for plant growth, under drought stomatal activities is affected decreasing leaf growth and development.

Researchers indicated that exposing wheat seedlings to osmotic stress using 20% Poly Ethylene Glycole (PEG) 6000 decreased shoot height, plant fresh weight and total protein concentration relative to the control treatments. However, inoculation of wheat seedlings with *Azospirillum* sp. could partially alleviate the drought stress on seedlings growth through adjusting plant water behavior and N fixation (Pereyra *et al*., 2006). *Azospirillum* sp. are able to affect plant growth by altering root metabolism and promoting plant hormones production (Dobbelaere *et al*., 1999; Pereyra *et al*., 2006). Effects of *Azospirillum* sp. on plant growth subjected to drought stress are previously documented, however there is little data related to such effects *in vitro* and in the darkness (Pereyra *et al*., 2006), which were addressed in this research .

Plant cultivar affected coleoptile growth very much, in seedlings inoculated with *Azospirillum*, in the darkness and in the presence of 20% polyethylene glycol after 72 h. Accordingly, compared with control, a 22% significantly increase in the coleoptile length of wheat (*T. aestivum*) seedlings was observed. However, such a difference was not significant for the cultivar *T. durum* (Creus *et al*., 1998). In addition, the amounts of

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magnesium and potassium were decreased in non-irrigated and un-inoculated treatments, while they were significantly increased in the grains of wheat plants inoculated with *Azospirillum* sp. (Creus *et al*., 1998).

Drought also decreases soil microbial population and activities. *Azospirillum* sp., which are among Plant Growth Promoting Rhizobacteria (PGPR) are able to survive in the rhizosphere of different plant species and also have a tendency to inoculate non-specific hosts. The results on the survival of different species of *Azospirillum* in non-rhizospheric soils and in tropical and temperate soils are controversial, however, it has been stated that their population under such conditions decreases. Under temperate and dry climates, the aging bacteria turn into C-form shapes resulting in their enhanced stress tolerance compared with regular cells. The bacteria are also able to produce fiber products, binding them to the surrounding environment in sandy soils. Such mechanism differentiates the bacteria from other PGPR such as *Pseudomonas* sp., which are leached into deeper soil by water (Bashan *et al*., 1995).

The reasons for the enhanced ability of plants inoculated with *Azospirillum* sp. under drought are increased cellular division in the roots, lateral roots and root hairs, decreased distance between root tip and root hairs and increased number of root hairs (Michiels *et al*., 1989) resulting in enhanced water and nutrients uptake by plant roots, especially from the deeper soil. However, in another research the results indicated that the adjusted nutrients uptake is not the only mechanism affecting the growth of inoculated plants in comparison with un-inoculated plants. In addition, in a non-nourished hydroponic experiment, inoculation with the species of *A. brasilense* 245 alleviated water stresses on the growth of seedlings as the rate of coleoptile growth increased (Creus *et al*., 1998).

Since, there is little data related to the alleviating effects of *Azospirillum* sp. on wheat growth under *in vitro* and in the darkness the presented research was conducted hypothesizing that *Azospirillum* sp. is able to alleviate drought stress on plant growth under such conditions. The objectives were to determine: (1) the effects of drought on wheat seedlings growth *in vitro* and in the darkness and (2) if inoculation of wheat seedlings with *Azospirillum* sp. can alleviate the unfavorable effects of drought on the wheat seedlings growth.

MATERIALS AND METHODS

Seed sterilization and germination: The experiment was conducted in 2007 at the College of Agriculture, Mazandarn University. Seeds of wheat (*Triticum aestivum* L.), cultivar Chamran, with similar size and weight were washed with tap water three times and soaked in alcohol 96% for 15 S. The extra alcohol was removed and seeds were washed with distilled water. Seeds were then sterilized for 5 min using soidium hypochlorite 3% and rinsed with distilled water 5 times. The sterilized seeds were soaked in distilled water for 3 h and 20 of them were grown in Petri dishes containing agar solution (10 g agar L^{-1} water) and were incubated for 48 h at 20°C. The grown seedlings were sandewhiched inside 6×12 cm Whatman filter papers (No. 42) with a 1×2 cm spacing from the nearby seedlings (six seedlings per each filter paper). The rolled filter appears were then transferred to laboratory tubes while moisturized with 1 mL of distilled water. The tubes were incubated for 48 h in the dark at 20°C. After this stage the length of coleoptiles and roots were 2 and 4 cm, respectively.

Inocolums preparation: Twenty five indigenous isolates including AZ1, AZ3, AZ5, AZ8, AZ9, AZ10, AZ11, AZ13, AZ17, AZ24, AZ25, AZ28, AZ30, AZ33, AZ35, AZ38, AZ40, AZ42, AZ43, AZ45, AZ47, AZ50, AZ53, AZ54, AZ71 and four standard isolates including *A. halopreaferanse*, *A. brasilense*, *A. irakense* and *A. lipoferum* (supplied by DSMZ Company) were selected for the experiment. The bacterial suspensions were prepared according to the method of Lin *et al.* (1983).

Seedlings inoculation with different strains of *Azospirillum* sp.: The tubes containing wheat seedlings were inoculated with 3 mL of bacterial inoculum with the population of 3×10^9 per mL. After 3 h the extra inoculum were removed from the tubes and the tubes were rinsed with distilled water. From each tube one seedling was selected for counting the bacterial population. The seedlings were then placed in tubes with distilled water for 3 days. The amount of distilled water in the tubes were kept constant by replacing the evaporated water. The distilled water in the tubes was then replaced with the solution of 2.41 and 1.64% of poly ethylene glycol 6000 on the fourth day and the tubes were kept under such conditions for 72 h. Such concentrations of poly ethylene glycol results in the osmotic potentials of 0.8 and 0.363 MPa (8 and 3.63 bars), respectively (Money, 1989). The control treatment was inoculated with the autoclaved AZ9 strain. After 72 h shoot water conditions (Creus *et al*., 1998) and shoot length were determined.

Plant measurements: Fresh (FW) and Dry Weight (DW) of seedlings and the weight of shoot part under turgesance, as well as the weight of coleoptile and primary leaves (made into pieces and soaked in distilled water for

72 h) were determined. The pieces, from the cut end were put in tubes containing distilled water in the dark for 4 h. The samples were then kept at 5°C for 24 h. After removing the extra water using tissue paper the samples were immediately weighed (TW). After placing the shoot samples in glass containers they were dried for 24 h at 65°C. The dried samples were weighed (DW). Relative Water Content (RWC) was calculated using the following formula:

$$RWC=(Fw-Dw)/(Tw-Dw)\times 100$$

where, Fw is shoot fresh weight, Tw is shoot weight under turgesanc and Dw is shoot dry weight (Creus *et al*., 1998).

Experimental design and statistical analysis: The experiment was a factorial including bacterial strains (25) and drought intensities (three levels) as the experimental factors in three replicates. Data were subjected to the analysis of variance using SAS (SAS Inc., 1999). Mean comparison was conducted using the Least Significant Difference (LSD) method at 5% level of probability (Steel and Torrie, 1980).

RESULTS and DISCUSSION

The data related to the number of *Azospirillum* sp. on the roots of young roots, un-inoculated, inoculated with bacterial inoculum and inoculated with autoclaved inoculum are presented in Table 1. Not any *Azospirillum* sp. was observed on the roots in treatments including the un-inoculated and autocalved inoculum before and after subjecting the seedlings to the drought stress. In the drought treatments the highest bacterial population ranged from 3.21×10^6 to 3.14×10^6 per gram root weight for the control and the highest drought intensity treatments, respectively. At the highest drought intensity the population of *Azospirillum* sp. decreased from 3.14×10^6 to 7.8×10^5 before and after subjecting the seedlings to drought, respectively.

Table 1: *Azospirillum* sp. population, grown in a semi solid NFB medium, on the roots of wheat seedlings using the Most Probable Number (MPN) method before and after subjecting to drought stress

Drought intensity	Inoculation treatments	*Azospirillum* population on the seedling roots (Cells g^{-1} fresh weight) Before	After
S_0	B_0	0	0
	B_1	0	0
	B_2	3.21×10^6	3.40×10^6
S_1	B_0	0	0
	B_1	0	0
	B_2	3.18×10^6	2.80×10^6
S_2	B_0	0	0
	B_1	0	0
	B_2	3.14×10^6	7.80×10^5

B_0: Un-inoculated, B_1: Inoculated with autoclaved bacteria, B_2: Inoculated with bacterial inoculum. S_0: Drought control treatment, S_1: First drought intensity (4 bar), S_2: Second drought intensity (8 bar)

Effects of experimental treatments on seedlings growth: Table 2 presents the analysis of variance related to wheat growth paramtres under the experimental treatments. Accordingly, the effects of drought intensity, *Azospirillum* sp. and their interaction were significant on most seedlings growth parameters at p = 0.05. The only non-significant results are the interaction effect of drought intensity and *Azospirillum* sp. on shoot dry weight and coleoptile length. Both the indigeneous and the standard strains of *Azospirillum* sp. enhanced the growth of seedlings under different drought stress, among which AZ1, AZ54 and AZ45 as well as *A. lipeoferum* are the most effective strains on seedling growth (root and coleoptile growth) in the first drought stress. In the second drought stress *A. lipeoferum*, AZ11 and AZ45 significanlty enhanced seedlings growth, compared with control and other strains. In the third harvest level also a similar pattern was observed, however the effects of *A. lipoferum* was not significantly different form control and other *Azospirillum* strains (Table 3-5). These results indicate the effectiveness of bacterial inoculation on drought stress relative to the uninoculated treatments (Table 6).

Although, with time plants have evolved different mechanisms to alleviate the unfavorable effects of different stresses on their growth and production, using biological methods such as soil symbiotic microbes have also been proved to be very effective on the alleviations of different stresses (Miransari *et al.* 2007, 2008, 2009a, b; Miransari and Smith, 2007, 2008, 2009).

There was a little decrease in the population of *Azospirillum* sp. before and after subjecting the bacteria to the drought stress. We have previously indicated that *Azospirillum* sp. can interestingly survive high drought intensities, or it is able to tolerate soil moisture fluctuations with very little population changes (unpublished data). Different mechanisms such as water adjustment or the ability to enhance water use efficiency by the bacteria may indicate such ability. Such abilities along with the enhanced plant growth when in symbiosis with the bacteria may indicate the superior performance of host plant under drought stress. These results are complementary to the resutls, previoulsy indicating the alleviating effects of *Azospirillum* sp. on wheat growth under drought.

There are previously documented results on the alleviating effects of *Azospirillum* sp. on plant growth under drought stress, however there is little data

Table 2: Analysis of variance for wheat (Chamran cultivar) growth parameters, grown in the dark

		Mean of squares				
SOV	df	Shoot fresh weight	Shoot turgor weight	Shoot dry weight	Shoot relative water content	Coleoptile length
Drought intensity (A)	2	0.02140864*	0.04395*	0.0003049*	16.841*	87.696*
Bacterial isolate (B)	26	0.000752393*	0.008165*	0.00011396**	187.204*	5.056*
Replicate	2	0.00084444**	0.008165**	0.00008272**	10.739**	0.169
A×B	52	0.00331847*	0.003934*	3.83E-05	49.523*	1.124
Error	160	0.000547	0.000917	2.86E-05	26.098	1.250203

*,**Significant at 5 and 1% of probability, respectively

Table 3: Effects of *Azospirillum* sp., isolates on wheat growth parameters in the first drought intensity

Bacterial strains	Shoot fresh weight (g)	Shoot turgor weight (g)	Shoot dry weight (g)	Shoot relative water content (%)	Coleoptile lenght (cm)
AZ1	0.330abcd	0.360bcdef	0.037abc	90.857a	17.71ab
AZ11	0.307abcd	0.347def	0.037bc	87.503ab	17.29abc
AZ40	0.283efghi	0.323efg	0.037abc	87.227abc	16.79abcde
AZ54	0.353a	0.400abc	0.043a	87.067abc	18.06a
AZ28	0.287defgh	0.327efg	0.033cde	86.517abcd	16.94abcde
AZ10	0.240ijkl	0.277gh	0.027de	84.680abcde	15.0fg
AZ45	0.300bcdef	0.360bcdef	0.037abc	81.187bcdefg	17.55ab
AZ10A	0.243hijkl	0.313fgh	0.030cde	75.107fghij	15.49efg
AZ45A	0.307abcd	0.373bcde	0.040ab	81.010bcdefg	16.67abcdef
Control	0.243hijkl	0.317gh	0.037abc	73.153ghij	14.72g
A. lipoferum	0.293cdefg	0.357cdef	0.040ab	80.000bcdefgh	17.21abcd
A. brasilense	0.287defgh	0.357cdef	0.030cde	77.683defgh	17.30abc
A. irakense	0.267efghij	0.347def	0.037abc	74.287ghij	16.92abcde
A. haloperaferance	0.300bcdef	0.353cdef	0.04ab	84.200abcdef	16.68abcdef
LSD	0.0434	0.0518	0.01	9.1172	1.7103
Mean sqaure of error	0.000703	0.000999	0.000037	30.96531	1.089686
df of error	52.0	52.0	52.0	52.0	52.0

Values with different letter(s) are not significantly different

Table 4: Effects of *Azospirillum* sp., isolates on wheat growth parameters in the second drought intensity

Bacterial strains	Shoot fresh weight (g)	Shoot turgor weight (g)	Shoot dry weight (g)	Shoot relative water content (%)	Coleoptile lenght (cm)
AZ1	0.267cdefg	0.317cde	0.033abc	82.803cde	16.51abcd
AZ11	0.317a	0.373ab	0.040a	84.007bcd	16.93a
AZ40	0.227hijklm	0.283defgh	0.030bcd	77.450efghi	16.09abcdef
AZ54	0.223ijklm	0.260ghi	0.030bcd	83.493cde	16.96a
AZ28	0.273bcde	0.297defg	0.033abc	89.690ab	16.77ab
AZ10	0.207klmn	0.250ghi	0.030bcd	78.940defgh	14.78efg
AZ45	0.290abcd	0.380a	0.037ab	74.05 ijk	16.87a
AZ10A	0.177n	0.230i	0.023d	73.877hijk	15.00defg
AZ45A	0.230hijkl	0.290defgh	0.033abc	74.783hijk	15.39abcdefg
Control	0.207klmn	0.283defgh	0.033abc	69.037k	14.57fg
A. lipoferum	0.260defgh	0.323cd	0.037ab	78.537defghi	16.42abcd
A. brasilense	0.250efghi	0.317cd	0.027cd	77.410efghi	16.20abcde
A. irakense	0.200lmn	0.250ghi	0.027cd	79.220defgh	16.71ab
A. haloperaferance	0.270bcdef	0.323cd	0.033abc	82.453cdef	16.58abcd
LSD	0.0354	0.047	0.0081	6.1921	1.6159
Mean sqaure of error	0.000466	0.000836	0.000024	14.28303	0.972667
df of error	52.0	52.0	52.0	52.0	52.0

Values with different letter(s) are not significantly different

regarding such effects in the absence of light and *in vitro*. Under such conditions plant growth can be evaluated irrespective of photosynthesis effects. This may be of great physiological significance as the mechanisms related to the stress alleviation can be elucidated under conditions, which are not interfering with light presence. Light is one of the most important parameters necessary for the process of photosynthesis and hence for plant growth and crop production. Interfering light presence with mechanisms related to the alleviation of stress may not exactly indicate the effects of such mechanisms on how the stress can be alleviated. This indicates the significance of this research.

Drought stress affects cellular membrane properties eventually resulting in enhanced membrane permeability. Accordingly, the rate of membrane leakage can determine the rate of membrane structural changes by drought stress (Blum and Ebercon, 1981). Furthermore drought alters the activity of enzymes like reductases, in the cellular membrane (Clemensson-Lindell, 1994).

According to the results different species of *Azospirillum* sp. have been able to alleviate drought stress on wheat growth relative to the control treatments. Although, according to the results *A. lipoferum* and AZ1 and AZ45 are the most influential strains on drought stress however, in some cases not similar patterns of

Table 5: Effects of *Azospirillum* sp., isolates on wheat growth parameters in the third drought intensity

Bacterial strains	Shoot fresh weight (g)	Shoot turgor weight (g)	Shoot dry weight (g)	Shoot relative water content (%)	Coleoptile lenght (cm)
AZ1	0.263defgh	0.327defgh	0.040ab	77.777abcde	16.07ab
AZ11	0.283cde	0.343bcdef	0.040ab	79.693abcde	16.18a
AZ40	0.240fghij	0.297fghij	0.033bcd	78.340abcde	15.38abcde
AZ54	0.333ab	0.387ab	0.037abc	84.550a	13.59efg
AZ28	0.270cdefg	0.323cdefgh	0.037abc	81.930abc	16.18a
AZ10	0.247fghi	0.287ghij	0.030cde	84.720a	13.99bcdefg
AZ45	0.360a	0.427a	0.043a	82.457abc	14.80abcdef
AZ10A	0.230hijk	0.307efghi	0.030cde	73.627cdefg	14.19abcdefg
AZ45A	0.243fghi	0.323cdefgh	0.033bcd	73.287cdefg	13.88defg
Control	0.207jkl	0.303fghi	0.030cde	65.700g	12.15g
A. lipoferum	0.303bc	0.353bcde	0.037abc	83.867ab	15.24abcde
A. brasilense	0.300cde	0.360bcd	0.037abc	81.677abcd	15.07abcdef
A. irakense	0.283cde	0.367bc	0.040ab	74.537bcdefg	13.66cfg
A. haloperaferance	0.230hijk	0.307efghi	0.033bcd	73.397cdefg	14.47abcdef
LSD	0.0359	0.0484	0.0078	9.3315	2.0937
Mean sqaure of error	0.00048	0.000873	0.000023	32.43809	1.632947
df of error	52.0	52.0	52.0	52.0	52.0

Values with different letter(s) are not significantly different

Table 6: Effects of different drought intensities on wheat growth parameters for un-inoculated treatment and autoclaved inoculated treatment (AZ10A)

Drought intensity	Shoot fresh weight (g)	Shoot dry weight (g)	Turgor weight (g)	Relative water content (%)	Coleoptile length (cm)
Un-inoculated					
S_0	0.243	0.037	0.316	73.15	14.72
S_1	0.206	0.033	0.283	69.04	14.57
S_2	0.206	0.030	0.303	65.70	12.15
Autoclaved treatment					
S_0	0.243	0.030	0.313	75.15	15.49
S_1	0.23	0.030	0.306	73.63	15.00
S_2	0.176	0.023	0.230	73.88	14.19

S_0: Drought control treatment, S_1: First drought intensity (4 bar), S_2: Second drought intensity (-8 bar), Values with different letter(s) are not significantly different

stress alleviation by *Azospirillum* strains were observed, which is also indicated by the significant interaction between drought intensity and *Azospirillum* sp. In addition, the ability of some microorganisms such as bacteria and arbuscular mycorrhiza to alleviate the stress increases with enhancing the stress level (Miransari and Smith, 2007, 2009; Miransari *et al*., 2007, 2008). These results are in agreement with the resutls of other researchers who have previously indicated that different *Azospirillum* strains may behave differently under different stress conditions. This has been partly related to different ecological and environmental conditions such as soil properties and bacterial abilities to establish symbiosis with the host plant. It should also be mentioned that the improved strains have the ability to compete with the indigenous soil bacteria, colonize and eventually enter the host root more efficiently (Ramos *et al*., 2002).

Scientists have indicated the mechanisms affecting the performance of host plant inoculated with *Azospirillum* sp. Usually the biochemical combination of inoculated plants under stress differs relative to the control plants. In addition more favorable water and elastic characters can also help alleviate the stress (Pereyra *et al*., 2006). It has been indicated that *Azospirillum* sp. enter the host roots from the place where the lateral roots emerge (Dobereiner, 1992; Egener *et al*., 1998). Hence, the higher the number of root hairs the higher the probability of root colonization by *Azospirillum* sp. Under stress plant allocate more carbon to the roots (Miransari and Smith, 2007, 2009; Miransari *et al*., 2007, 2008). Accordingly, the higher root growth under drought the higher the probability of root colonization. It has also been previously observed that inoculated wheat seedlings have a very developed root network under both control and drought conditions (Pereyra *et al*., 2006). The combination of such mechanisms by the host plant and the symbiotic *Azospirillum* sp. can alleviate the unfavorable effects of drought stress on plant growth and production.

With regrad to the presented results *Azospirillum* sp. are also able to alleviate drought stress on plant growth in the absence of light indicating that the related alleviating mechanisms are not related to light presence and hence photosynthesis process. This can be of great physiological and ecological significance. There are very little data on the alleviating effects of *Azospirillum* sp. on drought stress under darkness and *in vitro*. Almost all the previously presented results have been conducted under greenhouse or field conditions.

CONCLUSION

Because there is little data regarding the alleviating effects of *Azospirillum* sp. on wheat growth, subjected to drought stress, *in vitro* and in the darkness, the presented results are complementary to the previously little documented data. Strains *A. lipoferum*, AZ11 and AZ45 are the most effective ones alleviating drought stress on wheat seedlings growth. Furthermore, such results in combination with the previoulsy related results indicate that *Azospirillum* sp. are able to enhance plant growth and production under different physiological and ecological conditions. Apparently, there are some very interesting mechanisms developed under drought stress, which help *Azospirillum* sp. survive under such conditions.

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