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Effect of *Pseudomonas fluorescent* on Proline and Phytohormonal Status of Maize (*Zea mays* L.) under Water Deficit Stress

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ABSTRACT

Phytohormones play critical roles in regulating plant growth and its response to stress. This experiment was conducted to study the relationship between water deficiency and Pseudomonas fluorescent on proline amino acid and some phytohormones in maize (704 Hybrid). Experimental design was split plot in the form of randomized complete block design (RCBD) with three replications. Treatments included four Pseudomonas strains and a non-inoculated control treatment as sub plots in three levels of water deficit according to 40% (control), 60% and 75% of available soil moisture depletion. Results showed that drought stress triggered a change in plant phytohormonal balance, including an increase in leaf proline and abscisic acid (ABA) content, and a decline in auxin, gibberelline and cytokinin. However, inoculated plants had increased proline, abscisic acid, auxin, gibberelline and cytokinin content. Plants inoculated with P. fluorescens strain 153 showed the highest mean of proline, abscisic acid, auxin, gibberelline and cytokinin content in the leaves. This study indicates that application of PGPR can enhance phytohormones content of maize under water deficit stress condition.

Keyword: PGPR, Proline, Phytohormones, Maize, Water deficit.

INTRODUCTION

Limitation of groundwater resources is a widespread growing problem for cultivation of agricultural crops. In nature, any shortage of water occurs as a result of a water deficit or drought and therefore is called a water deficit stress (shortened to water stress) or drought stress [21]. Drought stress is one of the main limiting factors in crop production because it affects almost all plant functions [15]. Although the general effects of drought on plant growth are well known, its effect at the biochemical and molecular levels is not well understood [4]. Water stress tolerance is seen in all plant species but its extent varies from species to species. Improving the efficiency

of water use in agriculture is associated with increasing the fraction of the available water resources that is transpired, because of the unavoidable association between yield and water use [8]. For the last few decades, several scales of physiological works have been conducted under drought stress in crop plants, but it is not so with respect to medicinal plants [26, 35]. Osmotic adjustment is one of the most usual responses of plant to environmental stresses, especially osmotic variation of environment (as a result of drought or salinity stress).

In physiological mechanism, plant cell concentrate some ions in their vacuoles and some metabolites such as amino acids (mainly proline), monosaccharides, etc., in their cytozole. This will decrease osmotic potential and keep cell turgor pressure at high level to allow plants continue their physiological processes.

Numerous microorganisms live in the portion of soil modified or influenced by plant roots so called 'rhizosphere'. Among these microorganisms, some have positive effects on plant growth promotion constituting the plant growth promoting rhizobacteria (PGPR) such as *Azospirillum*, *Azotobacter* ,*Pseudomonas fluorescens*, several gram positive *Bacillus* sp. Certain bacteria like Pseudomonas survive under stress conditions due to the production of exopolysaccharides (EPS), which protects microorganisms from hydric stress and fluctuations in water potential by enhancing water retention and regulating the diffusion of carbon sources in microbial environment [32, 33]. In plants certain secondary metabolite pathways are induced by infection with Microorganisms.

The deleterious effect of auxin on root development is often mediated by ethylene. Auxin produced by bacteria in the rhizosphere can stimulate the activity of the 1-aminocyclopropane-1-carboxylate (ACC) synthase, an enzyme normally used by plants to form ethylene [14]. Another example of the necessity for adjustment of the auxin level is given by the isolation of a *P. putida* IAA producing strain which promotes the root elongation of its host plant [5]. Analysis of this PGPR effect revealed that an increased amount of ACC is produced by the plants exposed to bacterial auxin. Exuded ACC is hydrolyzed by an ACC deaminase, a bacterial enzyme known to be present in the PGPR *Enterobacter cloacae* and several *Pseudomonas* strains [29]. The uptake and subsequent hydrolysis of ACC by the PGPR decrease the amount of ACC outside the plant which must exude increasing amounts of ACC to maintain the equilibrium between internal and external ACC levels. The bacterium takes advantage of this situation by using ACC as a source of nitrogen and the plant shows a better root elongation as its internal level of ethylene decreases. As ethylene appears to be deleterious for plant growth, it is surprising that plants synthesize this compound. Since ethylene is not only a stress response hormone but also a growth response hormone, plants may have to deal with their physiological need of ethylene on one hand, and with the deleterious impact of ethylene on root elongation on the other hand. In fact, some bacteria have the ability to synthesize ethylene but, probably because of its damaging effect on root growth, reports concerning its production by bacteria are limited to deleterious interactions with the host-plant [13].

Relatively few mechanisms have been unequivocally demonstrated to explain the increased resistance to environmental stresses including water stress of plants treated with plant growth-promoting bacteria. The mechanisms that have been suggested include reduction of stress ethylene production via the action of ACC deaminase and increased expression of the ERD15 gene, which is responsive to drought stress [28]. Investigations into how drought stress affects plant hormone balance revealed an increase in abscisic acid (ABA) content in the leaves,

indicating that the reduction of endogenous cytokinin levels magnifies ABA content, eliciting stomata closure [17].

Auxin in combination with cytokinin stimulates cell division. Although reaction of auxin and cytokinin to drought stress is not well defined yet, some researchers represent that auxin and gibberllin levels in plants will decline under drought stress. Water stress can also make lower cytokinin level in xylem exudates and detach leaves.

Microorganisms could play a significant role in stress management, once their unique properties of tolerance to extremities, their ubiquity, and genetic diversity are understood and methods for their successful deployment in agriculture production are developed. These organisms also provide excellent models for understanding stress tolerance mechanisms that can be subsequently engineered into crop plants. The present work uses maize because it is an important human and animal food source, and there is a great need in Iran to narrow the gap between cereal crop production and consumption.

MATERIALS AND METHODS

Bacterial isolates and plant material

Four *Pseudomonas fluorescent* including *P. fluorescens* strains 153 and 169 and *P. putida* strains 4 and 108 were selected from Bacterial Culture Collection of Soil and Water Research Institute (SWRI) according to their plant growth promoting traits. Bacterial isolates were grown in nutrient broth for two days and 100 ml of culture suspension (population density ca. 10^8 $CFUml^{-1}$) was added to a polypropylene plastic bag containing 35 g of sterile powdered perlite and used as seed inoculant. Seeds of maize line SC 647 were obtained from Seed and Plant Improvement Research Institute and used as plant material in this study.

Experimental design and treatments

Field experiments were conducted at the research farm of Islamic Azad University, Miyaneh Branch, Iran, (located at 48º9´E, 37º43´N, elev. 1260m) for two consecutive years. The field lies in the semi-arid zone with a clay loam soil. The physico-chemical properties of the soil was 0.42 dS m^{-1} electrical conductivity, pH 7.3, 0.5% organic carbon, 0.154% N, and content of nutrients (mg kg^{-1}) including P, K, Zn, Fe, Mn and Cu were 14.61, 22.5, 1.6, 2.8, 10.0 and 1.3, respectively. Experiments were conducted in split plot arrangement in the form of randomized complete block design (RCBD) with three replications. Irrigation water deficiency at 40% (control, T40%), 60% (T60%) and 75% (T75%) of available soil moisture depletion was used as main factor. In order to measure the percentage of available soil moisture depletion, soil moisture blocks (chalk blocks) were installed in all plots, 30 cm below soil surface and connected to soil moisture meter by the means of fully isolated wires.

Field was prepared in conventional method (moldboard plow, 2 disks and leveler). Experimental plots measured 4.5 m × 9.0 m, with 0.7 m between rows, and plots separated by --- m terracing. Before sowing, plots received 150 kg urea ha^{-1}, 150 kg single super phosphate ha^{-1} and 50 kg potassium sulfate ha^{-1} according to soil analysis. At reproductive stage 150 kg urea ha^{-1} was also applied to plots. Four bacterial strains as well as a non-inoculated control were applied as sub-plot treatments. Seeds were inoculated by adding 10 g of inoculant for one kg of seeds following moistening the seeds with 15 ml of a 40% (w/v) gum-arabic solution to increase adherence and then planted in the rows. At the third leaf stage, plants were thinned to one plant per hill for the

appropriate final stand of 75000 plants ha^{-1}. Field was also weeded by hand continuously until maize canopy dominated weeds.

Determination of proline and phytohormones content in plants

In order to determine proline and phytohormones, one plant was harvested together with root at the beginning of flowering stage.

To determine auxin, gibberellin and cytokinin concentration, a Unicam model HPLC was used for extraction in Isocratic method. Auxin and cytokinin were extracted in a C18-HiqSil column (5µm × 4.6mm × 25cm) and gibberellin was extracted in a Zarbax SB-S18 column (3.5µm × 15cm) and standard solutions were for all hormones as 1g/Li in 20% methanol. Formic acid (1%) was added to these solutions and samples were kept at 4ºC.

For extraction of gibberellins, 1 g of plant leaf was placed in a solution containing methanol – water - acetic acid (30-70-1) and homogenized by the means of a homogenizer. The solution was then centrifuged at 3000 round for 15 minutes. At last, upper solution was injected in C18-SPE column and eluted at 10ml solution of ethanol-water-acetic acid (80-20-1). The extracted solution was dried in room temperature using a refrigerant and 1 ml of methanol was added again to make it the final solution for hormones extraction. For auxin and cytokinin extraction, 1.5 g of plant tissue was passed through 80% methanol. The extracted solution was dried at room temperature by refrigerant and 1 ml of 20% methanol, formic acid (1%) and 1 ml of 80% methanol were added. This final solution was used to measure hormones content.

Abscisic acid (ABA) was quantified according to Zhou *et al*., (2003) method. To do this, LC/MS HPLC column (3.5µm × 1.2mm × 50mm- Sun fire, waters USA) was used. To measure proline amino acid, bates (1973) method was used. Sample unit is micromole in gram of fresh leaf. Standard was prepared to measure proline and 1% pure proline was used to provide 1-160 µmol concentrations.

Data were subjected to combined analysis using SAS and means were compared by Duncan´s Multiple Range Test.

RESULTS AND DISCUSSION

Bacterial strains used in this study had obvious plant growth promoting traits. Some characteristic of bacterial strains have been shown in Table 1.

Results of the current study showed that drought stress have profound effect on proline and phytohormones content of maize plants. Inoculation by bacterial strains had similar significant effect on proline and phytohormones level of the plants (Table 2).

Proline

Proline content of the leaves was significantly affected by drought stress and increased by declining the water availability in the soil. The highest proline content was observed in T75% treatment and showed 63% and 8% increase compared to T40% and T60% treatments, respectively (Table 3).

Proline content of the leaves was also affected by PGPR strains used in this study. All strains were able to significantly increase proline content of the leaves as compared to control treatment.

P. fluorescens strain 153 was the most effective bacteria and increased proline content of the leaves by 133%. The highest amount of praline (75.06 µmol/g.fw) was observed in plants grown in 75% water depletion and inoculated with strain 4.

Proline is an important osmoregulator, accumulated as a consequence of drought stress. Creus *et al.,* (1998) studied the effects of *A. brasilense* Sp245 inoculation on water relations in two wheat cultivars [6]. They found that *Azospirillum* stimulated growth of wheat seedlings grown in darkness under osmotic stress, together with a significant decrease in osmotic potential and relative water content at zero turgor, in volumetric cell wall modulus of elasticity, and in absolute symplastic water volume and by a significant rise in apoplastic water fraction parameters. Increased production of prolinee along with decreased electrolyte leakage, maintenance of relative water content of leaves and selective uptake of K ions resulted in salt tolerance in *Zea mays* coinoculated with Rhizobium and Pseudomonas [1].

Auxin

ANOVA showed significant effect of interaction water deficit and pseudomonas strains on leaf auxin content at P≤0.05 (Table 1). Mean comparison of interaction water deficit and pseudomonas strains also showed that highest auxin content in T40% (normal irrigation) from S4 strain (759.6nmol/g.dw) and in T60% and T75% from S153 (708.6 nmol/g.dw and 586.6 nmol/g.dw) was obtained. Among three water deficit levels, lowest auxin content was observed in control (no inoculation). The impact of auxin on root morphogenesis ranges from beneficial to negative effects. The optimal concentration range may be extremely narrow as demonstrated by the isolation of a PGPR *Pseudomonas putida* strain producing indolacetic acid (IAA) and of a deleterious IAA overproducer mutant which produces four times the amount of IAA synthesized by the wild-type strain [14]. A similar conclusion could be drawn from the effect of several concentrations of *Pseudomonas thivervalensis* (an IAA-producing strain) on *Arabidopsis* root length and branching [11]. Many bacterial species are capable of producing auxin and/or ethylene, and synthesis of gibberellins and cytokinins has also been documented. Introduction of the rhizobacterial strain *Pseudomonas fluorescens* WCS417 in autoclaved soil promoted growth of Arabidopsis accession Col-0 by 33% [7]. In addition to the amount of bacterial auxin produced, the contrasting effects of this phytohormone on plant root development is linked to the sensitivity of the plant itself.

Gibberellin (GA)

ANOVA showed significant effect of irrigation on leaf gibberellin content at P≤0.01 (Table 1). Mean comparison also showed that T45% has produced 14.6% and 27.5% more gibberellins than T60% and T75%, respectively (Table 2). Among three water deficit, highest gibberellins content was observed in T45%. Little is known about the effects of drought stress on gibberellins. It is expected that during a period of slow growth, levels of growth promoters, such as GA, decrease. Although this happened in wilted detached lettuce leaves, did not happen in droughted intact sunflowers. Yang *et al*. (2001) that observed that although water stress treatments increase ABA, they cause reduction of GA [20].

Also, analysis of variances showed significant effect of pseudomonas strains at P≤0.01 on leaf gibberellins content. Means comparison showed that highest gibberellins content was observed in S153 and s4 with no significant difference and lowest content was observed in control treatment (Table 3). Sobieszczański et al. (1989) with comparsion abilities of seven *Pseudomonas fluorescens* strains to produce plant growth regulators on lettuce seedlings reported that among strains for producing IAA significant difference but not GA3 [18]. Whereas,

Pieterse and Van Loon [7] and Persello-Cartieaux [10] high level production of gibberellins in plant by Pseudomonas were reported. Totally, it can be concluded that effects of drought stress on gibberellins is complicated.

Cytokinin

According to ANOVA water deficit and bacteria significantly affected cytokinin content at P≤0.01 (Table 1). Mean comparison (Table 2) shows that cytokinin level as increasing of water deficit severity, cytokinin level decreased. Water deficit at 40% of available soil moisture depletion (T40%, control) produced the highest cytokinin level and water deficit at 75% (T75%), produced the least cytokinin content. When maize encounters water deficit, transition of cytokinin to shoot would probably reduce because more cytokinin would be stored in roots [28]. Yang *et al.*, (2004) reported that water stress treatments significantly decreased cytokinin level in the leaves during flowering stages [19].

Also, significant effect of bacteria on cytokinin content at P≤0.01was observed (Table 1). Means comparison showed that highest cytokinin content was observed in S153 and S4 with no significant difference and lowest content was observed in control treatment (Table 3). Cytokinins represent another class of phytohormones produced by microorganisms. One study indicated that as many as 90% of the microorganisms found in the rhizosphere are capable of releasing cytokinins when cultured in vitro [22]. Their production by plant associated has been well documented [23, 25], although the endogenous production of cytokinin by plants remains controversial [24]. Indeed the search for plant cytokinin biosynthesis genes has so far been unsuccessful [30]. Nevertheless, it is interesting to note the abundance of PGPR producing cytokinin in the phyllo-or rhizosphere. As cytokinins move from roots to shoots, root exposure to cytokinin could affect plant growth and development. Increases in yield and N, P, and K content of grains obtained after exogenous application of cytokinins in field trials with rice [36] support the hypothesis that bacterially supplied cytokinins to the soil can improve the growth and yield of treated plants. Bacterized potato plantlets grown in vitro were found to be greener and had elevated levels of cytokinins [12]. De Salamone *et al.*, (2001) reported that soybeans bacterized with *Pseudomonas fluorescens* produced the 35.5% more cytokinin in leaves than control plants [16].

Abscisic acid (ABA)

Analysis of variances showed that interaction water deficit levels and bacteria on abscisic acid have significantly affected ABA at P≤0.05 (Table 1). Means comparison (Table 4) also showed that highest and lowest ABA content of leaf was observed in T45% available soil moisture and S153 strain and T75% and control (no inoculation), respectively. Results showed that ABA content until 60% of available soil moisture depletion increased but with increasing of 75% available soil moisture depletion, ABA content decreased (Table 2). The accumulation of ABA was induced by water stress in rice, cucumber and bean [20, 31, 34]. But in this study maximum increasing of ABA content was observed in 60% of available soil moisture depletion, this result according to Domash *et al.*, (2006) results, they reported that there was a transient increase in the ABA content during the initial stage of adaptation to water stress in maize leaves, but it dropped sharply thereafter in response to water stress [34].

Inoculated plants also had increased ABA concentrations in leaf (which may also promote growth) in any three water deficit levels. Some PGPR strains produce cytokinin and antioxidants, which result in abscisic acid (ABA) accumulation and degradation of reactive oxygen species [9]. However, in drying soil, inoculated plants also had a higher xylem ABA concentration than

control plants, correlating with improved shoot and root growth [9]. Plants inoculated with a mutant bacterial strain with decreased ACCd activity had a similar xylem ABA concentration to control plants and shoot growth promotion was not observed [2]. Since under some circumstances ABA can promote rather than inhibit growth, the increase in ABA may have been causally related to the promotion of shoot and root growth (perhaps by suppressing the production of ACC and/or ethylene) in the inoculated plants [27].

Table 1- Some characteristics of pseudomonas bacteria used in this study

Bacteria	ACC-deaminase production	IAA production (mgL^{-1})	Siderophore production (halo diameter/colony diameter)
P. fluorescens strain 153	-	6.1	0.6
P. fluorescens strain 169	+	5.8	0.5
P. putida strain 4	+	9.6	0.5
P. putida strain 108	+	8.9	0.5

Table 2- Analysis of variances of measured traits

starch	DF	Abscisic acid	cytokinin	Gibberlin	Auxin	Proline
Year	1	883.60 ^{ns}	270.4 ^{ns}	650.7^{ns}	523.21 ^{ns}	25.22 ^{ns}
Rep(year)	4	174.05 ^{ns}	46.6 ^{ns}	1591.3 ^{ns}	2788.7 ^{ns}	19.9 ^{ns}
stress	2	17880.07**	22002.6**	105706.5**	294527.2**	3862.4**
Year*stress	2	496.30	347.4^{ns}	979.244 ^{ns}	15051.0*	75.9*
Error1	8	1762.256	468.7	273.922	1864.9	11.109
bacteria	4	27694.317**	15133.1**	85174.6**	139768.0**	4124.4**
Year*bacteria	4	682.07 ^{ns}	303.3^{ns}	196.8 ^{ns}	1403.8 ^{ns}	13.9 ^{ns}
Bacteria*stress	8	2810.80*	291.4^{ns}	1147.81 ^{ns}	14399.7*	328.7**
Year*stress*bacteria	8	173.43 ^{ns}	116.3^{ns}	987.022 ^{ns}	1022.3 ^{ns}	18.250 ^{ns}
Error2	48	1160.36	169.6	252.217	1800.5	13.178
CV%		16.69	10.3	4.45	7.31	7.80

*NS, nonsignificant; **, significant at p≤1%; *, significant at p≤5%.*

Table 3: Mean comparison of main effect of water deficit on measured traits

Water Deficit Levels	Proline µmol/g.fw	Auxin nmol/g.dw	Gibberlin nmol/g.dw	Cytokinin nmol/g.dw	Abscisic acid nmol/g.dw
T40% (control)	33.662c	657.5a	412.467a	150.233a	178.5c
T60%	50.907b	615.13b	363.6b	131b	227.1a
T75%	55.063a	468.67c	294.333c	96.767c	206.8b

Means in a column followed by the same letter are not significantly different at p≤0.05.

Table 4: Mean comparison of main effect of *Pseudomonas fluorescents* strains on measured traits

Pseudomonas strains	Proline µmol/g.fw	Auxin nmol/g.dw	gyberlin nmol/g.dw	Cytokinin nmol/g.dw	Abscisic acid nmol/g.dw
S153	63.13a	681.00a	426.50a	158.16a	251.89a
S4	59.72b	631.22b	412.20a	151.83a	227.94b
S169	46.22c	605.67b	375.50b	124.11b	211.44b
S108	35.97d	524.83c	296.60c	104.38c	173.44c
control	27.67e	459.44d	273d	91.50d	156.06c

Means in a column followed by the same letter are not significantly different at p≤0.05.

Table 5: Mean comparison of interaction effects of water deficit and *Pseudomonas fluorescents* strains on measured traits

Water Deficit Levels	*Pseudomonas fluorescents* strains	Abscisic acid nmol/g.dw	Cytokinin nmol/g.dw	Gibberllin nmol/g.dw	Auxin nmol/g.dw	Proline µmol/g.fw
T40%	S4	238.0^{abc}	176.6^{a}	495.0^{a}	759.6^{a}	36.30^{e}
T40%	S153	243.3^{ab}	175.0^{a}	487.6^{a}	747.6^{a}	48.43^{d}
T40%	S108	165.5^{def}	138.3^{b}	364.6^{c}	615.8^{b}	23.03^{h}
T40%	S169	224.1^{abcd}	160.6^{a}	426.3^{b}	687.6^{a}	35.00^{ef}
T40%	Blank	163.3^{def}	100.5^{c}	288.6^{e}	476.6^{ef}	25.56^{gh}
T60%	S4	269.1^{ab}	181.1^{a}	434.0^{b}	694.0^{a}	67.80^{b}
T60%	S153	280.8^{a}	168.8^{a}	428.3^{b}	708.6^{a}	72.53^{ab}
T60%	S108	209.5^{bcde}	94.8^{c}	286.6^{e}	563.3^{bcd}	37.83^{e}
T60%	S169	231.1^{abc}	126.3^{b}	368.6^{c}	602.6^{b}	46.70^{d}
T60%	Blank	144.8^{f}	83.8^{c}	300.3^{e}	507.0^{def}	29.66^{fg}
T75%	S4	176.6^{cdef}	97.6^{c}	307.6^{de}	440.0f^{g}	75.06^{a}
T75%	S153	231.5^{abc}	130.6^{b}	363.6^{c}	586.6^{bc}	68.43^{b}
T75%	S108	145.3^{f}	80.0^{c}	238.6^{f}	395.3^{g}	47.06^{d}
T75%	S169	179.0^{cdef}	85.3^{c}	331.6^{d}	526.6^{cde}	56.96^{c}
T75%	Blank	160.0^{ef}	90.1^{c}	230.0^{f}	394.6^{g}	27.80^{gh}

Means in a column followed by the same letter are not significantly different at p≤0.05.

CONCLUSION

From the results of this investigation, it can be concluded that bacterial elicitor like *Pseudomonas fluorescens* treatments had improved phytohormonal characters of maize (*Zea mays* L.) under water deficit. In conclusion, the *Pseudomonas fluorescens* can protect maize plants from drought stress by partial amelioration of drought induced growth inhibition, apart from their qualities as an efficient PGPR. Further studies are required to confirm whether proline, auxin, gibberellin, abscisic acid and cytokinin are involved in the changes associated with ACC deaminas production under treatment with this PGPR in drought stressed maize.

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