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Temporal Analysis of Al-Induced Programmed Cell Death in Barley (*Hordeum vulgare* L.) Roots

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Abstract. Aluminum (Al) is the third most elements found in the earth crust and Al toxicity is one of the most dangerous toxicants in terms of plants. As soil acidity increases due to a number of environmental factors, Al becomes soluble and transforms into toxic forms. In the present study, barley (*Hordeum vulgare* L.) roots were exposed to 100 µM $AlCl_3$ solution for short (1/2, 1, 2, 3, 4, 5, 6 and 7 h) and long (24, 48, 72 and 96 h) term to reveal time dependent programmed cell death evidences. At the end of time periods, Al^{+3} accumulations, loss of plasma membrane integrity and lipid peroxidation increased time dependently. On the other hand, increase in caspase-1 like enzyme activities were observed in Al toxicity beginning from ½ h. Similar to apoptosis seen in animals, cytochrome c release from mitochondria to cytoplasm was also determined quantitatively. As a result of our research, increase of cytochrome c release from mitochondria to cytoplasm was time dependent which is one of the indicators of programmed cell death. Finally, under Al stress, genomic DNA fragmentation was measured by Flow Cytometry, and it was determined that DNA fragmentation was visible at first hours, but it was more significant after long term application in barley roots. In conclusion; the presented study highlights the adverse effects of Al on barley roots and importance of clarifying the relationship between Al toxicity and time dependent programmed cell death mechanism.

Keywords. Aluminum, caspase-1 like activity, cytochrome c, DNA fragmentation, lipid peroxidation, programmed cell death.

INTRODUCTION

Aluminum (Al), as an abiotic stress factor, exists as third most abundant mineral forming 8% in earth's crust (Matsumoto 2000; Abate *et al.* 2013). It has been known that Al is the primary limiting factor on plant growth and development in acidic soils. Considering the 67% of the world's potential arable lands are acid soil, most of the agronomic plants are face to Al toxicity (Kochian *et al.* 2004; Abate *et al.* 2013; Ma *et al.* 2014). Non-toxic Al appears as insoluble aluminosilicates or oxides, but when soil pH decreases (pH<5) Al solubilized into reactive and phytotoxic forms being in a range of 10–100 µM (Matsumoto 2000; Ciamporova 2002; Vardar and Ünal 2007). Several

researches revealed that Al adversely affects the plant within a few minutes even at low micro-molar doses; as a result of this Al is considered as major constraint for crop yield (Vitorello *et al*. 2005; Abate *et al*. 2013).

Considering the whole plant, root apex (root cap, meristem and elongation zone) is the first target organ and accumulates more Al (Matsumoto 2000; Vardar *et al*. 2006). Several studies prove that Al induce morphological, biochemical and physiological alterations. Besides, it is a genotoxic agent leading adverse effects on DNA structure and function. Moreover, Al toxicity decreases mitotic index and causes chromosome aberrations (Frantzios *et al*. 2000; Vardar *et al*. 2011). Recent works also revealed that Al also adversely affects DNA methylation and polymorphism on LTR retrotransposons suggesting these alterations may be a defense mechanism to Al stress (Guo *et al*. 2018; Taşpınar *et al*. 2018).

There is a close relation to Al toxicity and ROS (reactive oxygen species) production triggering oxidative damage in the cell. Over accumulation of ROS cause damage on lipid, protein, carbohydrate, photosynthetic pigments and DNA leading to programmed cell death (PCD) (Darko *et al*. 2004; Sharma and Dubey 2007; Gupta *et al*. 2013).

PCD is considered as an alternative adaptive mechanism in plants to enable the survival of whole organism under extreme environmental stresses (Jackson and Armstrong 1999; Drew *et al*. 2000; Vardar *et al*. 2018). PCD is a genetically regulated cell suicide process and coordinated by specific proteases and nucleases (Wang *et al*. 2011; Vardar and Ünal 2012; Wituszyńska and Karpiński 2013; Petrov *et al*. 2015). It has been identified by common characteristics in eukaryotes such as cell shrinkage, vacuolization, cytochrome c release, specific protease activation, chromatin condensation, DNA fragmentation and finally breakdown of the cell (Vardar and Ünal 2008; Papini *et al*. 2011; Wang *et al*. 2012; Poor *et al*. 2013). There are several researches concerning abiotic stress induced PCD in plants, but Al stress-induced PCD is still need to be investigated to clarify its toxicity and tolerance mechanism. Even if there are a few research on temporal occurrence of Al-induced PCD (Vardar *et al*. 2015; 2016), more detailed time dependent analyses are of the essence. Therefore, we designed a detailed analysis addressing Al-induced PCD in the course of short and long term exposure in *Hordeum vulgare* roots.

MATERIAL AND METHODS

Plant material

The seeds of barley (*Hordeum vulgare* L. cv Çetin 2000) which were obtained from The Field Crops Central Research Institute (Ankara, Turkey) were sterilized with 1% sodium hypochloride solution for 10 min. After rinsing, seeds were placed on moistened filter paper in petri dishes for germination. The petri dishes were kept in a plant growth room with fluorescent tubes giving an irradiance of 5000 lux (day/night 16/8 respectively), temperature of 23 ± 2 °C, and relative humidity 45–50% for 48 h. The barley seedlings which reached 0.5-1 cm root elongation were immersed in 100 µM $AlCl_3$ (pH 4.5) with different time intervals. In the present study the exposure time designed in two groups: short (½, 1, 2, 3, 4, 5, 6 and 7 h) and long time (24, 48, 72 and 96 h) exposure. Distilled water was used for the control group. Fifty seeds were used for each experimental group. All of the analyses were assessed with three replicates for statistical validity. Analysis of variance of all the experimental data was performed with SPSS 13.0 computer program. Student t- test was used to determine the statistical significance of differences among the means at $P < 0.05$.

Determination of Al uptake

Al^{+3} ion uptake in control and treated roots was assessed by hematoxylin staining method (Ownby 1993). The intact barley roots were stained with solution containing 0.2% (w/v) hematoxylin and 0.02% (w/v) KIO_3 for 15 min in dark. Then the roots were washed with distilled water and 10 root tips (1 cm) immersed in 4 mL 1N HCl (v/v) for 1 h. After immersion, HCl solution was measured at 490 nm spectrophotometrically.

Determination of loss of plasma membrane integrity

The loss of plasma membrane integrity in control and treated roots was detected by Evans blue staining method (Pandey *et al*. 2013). The intact barley roots were stained with 0.25% (w/v) Evans blue solution for 30 min. After rinsing in distilled water for 10 min, 10 root tips (1 cm) were homogenized in 1 mL of 1% (w/v) sodium dodecyl sulphate (SDS) solution. After centrifugation at 13500 rpm for 10 min, the supernatant was measured at 600 nm spectrophotometrically.

Determination of lipid peroxidation

Lipid peroxidation was measured by the amount of malondialdehyde (MDA) produced after reaction with thiobarbituric acid (TBA) (Cakmak and Horst 1991). The

control and treated roots (0.4 g) were homogenized in 2 mL 0.1% (w/v) trichloroacetic acid (TCA). After centrifugation at 12000 g for 20 min, the supernatants (0.5 mL) were added on 0.6% (w/v) TBA in 20% (w/v) TCA (2 mL) and boiled at 95 °C for 30 min. The mixture was transferred to ice immediately. After color change the samples centrifuged at 12000 g for 10 min. Absorbance of the TBA-reactive substance was determined as TBA-MDA complex at 532 and 600 nm.

Determination of caspase-1 like activities

Control and $AlCl_3$ treated root tips (0.3 g) were ground in ice-cold mortar with 1 mL extraction buffer (50 mM HEPES-KOH – pH 7, 10% sucrose, 0.1% CHAPS, 5 mM DTT, and 1 mM EDTA) according to Lombardi *et al*. (2007). The homogenates were transferred on ice for 10 min and centrifuged at 14000 rpm (+4 °C) for 10 min. Protein concentration was determined by Nano photometer. For determination of caspase-1 activity, ENZO's Caspase-1/ICE Colorimetric Protease Activity Assay Kit was used. According to manufacturer's instructions, equal amounts of protein extracts were incubated at 37 °C for 90 min with p-NA (p-nitroaniline)-labeled substrates YVAD. The caspase-1 like activity was measured at 400 nm and calculated according to the standard curve. Comparison of the absorbance of p-NA from an apoptotic sample with an un-induced control allows determination of the fold increase in caspase activity.

Determination of cytochrome c release

Control and $AlCl_3$ treated root tips (0.2 g) were ground in ice-cold mortar with 3 mL 0.1 M phosphate buffer saline (PBS, pH 7.7). The homogenates were centrifuged (+4°C) for 15 min and the supernatant re-centrifuged at 16000 rpm for 15 min. After centrifugation, supernatant was collected as cytoplasmic phase and pellet is collected as mitochondrial phase. The pellet was re-suspended with 200 µL PBS (Huang *et al*. 2014). For determination of cytochrome c (cyt c) release, MyBioSource's Plant Cyt C ELISA Kit was used. According to manufacturer's instructions, both cytoplasmic and mitochondrial phases were incubated with HRP-conjugated reactive at 37°C for 60 min. After rinsing with washing solution, chromogen A and B solution were added and incubated at 37°C for 15 min. Along with stop solution, cytochrome c was measured at 450 nm and calculated according to the standard curve.

Determination of DNA fragmentation

DNA fragmentation was analyzed by flow cytometry in control and Al treated roots. For this purpose, *CyStain® UV Precise P* kit special for plants was used. The nuclei were isolated from 6 root tips by careful slicing with a razor blade in 0.4 mL Nuclei Extraction Buffer. The extract was collected with micropipette and stained with 1.6 mL DAPI in a micro centrifuge tube. The tubes were transferred on ice for 1-2 min and then filtered to test tubes. The DNA fragmentation of nuclei was analyzed with flow cytometry (Sysmex).

RESULTS

To determine the temporal effects of Al, the barley roots were exposed to 100 µM $AlCl_3$ (pH 4.5) for short (0, ½, 1, 2, 3, 4, 5, 6 and 7 h) and long (24, 48, 72 and 96 h) time points. After Al exposure Al^{+3} ion uptake, loss of plasma membrane integrity, lipid peroxidation, caspase-1 like activities, cytochrome c release and DNA fragmentation were analyzed in relation to time dependent programmed cell death (PCD) occurrence.

According to hematoxylin analysis Al^{+3} ion uptake increased by 8.7%, 30.4% and 73.9% at ½, 1 and 2 h, respectively. Ongoing times Al uptake raised by about 1.5 fold up to 5 h, 1.8 fold at 6 h and 2.1 fold at 7 h (Fig. 1a). After long term exposure, Al uptake increased exponentially by 4.6, 7.5, 11.3 and 12.1 fold at 24, 48, 72 and 96 h, respectively (Fig. 1b).

Evans blue analysis is frequently used to determine the loss of plasma membrane integrity. It has been known that the dye can penetrate through ruptured membranes and stains the damaged cells. In this respect after Al exposure plasma membrane rupture determined in barley root cells time dependently. Based on our results the dye uptake increased by 25.3%, 34.6%, 46.7%, 74.7%, 72%, 78.7%, 94.7% and 97.3% from ½ to 7 h respectively (Fig. 2a). After long time Al exposure Evans blue uptake increased progressively. It was increased by 2.4, 3.5, 4 and 4.3 fold at 24, 48, 72 and 96 h, respectively (Fig. 2b).

Under oxidative stress conditions, over production of reactive oxygen species (ROS) cause lipid peroxidation. After Al exposure MDA content one of the end product of lipid peroxidation showed an increment even at ½ h and it raised 6-7 fold up to 7 h (Fig. 3a). The increment showed 7.5, 9.7, 8.1 and 6-fold increase at 24, 48, 72 and 96 h, respectively. Although the MDA content was highest at 48 h, it decreased at 72 and 96 h with regard to 48 h (Fig. 3b).

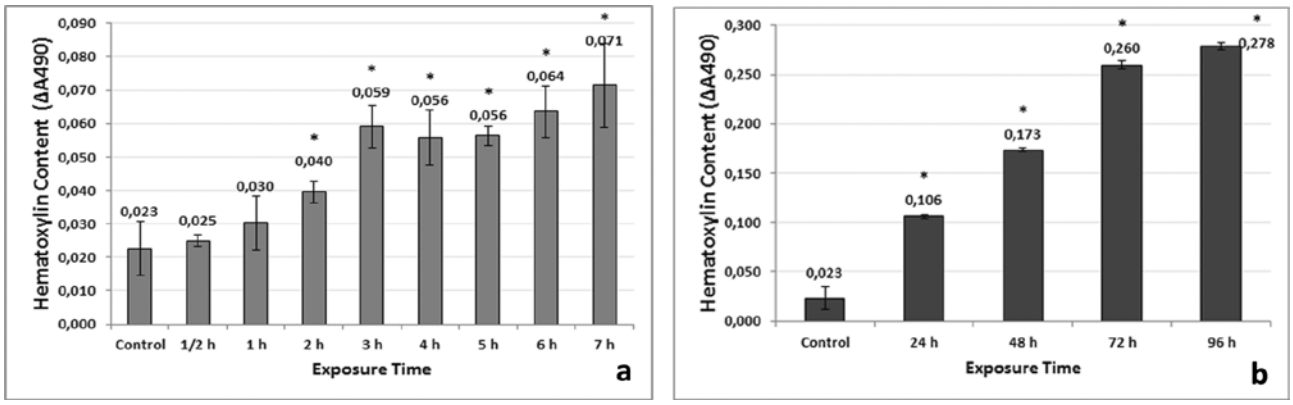

Figure 1. Hematoxylin content of control and 100 μM $AlCl_3$ treated barley roots at short (a) and long (b) time points. The data with (*) are significantly different from the control at $P < 0.05$ level.

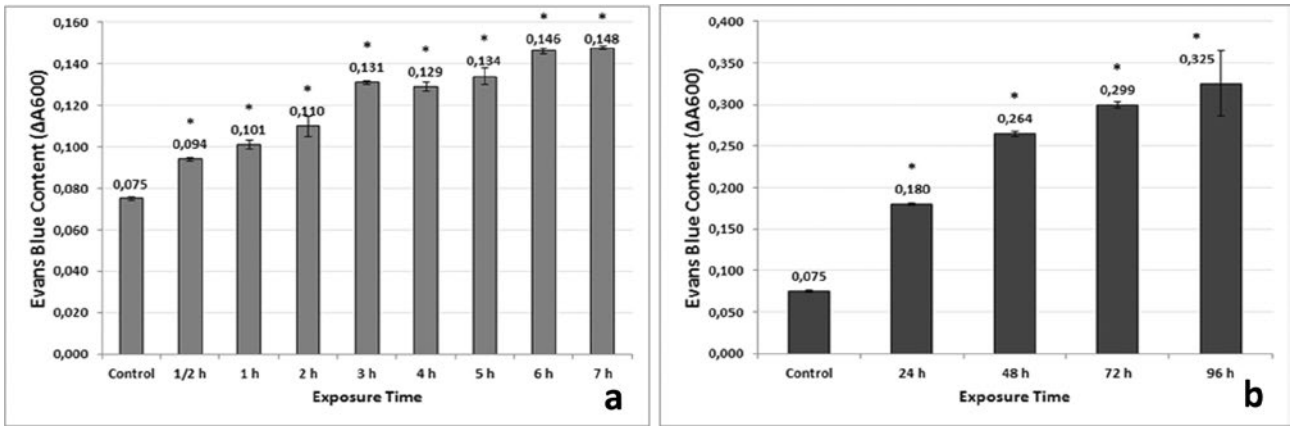

Figure 2. Evans blue uptake of control and 100 μM $AlCl_3$ treated barley roots at short (a) and long (b) time points. The data with (*) are significantly different from the control at $P < 0.05$ level.

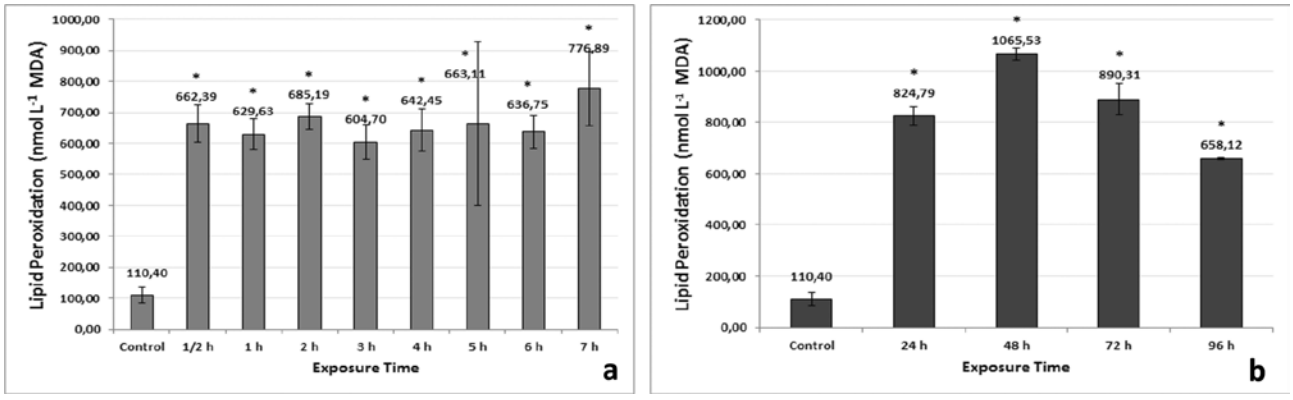

Figure 3. Lipid peroxidation of control and 100 μM $AlCl_3$ treated barley roots at short (a) and long (b) time points. The data with (*) are significantly different from the control at $P < 0.05$ level.

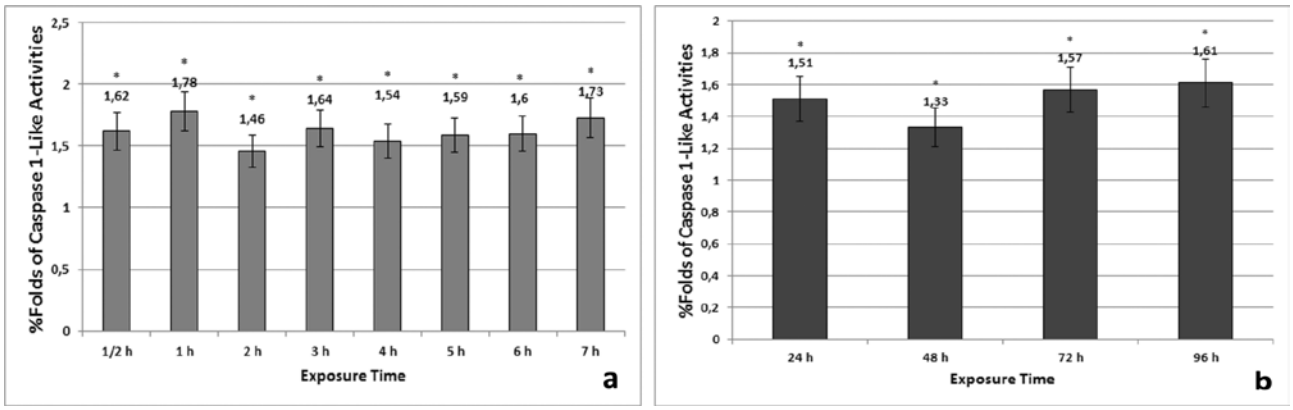

Figure 4. % fold caspase-1 like activities of control and 100 µM $AlCl_3$ treated barley roots at short (a) and long (b) time points. The data with (*) are significantly different from the control at $P < 0.05$ level.

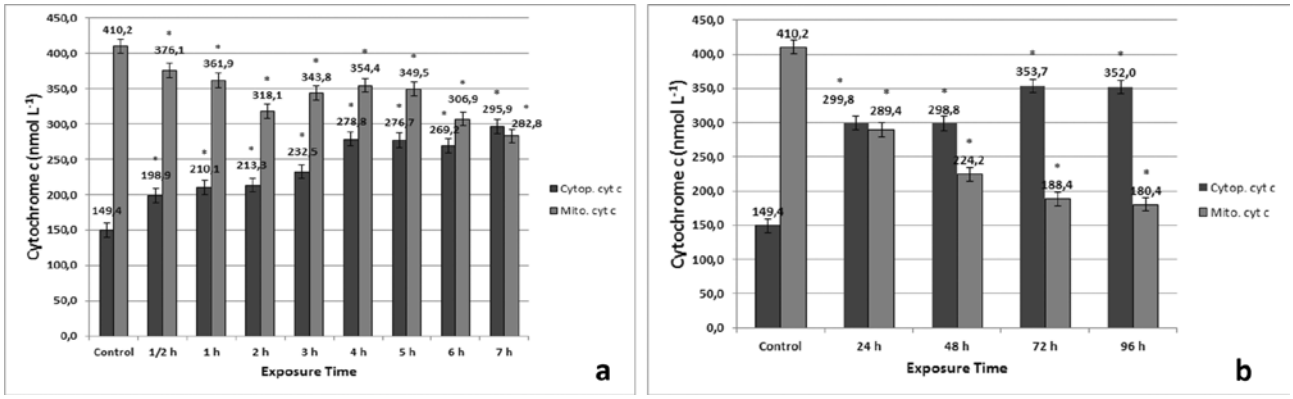

Figure 5. Cytochrome c level in mitochondria and cytoplasm of control and 100 µM $AlCl_3$ treated barley roots at short (a) and long (b) time points. The data with (*) are significantly different from the control at $P < 0.05$ level.

Although there are no functional homologs of animal caspases, there are true caspase-like activities in plants during PCD. Although the results of caspase-1 like activities showed fluctuations between exposure groups, Al caused increase both at short and long time points. The increased activities were among 1.33 – 1.78 than that of control (Fig. 4a, b).

It has been known that cyt c is released from inner mitochondrial membrane to cytoplasm during PCD. In barley roots, after Al exposure cyt c release started from ½ h and increased exponentially. It was 33.1% in ½ h, 40.6% in 1 h, 42.8% in 2 h, 55.6% in 3 h, 86.6% in 4 h, 85.2% in 5 h, 80.2% in 6 h and 98.1% in 7 h (Fig. 5a). After long term exposure it increased about 2 fold in 24 and 48 h, and about 2.4 fold in 72 and 96 h (Fig. 5b).

DNA fragmentation is one of the significant markers of PCD and flow cytometry is one of the analyses that used. The fragmented DNA can be seen as peaks at the left of G1 in the histogram. After the data analysis, the cell rate of having fragmented DNA was 23.7%, 17.3%, 31.4%, 37.1%, 15.0%, 37.8%, 25.1% and 30.6% between ½ - 7h, respectively (Fig. 6). At long term exposure DNA fragmentation rate was more significant. It was 63.7%, 49.6%, 62.6% and 64.4% between 24-96 h, respectively (Fig. 7). According to the flow cytometry Al caused DNA fragmentation both in short and long term exposure.

DISCUSSION

Al toxicity is one of the major inhibitors of plant growth and development in acidic soils (Abate *et al*. 2013; Ma *et al*. 2014). Although multiple studies have been performed in understanding physiological and molecular mechanism of Al toxicity and tolerance in the last few decades, there is limited studies concerning time dependent occurrence of Al-induced PCD. Therefore, we

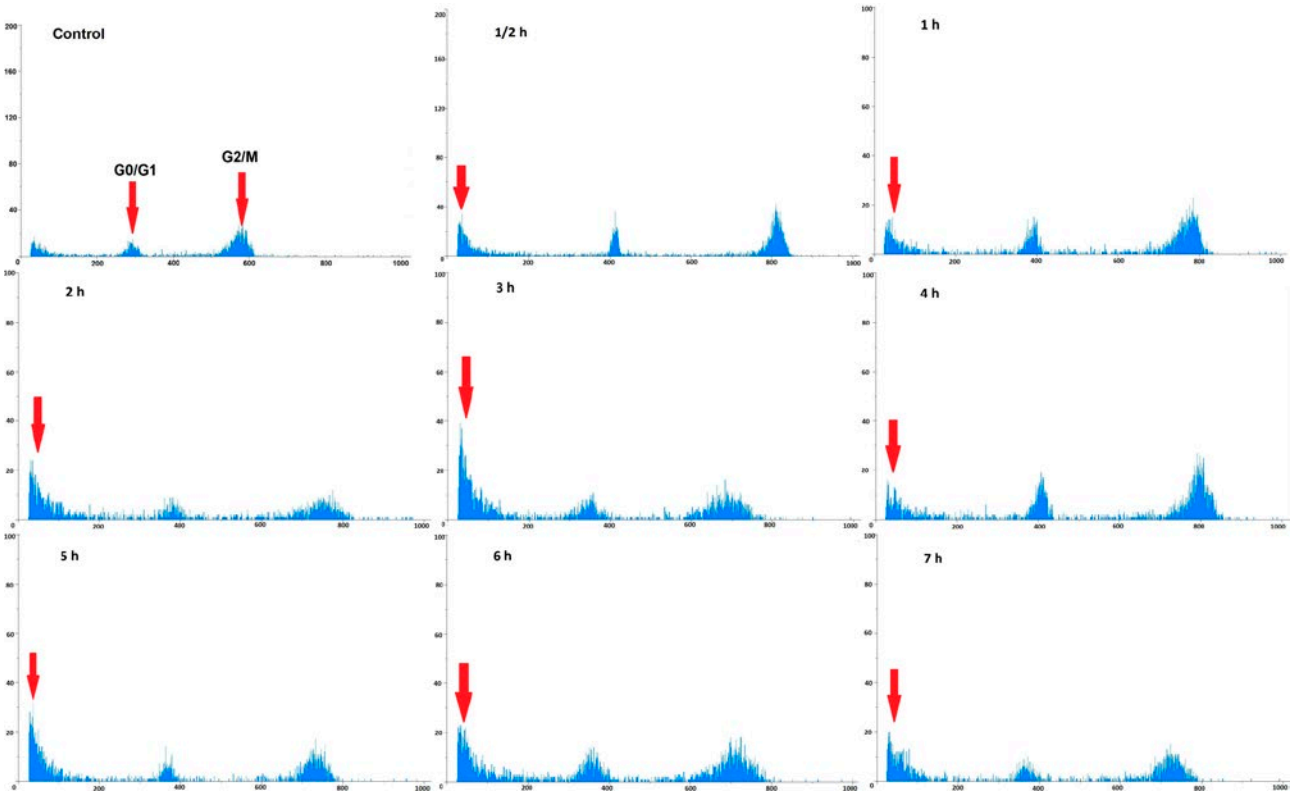

Figure 6. DNA fragmentation of control and 100 µM $AlCl_3$ treated barley roots at short term exposure. (x) axis denotes cell counts and (y) axis denotes fluorescence intensity.

determined the temporal effects of Al correlating with Al^{+3} ion uptake, loss of plasma membrane integrity, lipid peroxidation, caspase-1 like activities, cyt c release and DNA fragmentation in relation to PCD.

Al toxicity adversely affects the cellular processes due to the strong and rapid interactions of Al with apoplasmic and symplasmic targets. It has been widely known that Al accumulates more in root apex and strongly binds to negative charged materials such as cell walls and membranes (Matsumoto 2000). Several researches revealed that primer cellular responses to detrimental effects of Al could arise within second to minutes along with Al penetration (Kochian *et al.* 2005; Singh *et al.* 2017). Based on our Al^{+3} ion uptake results, Al penetrated into root apex beginning from ½ h and increased on the advancing hours. Although the Al uptake was very slight (8.7%) at the beginning, its impact was very severe in barley roots. Considering the loss of plasma membrane integrity and lipid peroxidation, the toxicity was very significant even at ½ h Al exposure. Al uptake increased gradually depending on time and at the same time loss of plasma membrane integrity enhanced. Considering the sharp increase of MDA even at ½ h suggests that Al is not detrimental on only plasma membrane. It's also very injurious on whole cellular membrane system including organelles. Although MDA content rose sharply up to 48 h, the reduction of MDA at 72 and 96 h may be related to increased generation of the other end product aldehydes such as 4-HNE.

According to general consensus Al induces oxidative stress as an abiotic stress factor in plants (Matsumoto 2000). Whereas Al is not a transition element, it catalyzes formation of ROS inducing oxidative stress (Gupta *et al.* 2013). Over-accumulation of ROS induces damage of biological molecules such as proteins, DNA and lipids. Membrane lipids are the more significant targets of ROS and culminate in lipid peroxidation eventually affecting normal cellular functions such as reduction in membrane fluidity, increase in phospholipid exchange, leakage of membrane and membrane rupture. Besides lipid peroxidation is considered as one of the hallmarks of PCD (Gill and Tuteja 2010; Woo *et al.* 2013; Nath and Lu 2015). Moreover, it has been reported that Al induces swollen mitochondria with several vacuoles, disrupted plasma membrane, and nuclear deformations following

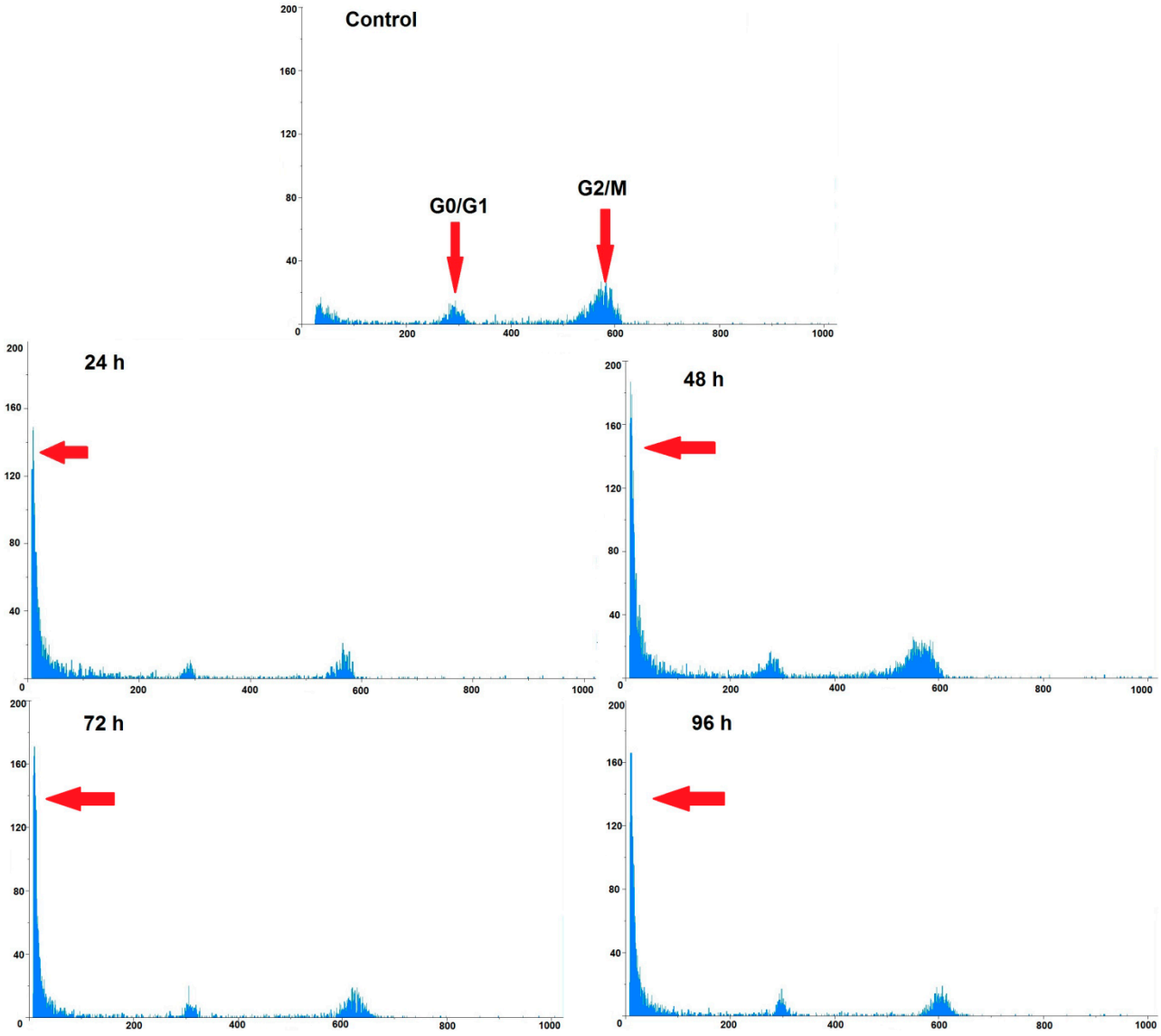

Figure 7. DNA fragmentation of control and 100 µM $AlCl_3$ treated barley roots at long term exposure. (x) axis denotes cell counts and (y) axis denotes fluorescence intensity.

the mitochondrial pathway of PCD (Gupta *et al.* 2013; Singh *et al.* 2017).

There is a close relation to ROS triggered cyt c release from the inner mitochondrial membrane to cytoplasm constituting mitochondrial pathway of PCD. ROS provokes the cardiolipin oxidation in mitochondrial inner membrane weakening the bonds of cyt c. ROS also triggers the Ca^{2+} transition as a secondary signal from ER to mitochondria reducing the mitochondrial membrane potential (ΔΨm). After ΔΨm decrease, cyt c released to intra-membrane space translocate to the cytoplasm from the mitochondrial permeability transition pores (MPTP) in the outer mitochondrial membrane (Ott *et al.* 2007; Williams *et al.* 2014). In PCD cyt c release stimulates caspase-like activities resulting in execution of PCD (Li and Xing 2010; Petrov *et al.* 2015; Gunawardena and McCabe 2015).

Huang *et al.* (2014) indicated that Al-induced ROS accumulation and PCD is closely related. According to their results 100 µM $AlCl_3$ caused increase in MPTP opening, reduction of ΔΨm, cyt c translocation to cytoplasm and caspase 3-like activity subsequent to ROS accumulation in *Arachis hypogaea* roots. It has been concluded that Al induced PCD is mediated by ROS

related cascade of cellular events resulting in mitochondrial alterations and caspase like activities. Simultaneously, Zhan *et al*. (2014) established the relation between ROS accumulation and mitochondrial alterations during Al-induced PCD in *Arachis hypogaea*. The researchers reported that increase in mitochondrial ROS induced reduction of mitochondrial Ca concentration, opening of MPTP, collapse of ΔΨm and cyt c release were more extensively in Al sensitive cultivar depending on Al concentration. Similarly, in the present study Al application caused cyt c release to the cytoplasm depending on time. The best of our knowledge all of the researches subjecting cyt c release were qualitative analysis and no report has been published of quantitative analysis of cyt c release during PCD in plants except our study.

In addition to alterations of mitochondria several researchers reported caspase like activities induced by ROS increase and cyt c release after Al application (Li and Xing 2011; Huang *et al*. 2014; Aytürk and Vardar 2015; Yao *et al*. 2016). Caspases are cysteine proteases initiating and amplifying PCD and cleave their cellular substrates at certain aspartic acid residues (McIlwain *et al*. 2013). Whereas there are no functional homologs of caspases in plants, caspase-like activities have been elucidated. According to recent reports, vacuolar Processing Enzymes (VPEs) belonging to cysteine protease family cleave synthetic caspase-1 substrate YVAD (Tyr-Val-Ala-Asp) in plants (Hatsugai *et al*. 2004; Cai *et al*. 2014; Rocha *et al*. 2017). Besides synthetic caspase-1 inhibitor (ac-YVAD-CHO) blocks VPE activation (Sexton *et al*. 2007; Misas-Villamil *et al*. 2013). Kariya *et al*. (2013; 2018) reported Al-induced dose dependent VPE activity at transcriptional level in *Nicotiana tabacum*. The researchers also indicated that the presence of VPE inhibitor (Ac-YVAD-CHO) suppressed PCD symptoms. Aytürk and Vardar (2015) revealed that short term Al toxicity also increased caspase-3, -8 and -9 like activities which are responsible for DNA fragmentation and initiation of apoptosis in animal systems in rye, barley, oat and triticale roots. Similar to the stated studies, Al application caused caspase-1 like activity from ½ h to 96 h in barley roots. Although it has been reported that Al toxicity induces different caspase like activities, using specific caspase-1 substrate is more accurate approach because VPE specific to plants has caspase-1 like activity.

DNA fragmentation which is one of the important signatures of PCD originates from internucleosomal DNA cleavage by specific proteases and nucleases. In light and electron microscopy fragmented DNA can be seen as a compact mass at the periphery of the nucleus (Vardar and Ünal 2012). DNA fragmentation can also be analyzed by TUNEL reaction, comet assay, laddering on the agarose gel and flow cytometry (Tripathi *et al*. 2016).

Flow cytometry is based on DNA staining with a florescence dye and analyze of the cell cycle (Shen *et al*. 2017). In the histogram of flow cytometry, the first peak indicates G0/G1 cycle and the second peak indicates G2/M. During cell death DNA was cleaved into oligonucleosomal fragments. As a result of this, DNA content decreases in apoptotic cells and fragmented DNA accumulates at the left of G0/G1 peak (Yamamada *et al*. 2006).

Vardar *et al*. (2015; 2016) revealed DNA fragmentation using comet assay and agarose gel electrophoresis under Al stress in early hours in wheat, rye, barley, oat and triticale. According to our results the accumulation of fragmented DNA was also visible after short term Al application. The fluctuation in the rate of cells having fragmented DNA may suggest a recovery effort in short term exposure. However the rate of dying cells is up to 50% and DNA fragmentation is more severe in long term exposure. As distinct from the previous results, we put forward the long term effect of Al on DNA fragmentation. Although there are several studies concerning DNA fragmentation revealed by flow cytometry during senescence (Yamada *et al*. 2003; 2006), there is no study available during abiotic stress as well as Al stress. However, Jaskowiak *et al*. (2018) examined the effect of Al on the cell cycle of barley roots by flow cytometry. The researchers revealed that the DNA replication and mitotic index reduced, but G2/M phase increased. Besides

In conclusion; Al caused loss of plasma membrane integrity, lipid peroxidation, cyt c release, caspase-1 like activity and DNA fragmentation which are characteristic features of PCD. Although Al uptake, plasma membrane integrity, lipid peroxidation, cyt c release increased time dependently, caspase-1 like enzyme begun to activate at ½ h and did not represented very wide difference during short or long term Al application. Moreover, DNA fragmentation was progressive at long term exposure during Al-induced PCD.

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CONFLICT OF INTEREST

The authors declare that they have no conflict interest.

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