

Determination of Aluminum Stress Tolerance Threshold During Seed Germination of Wheat

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Abstract

Environmental stresses are the most important factors that reduce plant growth in stages of development. The presence of aluminum in acidic soils as an environmental stress has an impact on different parts of the plant and reduces root growth, water absorption and nutrients and increases susceptibility to drought. In order to evaluate the effect of aluminum stress levels on wheat at germination stage, an experiment was conducted as factorial based on completely randomized design with three replications. The root and shoot characters of four wheat cultivars (Arta, Gascozen, Moghan3, Mihan) at seedling stage were studied at eight stress levels (control, 0.5, 1, 1.5, 2, 2.5, 5 and 10 mM Al³⁺). The root and shoot fresh weight, root and shoot length, root volume and area, root number and Root/Shoot ratio were measured. Results showed that stress level had significant effects on all studied traits. Also, wheat cultivars were significantly different for number, volume, weight and area of root and also shoot length. Interaction between cultivars and stress levels was significant for root number. The Mogha3 variety had higher root number at 2.5 mM Al³⁺ stress level. In all of varieties, the root number increased by increasing of Al³⁺ concentration. Comparison of means showed that Al³⁺ stress caused significant reduction in all studied traits except root number. The Moghan3 variety had higher means for studied traits as compare with other varieties.

Keywords: Aluminum; Germination; Stress; Tolerance; Wheat

Introduction

In developing countries such as Iran, about half of daily energy is obtained directly from wheat (Cakmak 2008). This plant is affected by different biotic and abiotic environmental stresses in growing stages (Alavi Siney and Saba 2015). One of these stresses is aluminum toxicity in acidic soils. About 30-40% of cultivated lands in the world are acidic (Lilienfein *et al.* 2003). Aluminum is a trivalent metal and due to high affinity to bound to hydrogen easily passes from biological membranes. A physical and chemical property of aluminum reflects the higher strength of connection and slower reaction kinetics than the divalent metals such as calcium and magnesium. Therefore, aluminum acts as a strong inhibitor of

the many biological processes related to the cations (Exley and Brichhal 1992). Soil, water and food chain contains aluminum that causes different diseases in human and also reduces agricultural products. Aluminum production sources are mines, factories smelt metals containing ammonium hydroxide, kitchen utensils, cosmetics, medical and acidic rain (Barabasz *et al.* 2002; Krewski *et al.* 2010). Aluminum mainly exists in aluminosilicate form in the soil minerals. Small amount of it appears in solution forms and can affect the living organisms. The various forms of aluminum in the soil includes Al(OH)₂⁺ and Al(OH)₂²⁺ at pH less than 5, Al³⁺ at pH about 5 to 7 and Al(OH)₄⁻ at pH 7 to 8. Many aluminum cations bind to ligands such as PO₄³⁻, SO₄²⁻, organic acids,

proteins and lipids. The order of aluminum toxicity for wheat root is: $Al^{3+} > AlF^{2+} > AlF_2^+$ (Kinraide 1997).

Aluminum toxicity depends on various factors. The important factors of aluminum toxicity in the environment include: increasing the ratio of evaporation to precipitation of soil, pyrite oxidation, soil acidification, application of nitrate and ammonia fertilizers in agriculture and reaction of clay particles with hydrogen ions. These factors lead to the release of aluminum ions from soil particles and increases its concentration in the environment. Oxidation of sulfuric compounds in the soil can cause aluminum release from the soil particles and increase the exchangeable aluminum of the soil (Egli *et al.* 1999). Increase in the solubility of ammonium compounds is based on the soil acidification that occurs by washing alkali metal ions such as sodium, potassium, calcium and magnesium from soil and consequently the reduction of pH of the soil solution which causes the aluminum ions move to the upper parts of the plant (Ma *et al.* 1997). These elements belong to unessential elements group. This element don't have any known function in the plant metabolism (Wang and Kao 2004). Aluminum can bind to the cell wall and nucleus and creates an effect called aluminum toxicity (Arroyave *et al.* 2011). The important symptom of aluminum toxicity in plants is the inhibition of root growth in the root tip of sensitive plants. These symptoms appear about 30 minutes to 2 hours after the aluminum treatment (Ryan *et al.* 1993). The decrease in Fe concentration of stems and Ca and Mg concentrations in stems and roots are the result of accumulation of Al in the roots (Cumming *et al.*

1985). Lateral roots get thick and brown and root tip curves, swells and becomes woody (Vardar and Unal 2007). Aluminum affects the root tip cell and leads to the reduction of water and nutrient absorption from the soil (Ma *et al.* 2001). Depending on the acidity of lower horizon of soil, aluminum toxicity reduces root penetration, increases sensitivity to drought and reduces the utilization of soil nutrients (Poot-Poot *et al.* 2011). In the sensitive plants, root tip meristem immediately is affected by aluminum and it affects root elongation (Meda and Furlani 2005). The accumulation of Al^{3+} , increases double helix of DNA, and decreases cell division in the meristem (Meriga *et al.* 2010).

Shoot responses to the aluminum are cellular and ultra-structural changes in the leaves, increase in the diffusion resistance, decrease in the stoma, decrease in the photosynthetic activity, decrease in the number and size of leaves and decrease of the shoot biomass (Rout *et al.* 2001). The plants under aluminum stress have yellow leaves (Cumming *et al.* 1985). In some cases, aluminum toxicity leads to deficiency of calcium or inhibition of its transport. Calcium deficiency cause the torsion of young leaves, loss of leaflets and leaf growth stalling (Furlani and Clark 1981). It also, stimulates the phosphorus deficiency symptoms such as dwarf, dark green leaves and purple stem (Rout *et al.* 2001).

Cell wall is the main site of toxicity and also the first binding site for the heavy metals (Harvey *et al.* 2002). Aluminum changes cytoplasmic levels of calcium and activates calcium-dependent nucleases. Finally, these enzymes cut the DNA and lead to DNA fragmentation (Yakimova *et al.*

2007). Plasma membrane is one of the aluminum binding sites (Takabatake and Shimmen 1997). Aluminum interferes with the normal function of the Golgi apparatus and inhibits mitotic activity and DNA synthesis (Frantzios *et al.* 2000). Researchers have reported that a part of aluminum toxicity is due to oxidative stress caused by this ion. Therefore, plant tolerance to the aluminum may depend on the activity of antioxidant system (Xu *et al.* 2012).

In this study, the seeds of four varieties of wheat were treated with different concentrations of aluminum and the responses of these varieties to aluminum stress during seed germination and early stages of seedling growth for identifying the tolerance threshold of varieties was investigated.

Materials and Methods

To evaluate the effect of aluminum stress on wheat root characteristics at the germination stage and to determine tolerance threshold of wheat varieties to aluminum toxicity, an experiment was performed at Faculty of Agricultural Science of Mohaghegh Ardabili University in 2015. The root and shoot length, root and shoot fresh weight, root volume, root area, root length to shoot length ratio of four wheat varieties (Arta, Gascogne, Mihan, Moghan3) were studied at eight levels of aluminum (Al^{3+}) stress (0, 0.5, 1, 1.5, 2, 2.5, 5 and 10 mM). For this purpose, a factorial experiment based on completely randomized design with four replications was conducted. To create the stress levels of Al^{3+} , $AlCl_3$ was used. The seeds after sterilization with 1% sodium hypochlorite for 15 min and rinsing three times with sterile distilled water, transferred to Petri dishes containing sterile

Whatman filter paper. Fifty seeds were planted in each Petri dish. Then, distilled water containing different concentrations of aluminum were added. Petri dishes maintained in a growth chamber at 20 ± 2 °C. Every day, the seeds were evaluated if they needed to add solution. On the 14th day, 12 seedlings randomly selected from each petri dish and number of radicle, shoot length (from collar to apical bud) and root length (from collar to primary root tip) were measured. Also, the root and shoot fresh weight was measured with a digital scale. Root volume was measured through immersion in distilled water in the graduated cylinder. Root area was calculated by Etkinson's formula (Alizadeh 2009) as follows:

$$\text{Root area} = 2 \times (\text{root volume (cm}^3\text{)}) \times 3.14 \times (\text{root length (cm)})^{0.5}$$

Data analysis was performed using IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY, USA). Mean comparisons were carried out using Duncan's multiple range test at a probability level of 0.01. Diagrams were drawn using Excel software.

Results and Discussion

The analysis of variance showed significant difference between levels of aluminum stress for all traits. The studied varieties were significantly different in terms of volume, weight, number and area of root and also shoot length. Variety by stress interaction was significant for root number (Table 1). With increasing aluminum concentration there was a reduction in root length. The highest amount of root length was observed in the normal condition (control). AT 10 mM aluminum stress, 83.21% reduction in root length was observed as compared

to the control (Table 2). The genotypes were not significantly different in terms of this trait. The decrease of roots at even the lower concentrations of aluminum stress (0.5, 1, 1.5, 2, 2.5) was evident. At 5 mM stress, the root and shoot growth extremely decreased. Severe stress level (10 mM aluminum) inhibited the seed germination and root and shoot growth. At high levels of aluminum stress, roots were fragile and their tip was brown (Figure 1). Previous studies have reported the effect of short-term aluminum stress on the rapid and lateral roots (Wagatsuma and Kaneko 1987). At aluminum stress condition the lateral roots are thick and brown and root tip is torn, swollen and lignified (Vardar and Unal 2007). Reports have shown that aluminum increases ferulic acid and diferulic acid of the cell wall, which make cell wall thick and hard. These acids involve in the lignification processes and subsequently inhibit root growth. Lignification and suberization of cell wall is an immune response that limits the entry of toxic elements to the roots and defensive responses

are associated with growth inhibition (Haluskova *et al.* 2010). Primarily aluminum accumulates in the root tip, which interferes with cell division and proliferation. Evidence suggests that many dicot and monocot plant species prevent aluminum entrance to root cells by secretion of dicarboxylic acid and tricarboxylic acid. Therefore, aluminum accumulates in the root tip by creating stable and non-toxic compounds (Pineros *et al.* 2008). The aluminum toxicity inhibits root growth and induces the synthesis of β -1, 3-glucan after short-term treatment. Both events are related to the oxidative stress induced by aluminum treatment (Yamamoto *et al.* 2003). Binding of aluminum to the cell wall changes cell membrane potential. Increase in aluminum level cause formation of β -1, 3-glucan and destruction of the cytoskeleton (Romero Puertas *et al.* 2004). β -1, 3-glucan decreases permeability to water and blocks plasmodesmata and thereby inhibits cell-cell interactions (Ishikawa and Evans 1995).

Table 1. Analysis of variance for studied traits in wheat under Al³⁺ toxicity condition

SOV	df	Mean squares							
		Root length	Root fresh weight	Root area	Root volume	Shoot length	Shoot fresh weight	Root/Shoot ratio	Root number
Stress	7	184.71**	10.803**	728.924**	15.158**	119.49**	5.416**	1.196**	38.639**
Variety	3	3.879	0.654**	15.32**	0.559**	4.802**	0.008**	0.120	2.078**
Stress*Variety	21	1.776	0.111	5.445	0.146	0.810	0.025	0.089	0.568**
Error	96	1.817	0.089	4.449	0.153	0.966	0.048	0.065	0.182
CV%	-	23.86	24.51	23.3	29.2	13	15	31	8

**Significant at 1% probability level

Table 2. Means of aluminum stress levels for studied traits in wheat

Stress levels	Root - length (cm)	Root fresh weight (gr)	Root area (cm ²)	Root volume (cm ³)	Shoot length (cm)	Shoot fresh weight (gr)	Root/Shoot ratio	Root number
Control	11.11 ^a	2.43 ^a	18.1 ^a	2.61 ^a	9.12 ^a	1.90 ^a	1.22 ^a	5.35 ^d
0.5 mM	8.52 ^b	2.12 ^b	15.96 ^b	2.41 ^a	9.14 ^a	1.87 ^{ab}	0.928 ^b	5 ^c
1 mM	8.42 ^b	1.75 ^c	13.62 ^c	1.79 ^b	8.7 ^a	1.73 ^b	0.96 ^b	5.30 ^c
1.5 mM	5.40 ^c	1.13 ^d	8.75 ^d	1.16 ^c	8.46 ^{ab}	1.63 ^b	0.66 ^c	5.67 ^b
2 mM	4.42 ^d	0.97 ^{ef}	7.41 ^{de}	1 ^c	7.81 ^{bc}	1.48 ^c	0.57 ^c	5.86 ^b
2.5 mM	3.58 ^d	0.91 ^f	6.81 ^f	1.04 ^c	7.60 ^c	1.44 ^c	0.47 ^c	6.18 ^a
5 mM	2.29 ^e	0.41 ^g	1.05 ^g	0.41 ^d	4.36 ^d	0.80 ^d	0.53 ^c	3.85 ^e
10 mM	1.3e	0.04 ^h	0.04 ^g	0.004 ^d	1.52 ^e	0.20 ^e	1.04 ^{ab}	1.39 ^f
Percent of decreasing for 10 mM stress level	83.21	98.36	97.30	99.98	83.40	89.74	-12.68	73.63

Means with the same letter in each column are not significantly different at 1% probability level



Figure 1. The effect of Al³⁺ toxicity on wheat root characters: a) Decreasing root length, b) Black root tips, c) Increasing number of root, d) increasing branch of roots and e) Germination of Moghan3 variety in 10 mM Al³⁺ concentration

The results showed that root weight significantly decreases by increasing aluminum stress levels. The highest amount of root weight was obtained in the control condition. Aluminum stress (10 mM) decreased root weight 98.36% as compared to the control (Table 2). Moghan3 variety had the highest amount of root weight than other varieties. Root length was significantly and negatively affected by aluminum levels. Due to the correlation between root length and weight, with increasing stress level, root weight decreased. Aluminum toxicity damaged the epidermal cells and outer skin of the corn (sensitive to aluminum) at approximately 1 cm of the root tip has been and the cell wall of these cells was abnormal. In oat after six days of treatment with aluminum, few skin cells survived (Rout *et al.* 2001). With increasing aluminum concentration, root area decreased. Aluminum stress at 10 mM decreased root area 97.30% as compared to the control (Table 2). Moghan3 variety had the highest amount of root area than other varieties (Table 3). Since the root area has direct correlation with root length and volume, therefore, aluminum stress significantly decreased this trait. Rout *et al.* (2001) reported that aluminum interferes with cell division at root tip and lateral roots, decreases root respiration and

stabilizes soil phosphorus to less accessible and causes disturbance of rootlet size and rootlet area.

The means with same letters in each column don't have significant difference at 1% probability level. With increasing aluminum concentration, root volume decreased. The highest amount of root area was obtained in the control condition. Aluminum stress (10 mM) decreased root volume by 99.98% as compared to the control (Table 2). Moghan3 variety had the highest root volume (Table 3). Due to reduction of root area and weight by toxic effects of aluminum, root volume was also significantly decreased by increasing the stress level and at 10 mM stress level the root volume highly decreased. Matsumoto (1991) reported the aluminum toxicity due to the formation of a complex of DNA-Al. According to the other reports, inhibition of root growth was observed before inhibiting DNA synthesis (Wallace and Anderson 1984). Identifying DNA specific markers for aluminum tolerance can help to select genotypes that remain fertile at the aluminum stress condition. In order to obtain tolerant plants efforts have been made by using biotechnology techniques leading to the production of rice, tobacco and Arabidopsis transgenic plants (Mossor-Pietraszewska 2001).

Table 3. Means of wheat varieties for the studied traits at different aluminum stress conditions

Varieties	Root volume (cm ³)	Root fresh weight (gr)	Root number	Shoot length (cm)	Root area (cm ²)
Arta	1.160 b	1.76 b	4.884b	7.361a	8.574b
Gascogne	1.215 b	1.162 b	4.764b	6.8542b	8.540b
Mihan	1.198 b	1.216 b	4.521b	7.367a	9.152ab
Moghan3	1.451 a	1.413 a	5.134a	7.090a	10.020a

The means with same letters in each column don't have significant difference at 1% probability level

Table 4. Results of regression analysis between studied traits and Al stress levels in wheat

Traits	Type of relation	Intercept	Regression coefficient			R ²
			b ₁	b ₂	b ₃	
Root length	Quadratic	10.21**	-2.86**	0.2**	-	0.89
Root fresh weight		2.29**	-0.64**	0.04**	-	0.93
Root volume		2.57**	-0.81**	0.056**	-	0.96
Root area		2.18**	-5.56**	0.038**	-	0.96
Root/Shoot ratio		1.1**	-0.29*	0.3*	-	0.81
Root number	Cubic	4.8**	1.24**	-0.41*	0.03*	0.96
Shoot length	Linear	9.39**	-0.82**	-	-	0.97
Shoot fresh weight		1.68**	-0.18**	-	-	0.97

With increasing aluminum levels shoot length decreased. Aluminum stress (10 mM) decreased shoot length about 83.40% (Table 2). Mihan genotype had the highest amount of shoot length than other varieties (Table 3). By increasing the stress level, shoots became extremely short and some seeds lacked root and shoot growth. At the higher concentration of aluminum, the shoot of some seeds grew just 2 mm. Aluminum toxicity induced phosphorus deficiency and led to atrophy of leaves, delay in the maturation of leaves and loss of leaf tips. Also, calcium deficiency or inhibition of its transfer cause young leaves to twist and destruction of leaflets (Foy and Fleming 1982). Rubisco is a key enzyme in the carbohydrates syntheses. When aluminum binds to phosphate compounds, it reduces the access of Rubisco enzyme to the phosphate and reduces the activity of Rubisco (Chen 2006). Furthermore, aluminum reduces biomass production by preventing the absorption of phosphorus by cell (Palma *et al.* 2002). Studies have shown that in plants higher amount of aluminum ions are absorbed through the roots and only a very small amount of that penetrate from leaves. Most researchers believe

that in the process of absorption of metals, ion transporters are involved by energy expenditure. So far, no aluminum-specific transporter is known. The plasma membrane is the primary target of aluminum toxicity (Mossor-Pietraszewska 2001). Nonetheless, tea (*Camellia sinensis*), hydrangea and Rubiaceae family members are examples of plants that accumulate aluminum at their shoots. These plants are woody and mostly tropical or subtropical (Vitorello *et al.* 2005).

Increasing aluminum stress levels decreased the shoot weight. The highest amount of shoot weight was obtained in the control condition. Aluminum with 10 mM concentration decreased shoot weight by 89.74% as compared to the control (Table 2). Thornton *et al.* (1987) reported that aluminum toxicity causes the cellular and structural changes in leaf, increase the resistance against the release of the materials, decrease the stomata diaphragm, decrease the photosynthesis activity (yellowing and necrosis of the leaves), decrease number and sizes of the leaves and decrease shoot biomass. During the aluminum stress, soluble proteins of the cell such as antioxidant enzymes and polyamine synthesis

enzymes increase, but this increase does not promote the growth and photosynthesis (Minocha *et al.* 2001). According to some reports, aluminum decreases growth and biomass by disturbance with photosynthesis, nitrogen metabolism, respiration and damaging the cell membrane of the plant. Also, it is known that decreasing of root and shoot weight is due to disturbance in the nutrients and water absorption (Wang *et al.* 2006).

The root/shoot ratio decreased by increasing the aluminum stress levels. The highest ratio was obtained at the control condition (Table 3). Aluminum is toxic for various plants and prevents the root and stem growth. The highest effect of aluminum toxicity appears on the roots (Chang *et al.* 1999). The results showed that the effect of aluminum on the root length is higher than shoot length. As noted above, the aluminum had the highest damage on root and the decrease of the Root/Shoot ratio can be the reason of this damage. The shoot is less affected than the root, because it may slow down elements movement to the shoot. Comparison of varieties at different stress levels indicated that the root number at different concentrations of aluminum stress was not the same and genotypes showed different behavior (Figure 2). Moghan3 had the highest number of roots at 1.5 mM stress level and the lowest number at 10 mM level. In the control condition this variety had the highest value. By increasing the stress level to 2.5 mM in Arta, Gascogne and Moghan3, the number of roots increased. At 5 and 10 mM concentrations, number of roots was much lower. In fact, the increase in root number is a mechanism for resistance to the higher levels of aluminum stress up to 2.5 mM. By increasing the

concentration of aluminum up to 2.5 mM root number and length was strongly reduced. Some seeds at 10 mM aluminum stress level had single root with two branches. Investigation of the effect of aluminum on the canola roots in a nutrient solution with pH 4.5 showed that at 40 mM aluminum, root growth of seedlings enhanced and number and sizes of the cells in the central cells of the root cap increased (Kafi *et al.* 2009). The roots of several plant species in response to aluminum, secrete organic acids by membrane transporters which lead to the formation of non-toxic complexes. Therefore, this mechanism prevents aluminum transfer from plasma membrane to symplast (Simões *et al.* 2012). Studies showed that in the coffee cells, aluminum leads to the production of growth limiting compounds at different concentrations. Aluminum involves in different biochemical processes including enzymatic and membranous phospholipids such as phospholipase (Munnik and Testerink 2009). In aluminum-sensitive plants more absorption of this element causes plant toxicity and thus rapid cell death. However, in tea, moderate treatment with aluminum can even be beneficial to the plant and effective in increasing the viability of the cells (Ghanati *et al.* 2005). Houd and Diallo (2008) identified 83 genes related with aluminum stress and 25 genes related with aluminum stress tolerance in wheat. Buckwheat (*Fagopyrum esculentum* Moench CV. *Jianxi*) has a high resistance to aluminum, but the mechanism responsible for resistance is not known. *Juanxi* variety of buckwheat is resistant to aluminum and secrete oxalic acid in response to this element (Feng *et al.* 1997).

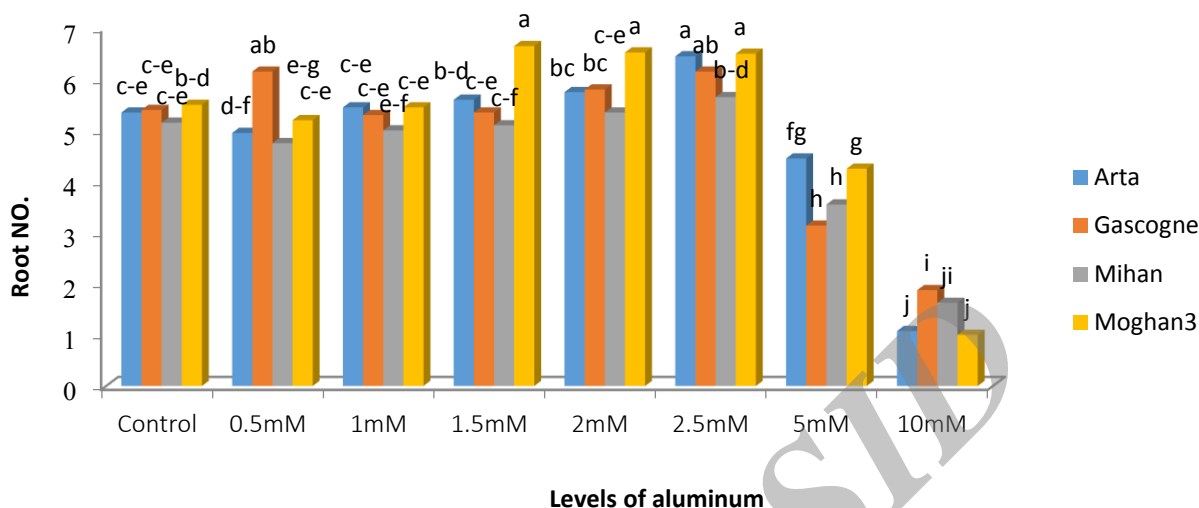


Figure 2. Mean root number of wheat varieties at different aluminum stress levels

Studying the regression relation between traits under investigation and different concentrations of aluminum showed that for most traits except root number and Root/Shoot ratio with increasing aluminum concentrations the magnitude of traits were decreased. Up to 5 mM concentration, the reduction trend of was linear, but from 5 mM to 10 mM a sharp decrease occurred for most traits. Therefore, the amount of most traits was close to zero and this concentration was not tolerable for all genotypes. The length and weight of shoot increased linearly and the length and weight of root increased quadratically by increasing the aluminum concentration. Up to 5 mM the reduction in the root characters was higher than the shoot, but at 10 mM stress level, the reduction in the shoot characters such as shoot length was higher than the root. Therefore, the Root/Shoot ratio increased after 5 mM (Figure 3). Up to 2.5 mM the number of root significantly reduced (Figure 3 and Table 4). The results indicated that 2.5 mM aluminum is

suitable for selection of aluminum-tolerance seedlings. At lower concentrations, the difference between genotypes was not significant and after 5 mM excessive stress occurred. Under aluminum stress the root tip was tight and black (Figure 3). Varder and Unal (2008) reported that at aluminum stress conditions number of roots, black roots and root branches increased.

Conclusion

The results showed that the different levels of aluminum stress had negative and significant effects on all studied traits. The highest amount of root length, root area and root volume were obtained at the control condition and 0.5 mM aluminum stress. Furthermore, varieties differed significantly for some traits under the stress condition. Moghan3 had the highest number of roots at most stress levels than other varieties. This variety showed more growth rate than other genotypes up to 2.5 mM Al^{3+} level, but at 5 and 10

mM stress levels the number of roots decreased drastically. Moghan3 had higher mean than other varieties for most traits. Also, we suggest the

suitability of 2.5 mM aluminum level for discriminating tolerant and sensitive genotypes of wheat at the seedling stage.

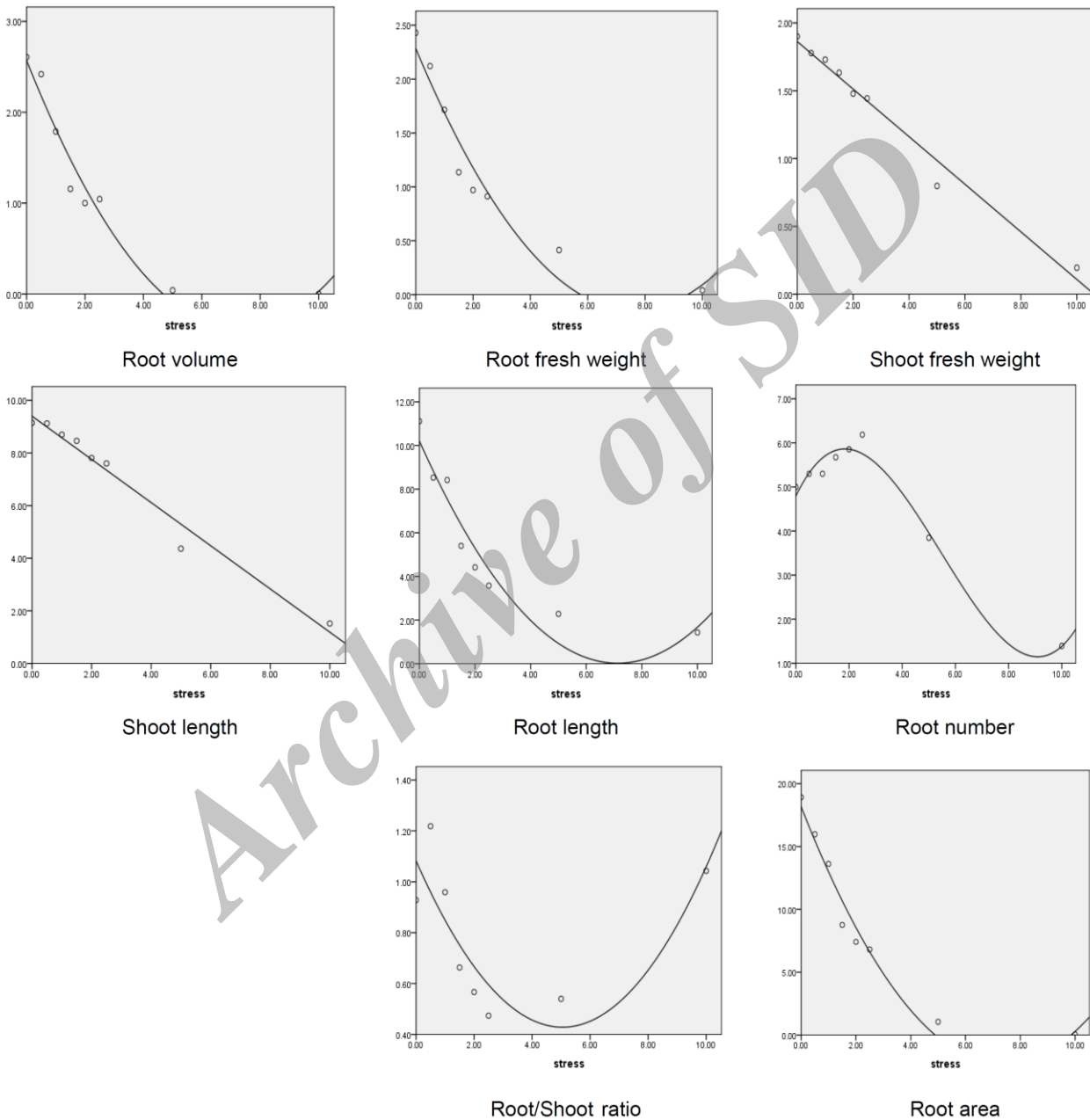


Figure 3. Regression relations of studied traits with aluminum stress levels in wheat

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