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DETERMINATION OF *IN VITRO* RESPONSES OF TURKISH BARLEY CULTIVARS TO PRESENCE OF ALUMINIUM

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ABSTRACT

Aluminium (Al) is a limiting factor for plant growth in acidic soils due to its toxic property under acidic conditions. Highest Al sensitivity of barley among other cereals is important for cultivation of this crop. In this study, *in vitro* responses of four Turkish barley cultivars to presence of Al were analyzed based on the changes in root length, shoot length, pH of growth medium and eriochrome cyanine R staining. All cultivars were grown in MS medium with agar containing different Al concentrations (0, 10, 20, 40, 60, 80, 100 µM). Average root length measurements obtained daily over 6 d periods showed that variations in lengths were present up to 60 µM Al. However, root lengths were decreased numerically for all cultivars at 80 µM Al. Based on the root growth inhibition level at an increase of Al concentration from 60 to 80 µM, a numerically lowest root growth inhibition was observed for Konevi-98 (37.6 vs. 33.4 mm). Therefore, Konevi-98 was determined as the most tolerant variety, while Kıral-97 (32.3 vs. 29.9 mm) was followed by Konevi-98. Beyşehir-98 (31.5 vs. 21.4 mm) and Karatay-94 (31.3 vs. 23.8 mm) were determined as the most sensitive cultivars. In accordance with the root growth inhibition results, Konevi-98 had the highest shoot growth (97.3 mm; $P< 0.05$) and caused the least pH change (4.32; $P< 0.05$) on the growth medium. On the other hand, eriochrome cyanine R staining results of roots grown under various Al concentrations was not definitive for the separation of cultivars based on Al tolerances.

Key words: barley, aluminium tolerance, root growth, pH of medium.

INTRODUCTION

Aluminium (Al) is commonly toxic on acidic soils and become a restricting factor for cultivation of many crops such as wheat, barley, rice, and maize by inhibiting root cell division and elongation, thus reducing water and nutrient uptake (Foy, 1983; Kochian, 1995; Khan *et al*. 2001). Among cereals, barley is the most sensitive crop to Al toxicity (Foy, 1983). In addition, genetic variation for Al toxicity exists within barley species (Foy *et al*. 1965). Therefore, selection of cultivars is essential for increasing barley production on acidic soils. Moreover alternative protection ways against soil acidity such as conventional fertilization and liming practices are not economical.

Many screening methods based on soil, laboratory, field and combination of those were used for determination of Al toxicity level in different plants (Tamas *et al*. 2006; Wang *et al*. 2006). Some of those methods were specifically adapted for screening Al tolerance in barley (Ma *et al*. 1997; Echart *et al*. 2002; Tamas *et al*. 2006). Most of those have been relied on visual scoring of colour development in root tips after staining roots with dyes showing Al accumulation such as haematoxylin (Polle *et al*. 1978) and eriochrome cyanine R (Ma *et al*. 1997) following to growth in nutrient solution containing Al. However, this staining technique might cause false scoring due to biased estimation of colour intensities. Another common way of discriminating cultivars for Al tolerance has been demanded the inhibition of root growth or re-growth following to Al shock (Hede *et al*. 2002; Raman *et al*. 2002; Ninamango-Cardenas *et al*. 2003).

Currently, two main Al tolerance mechanisms have been identified in plants. One of those mechanisms was referred as external exclusion where Al is prevented from entering the symplasm and reaching sensitive metabolic sites. The events induced by this mechanism can vary such as formation of a plant induced pH barrier in the rhizosphere or root apoplasm, exudation of chelate ligands (organic acids), and exudation of phosphate (Taylor and Foy, 1985; Delhaize *et al*. 1993; Kochian, 1995; Degenhardt *et al*. 1998; Ma, 2000; Prijambada and Proklamasiningsih, 2010). Exudation of organic acids from roots, mainly citric and malic acids, were identified as the external detoxification factor against Al. Moreover many Al-activated transporter genes responsible from organic acid exudation were identified in cereals (Sasaki *et al*. 2004; Furukawa *et al*. 2007). The other tolerance mechanism is achieved by internal exclusion mechanism involving detoxification, immobilization or changes in the metabolism after Al was entered to the symplasm (Kochian, 1995).

In our study, *in vitro* responses to Al was determined for four Turkish barley cultivars by analyzing changes in the root growth, shoot growth and pH of growth medium. Moreover, eriochrome cyanine R

staining was performed on the roots grown in the presence of Al.

MATERIALS AND METHODS

Plant material: Four Turkish barley cultivars (Beyşehir-98, Karatay-94, Kıral-97, Konevi-98) were assayed in this study. All cultivars were obtained from Bahri Dagdas International Agricultural Research Institute.

Growth conditions: Surface sterilization was performed with some modifications as described by Battke *et al*. (2003). All seeds were kept in 70% ethanol for 1 min. Seeds were rinsed for 1 min in sterile distilled water and subsequently kept in 10% NaOCl for 5 min. Finally, seeds were rinsed in sterile distilled water 10 times. The final rinse was extended to 30 min and excess water was removed using filter paper. Murashige Skoog (MS) medium (Sigma 5519, Germany) (4.3 g/l) was prepared by sucrose (10 g/l) and agar (10 g/l) addition (Murashige and Skoog, 1962). The pH of medium was adjusted to 4.5 by NaOH. After autoclaving, liquid medium and agar constituents were combined. For the experiment group, Al as in the form of $AlCl_3$ (MW of 133.34 g) were used to prepare 100 mM Al stock solution in distilled water. After filter sterilization of Al stock solution, it was added to various final concentrations (10 μM, 20 μM, 40 μM, 60 μM, 80 μM, 100 μM) onto autoclaved and cooled (55^{o}C) MS medium. The prepared medium was poured to sterile petri plates. Following to surface sterilization, two replications of 8 seeds from the same cultivar were seeded to each petri dish twice. Petri plates were kept in an incubator at vertical position under darkness at 25^{o}C for 40 h. Following to incubation, petries were placed at the same position to the growth chamber and was grown at 25°C with the photoperiod of 16 h/8 h (light/dark) for 6 d (Hossain *et al*. 2005). Daily root length measurements were performed with ruler, while daily pH measurements of medium were done with pH indicator paper. During 6 d, neither pH adjustment nor renewal of fresh media was done. Eriochrome cyanine R solution 0.1% (w/v) was prepared in distilled water. After Al treatment, roots were placed in distilled water for 30 min and stained with 0.1% eriochrome cyanine R (v/w) for 10 min. Following to staining, excess dye was removed with washing roots in distilled water for 10 min (Ma *et al*., 1997). After staining, the roots were examined with binocular microscope. Shoot length measurements were performed once at the end of growth period.

Statistical analysis: Daily root growth measurements were performed for control and experimental groups at the same time over 6 d. Root growth of each seed was determined by averaging the root length measurements. Those values were then used to estimate the daily average root growth for each cultivar. Statistical analysis was performed based on the randomized block design. All analyses were performed using PROC GLM procedure of SAS (version 8.1., SAS Inc., Cary, NC) statistical program. Treatment comparisons were done with Duncan test at $P< 0.05$.

RESULTS AND DISCUSSION

Al inhibits root growth by affecting root tips, therefore root re-growth measures were frequently used for Al tolerance screening of different cereals (Echart *et al*., 2002; Pineros *et al*., 2005). Root growth measurements are presented in Table 1. Root lengths were fluctuated up to 60 μM Al concentration for each cultivar and in some cases even greater root growth was observed in higher Al than lower Al containing medium. A common numerical root growth trend was observed for all cultivars when changing the concentration of Al from 60 to 80 μM. For instance, root growth of Beyşehir-98 (31.5 vs. 21.4 mm) and Karatay-94 (31.3 vs. 23.8 mm) were drastically decreased during the shift of Al concentration from 60 to 80 μM. Similar root growth differences were also reported in the presence of various Al concentrations for maize cultivars (Pineros *et al*., 2005). However, gradual or dose dependent inhibition of root growth by increasing Al concentration was reported more frequently during Al stress conditions (Ezaki *et al*. 2000). The degree of root length decrease during the concentration shift from 60 to 80 μM was used for the distinction of cultivars based on Al response differences. On the other hand, Echart *et al*. (2002) tested 15, 30, 45 and 75 μM of $AlCl_3$ in nutrient solution and found 30 μM was the most efficient concentration for classifying two parental barley cultivars as tolerant or sensitive. Moreover, Tamas *et al*. (2006) was found that 4 mM of Al was highly toxic during germination of barley in a filter paper based system.

Change in pH of medium for all Al concentrations was significantly different from the control group (Figure 1). This might indicate that there was no drastic pH change difference for rest of the tested Al concentrations. pH change of growth medium was also analyzed daily over 6 d period. As a result, no significant reduction in medium pH was observed during the first 3 d (data not shown).

Nutrient solution culture is the most common screening medium for Al tolerance or intolerance. This medium provides easy access to root system and control over nutrient availability and pH (Baier *et al*. 1995). However many other factors, such as variation in temperature in the growth chamber and minor fluctuation of pH of the nutrient solution, have been reported to affect repeatability of the screening results (Moore *et al*., 1977). Moreover, the concentration of Al and duration of exposure have been reported to vary inversely (Shuman *et al*. 1993). In our study, agar based MS medium was

employed for determination of Al responses of barley cultivars. Indeed, this medium was used for growing barley which has been aimed to monitor cadmium (Cd^{2+}) and mercury (Hg^{2+}) accumulation when compared to hydroponic system (Battke *et al*., 2003). Another example of using MS medium as diluted modified form has been known for Al sensitivity test of *Arapidopsis* (Ezaki *et al*., 2000). However basal MS medium has a phosphorous containing component such as KH_2PO_4 (170 mg/L) in the present study and this might be undesired property for Al tolerance screening. Since higher concentrations of phosphorus has been proposed to be precipitated with Al as Al-phosphate and protect plants against Al toxicity (Wagatsuma and Yamasaku, 1985). To support this explanation, several studies were stated that the changes of nutrient composition could alter the intensity of Al stress at a given concentration (Foy *et al*. 1988; Little, 1988; Scott and Fisher, 1989).

Plant-induced pH increase around root was proposed to be another Al exclusion mechanism in plants (Foy *et al*. 1965). The rhizosphere can not be observed clearly in the hydroponic experiments. Therefore, pH changes around roots were observed by different ways, such as addition of bromocresol purple as a pH indicator on agar medium (Chen and Shen, 2008). In another study, a microelectrode system was used to simultaneously measure rhizosphere pH, K^+ and H^+ fluxes, and membrane potentials (*Em*) along the root of different wheat cultivars at various distances from the root apex either in complete nutrient solution or 0.6 mM $CaSO_4$, with and without Al at pH 4.50 (Miyasaka *et al*. 1989). In the present study, a daily pH change of growth medium was detected and this information was used for determining the rhizosphere's pH change. Results in Figure 2 indicate that Konevi-98 had a significantly lower pH change (4.32) in the growth medium than the other cultivars ($P< 0.05$). Therefore, H^+ secretion activities of cultivars in our study were varied which support the presence of plant-induced pH increase for barley (Taylor and Foy, 1985). In addition, shoot length results (Figure 3) at d 6 showed that Konevi-98 had the significantly highest shoot length (97.3 mm) compared to shoot lengths of Beyşehir-98 (74.7 mm), Karatay-94 (71.0 mm), and Kıral-97 (68.0 mm). Moreover the medium pH differences for cultivars were correlated with different Al responses observed for root growth length inhibition of concentration shift from 60 to 80 µM Al. The plant induced pH change mechanism still needs more investigation to understand the details. There have been conflicting results obtained from different plant species including barley (Foy *et al*. 1965; Wagatsuma and Yamasaku, 1985; Hayashi *et al*. 2005; Chen and Shen, 2008). Eriochrome cyanine R staining results were not definitive for discrimination of cultivars with different concentration of Al treatments (data not shown).

Table 1. Mean root length of barley cultivars during the exposure of various Al concentrations (pooled SEM= 5.8).

Cultivars	$AlCl_3$ concentration (µM)	Root length (mm)
Beyşehir-98	0 (control)	31.5
Karatay-94		25.2
Kıral-97		27.3
Konevi-98		40.9
Beyşehir-98	10	24.0
Karatay-94		25.3
Kıral-97		36.0
Konevi-98		28.1
Beyşehir-98	20	22.2
Karatay-94		27.9
Kıral-97		34.9
Konevi-98		29.9
Beyşehir-98	40	30.1
Karatay-94		30.0
Kıral-97		31.0
Konevi-98		26.7
Beyşehir-98	60	31.5
Karatay-94		31.3
Kıral-97		32.3
Konevi-98		37.6
Beyşehir-98	80	21.4
Karatay-94		23.8
Kıral-97		29.9
Konevi-98		33.4
Beyşehir-98	100	32.4
Karatay-94		24.6
Kıral-97		35.4
Konevi-98		40.3

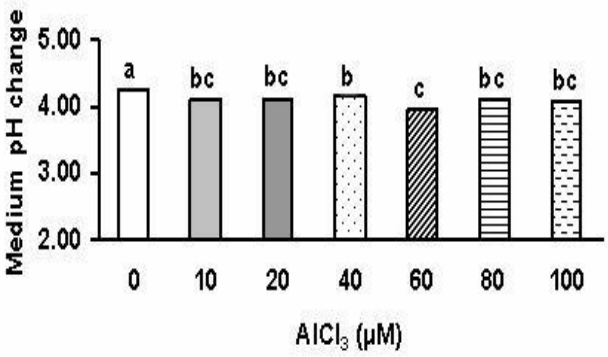

Figure 1. Mean growth medium pH change in the presence of various Al concentrations tested for barley cultivars (n= 24, pooled SEM= 0.10, P< 0.05).

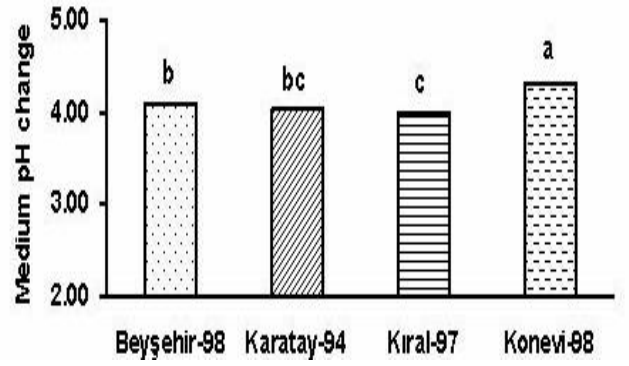

Figure 2. Mean growth medium pH change for barley cultivars exposed to various Al concentrations (n= 42, pooled SEM= 0.08, P< 0.05).

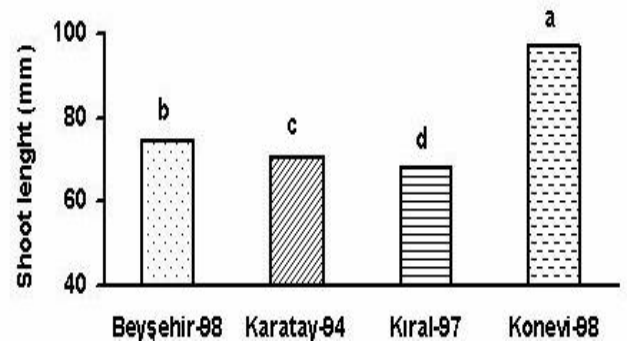

Figure 3. Mean shoot length of the barley cultivars at d 6 grown in various Al concentrations (n= 14, pooled SEM= 2.3, P< 0.05).

Conclusion: Acidity is not a major problem for soils in Turkey, however to our knowledge there has not been any study for addressing Al responses of Turkish barley cultivars. Therefore, the results of this study might aid the determination of desired cultivars grown in acidic conditions. Results suggest that Konevi-98 has the highest ability of coping with presence of Al compared to other cultivars. Moreover, different parameters such as pH change of MS medium, and inhibition of shoot growth might be employed in combination to other parameters (inhibition of root growth) for reliable selection of cultivars for Al response. However; reliability of MS medium for Al tolerance determination of barley and correlation of root growth results with Eriochrome cyanine R staining need further investigation.

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