Effects of some Stress Factors (Aluminum, Cadmium and Drought) on Stomata of Roman Nettle (\textit{Urtica pilulifera} L.)

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Abstract

In this study, Roman nettle (\textit{Urtica pilulifera} L.) seedlings grown singly in standard pots containing compost were exposed to two different levels of aluminum and cadmium (100 µM and 200 µM) and water stress (moderate and severe stress) treatments. Measurements of stomatal perimeters, diameters and areas from the epidermal sections in lower surfaces of young expanded leaves of main stem and first lateral branches were examined by image processing and analysis software. The data proved that all stomata were affected significantly, but with varying responses, in all treated plants compared to control plants. Excluding severe water stress (WS 2), the data from first lateral branch leaves showed slight sensitivity to all stress treatments. Nevertheless, there were no statistically significant differences between stomatal measurements from main stem and first lateral branch leaves. Particularly, reduction in stomatal diameters of both main stem and first lateral branches in severe water stressed plants, reducing by 26.45% and 48.09% respectively; suggest that this could be a response of \textit{U. pilulifera} to drier environments.

Keywords: Stomata, Al, Cd, water stress, drought, nettle, \textit{Urtica pilulifera} L.

Introduction

Stomata are the principal means of gas exchange in vascular plants. They are small pores, found epistomatically, hypostomatically and amphistomatically on leaves that are fully/partly opened or closed under the control of a pair of kidney-shaped cells called guard cells (Fitter and Hay, 1978; Grant and Vatnick, 1998; Adedeji and Jewoola, 2008). In nature, the opening and closing of stomata involves feedback and feed-forward loops, and is affected by decreased CO$_2$ in the intercellular air space, too much transpiration and some environmental conditions such as water stress (Garbutt et al., 1990; Jones, 1992). The stomatal mechanism is also affected by the plant hormones; abscisic acid (ABA), cytokinins, auxins, and possibly gibberellic acid. ABA plays an important role in stomatal closure, seed dormancy and plant adaptation to environmental stresses (Tal and Imber, 1970; Davies, 1987). In addition, stress factors like high salinity and drought are among the most crucial factors for the growth of plants and water stress induces a rapid decline in stomatal conductance, rate of transpiration and net photosynthesis (Davies, 1987; Kozlowski 1997; Munns, 2002; Buckley, 2005). Some toxic and heavy metals affect soil pH and uptake of the nutrients from the soil, which influence plant growth and development (Matsumoto, 2000; Neil and Gregory, 2001; Nocito et al., 2002; Vitorello et al., 2005).

Al is the most abundant metal in the earth's crust and one of the most important components of the soil (7%), and also it is soluble as a trivalent ionic form is highly active in acid soil (< pH 5.0) and toxic to plant growth (2-3 ppm) causing reductions in crop production (Thornton et al., 1986; Kochian, 1995; Matsumoto, 2000; Vardar et al., 2006). The molecular mechanisms of Al toxicity are still poorly understood, despite extensive studies (Rengel, 1992; Delhaize and Ryan, 1995). Among the common effects of Al are: decrease in total leaf number and size, a decrease in shoot biomass, inhibition of root elongation, chlorosis and necrosis of leaves leading to decreased photosynthetic activity (Thornton et al., 1986; Kochian, 1995; Jones and Kochian, 1995). Al also causes ultrastructural and cellular changes in leaves, as cell division and elongation are inhibited, and reduces stomatal aperture (Rengel, 1992; Kochian, 1995; Delhaize and Ryan, 1995; Vardar and Unal, 2007).

Cadmium is considered a trace element, and is one of the heavy metals with an occurrence in natural and agricultural environments mostly resulting from human activities, such as industrial processes like mining and refining (Wagner, 1993; Sandalio et al., 2001; Akgüç et al., 2008). Cd is a strong phytotoxic element, which inhibits vegetative plant growth and even causes plant death (Sandalio et al., 2001). The mechanisms involved in cadmium toxicity still require more research, despite intensive studies on its toxicity in a variety of plants. Common effects of Cd include; affecting water balance of plants by reducing root growth, limiting water uptake via a reduction in vessel size, and causing partial stomatal closure (Barcelo and Poschenrieder, 1990;
Prasad, 1995). It also causes a decrease in tissue biomass, chlorosis, and effects on specific physiological (e.g., xylem transport) or biochemical (e.g., nitrogen fixation) processes (Kosma et al., 2004).

Water stress is also one of the most important environmental factors causing to reduction in plant growth and development as well as plant productivity and crop yields (Boyer, 1982; Jones and Famjul, 1982; Akinci, 1997). The effect of water stress can be manifest in many ways, as varied morphological, physiological and biochemical changes in plants under different water stress. For instance changes in leaf morphology (Parker, 1968; Morgan, 1980; Hsiao et al., 1984; Blum, 1989; Akinci, 1997), effects on shoot and root growth and development (Sharp and Davies, 1979; Rambal and Debussche, 1995; Akinci, 1997), limiting photosynthetic activity by decreasing CO₂ influx, decrease in carboxylation, electron transport chain activities of the chloroplasts in the mesophyll cells (Akinci, 1997). It also affects many other metabolic pathways, mineral uptake, membrane structure (Schulze, 1986; Davies and Zhang, 1991; Tardieu and Davies, 1993; Davies, 1995) stomatal structural changes and conductance (Huber et al., 1984; Wong et al., 1985; Raschke and Reesmann, 1986; Cornic et al., 1989; Akinci, 1997), and CO₂ uptake (Hsiao, 1973; Quick et al., 1992; Akinci, 1997).

Water deficit in plants causes the closure of stomata (Hsiao, 1973; Epstein and Grant, 1973; Quick et al., 1992; Akinci, 1997), which decreases both transpiration and photosynthesis in many plant species (Zelitch, 1971; Shekhvar and Iritani, 1979; Fatemy et al., 1985). Stomatal closures occur via the distress signal "abscisic acid" and lead to a decreased rate of transpiration from the mesophyll chloroplasts to the guard cells of the stomata during water stress conditions (Wright, 1969; Wright and Hiron, 1969).

Urticaceae family members are very common and widespread species found in the margins of arable fields, gardens and countryside throughout Europe, Asia and Northern Africa (Firbank et al., 2002). They have high nutrient requirements demonstrated by leaves, which contain high levels of N, Ca, Mg (Grime et al., 1988; Wilman and Riley, 1993) and Fe (Salisbury, 1962). Urtica dioica L. (stinging nettle) and Urtica urens L. (dwarf nettle) are well-known Urticaceae family member species and they have been used as medicinal plants all over the world for years (Kavalalı et al., 2003). They have used as expectorant, purgative, diuretic, hemostatic, vermifuge and for the treatment of eczema, rheumatism, hemorrhoids, hyperthyroidism, bronchitis and cancer (Barker, 2001; Kavalalı et al., 2003). Furthermore, their stems have also used for making linen and ropes (Bond et al., 2006). A less known Urticaceae family member U. pilulifera L. (Roman nettle) locally, named “Kara Isırgan” is one of the most important traditional drugs in Turkey. All parts of the plant bristle with stinging hairs and it flowers from May to August (Davis, 1982). In Turkish traditional folk medicine, this plant is commonly used as a remedy for diabetes mellitus (Baytop, 1999). Up to this day, such use of U. pilulifera L. is quite prominent in the Black Sea region of Turkey (Kavalalı et al., 2003).

The objectives of this research were to investigate the effects of different levels of Al (100 µM-200 µM), Cd (100 µM-200 µM) and water stress (moderate stress and severe stress) exposure to Roman nettle seedlings and to observe the relationship between some stomatal parameters (pore diameter, perimeter and area) and various stress types under growth room conditions.

Materials and methods

Growing seeds

The surface of Roman nettle seeds were soaked by immersion in ethyl alcohol (50%) for 1 minute followed by deionized water for 5 minutes. They were then transferred into small vessels containing sterilized compost for germination. During the germination period (2 weeks), the seeds were moisturized with deionized water. When the shoot lengths of the young plantlets reached 3-4 cm, they were transferred into standard plastic pots containing sterilized compost and maintained under growth-room conditions. The plants were grown under fluorescent tubes giving an irradiance of 5000 lx (day/night-16/8 respectively), and a temperature of 23±2 °C and relative humidity 45-50%. Each of the experimental groups of eight replicates were watered with Hoagland’s nutrient solution (Hoagland and Arnon, 1950) at two-day intervals for the 2 months during which the stress treatments were applied.

Stress Treatments

Application of Al and Cd

While control plants were watered only with Hoagland solutions, the experimental groups were watered with spiked Hoagland solutions (prepared as 100 and 200 µM AlCl₃ or CdCl₂). Each treatment was watered with 40 ml of solution at two-day intervals. The soil pH was adjusted to 4.5 for Al treatments using 2% H₂SO₄.

Water stress

The gravimetric determination of water content by weighing soil samples before and after oven drying to constant weight at 85 °C was used to calibrate all measurement of the moisture content of compost in pots. The pot weights corresponding to soil moisture contents after 12 and 18 days were calculated according to the equation of Paquin and Mehuys (1980). After determining the stress levels as 52% and 45% moisture content for moderate (MS) and severe stress (SS) levels respectively, the seedlings were watered at two-day intervals to maintain the moisture levels.
After 2 months of stressing with Al, Cd and water, plants were harvested and microscopic preparations were arranged for stomata studies. The two youngest fully-expanded leaves from shoots and first lateral branches were harvested from each plant, and for each leaf 20 stomata from the abaxial leaf epidermis were measured for stomatal apertures (pore diameter, perimeter and area) (Fig. 1). The preparations were photographed with an Evolution LC Color camera and an Olympus BH-2 microscope. The images were analyzed with Image-Pro express version 6.0 scientific image processing and analysis software. The stress treated plants are abbreviated as Al 1 (100 µM AlCl₃), Al 2 (200 µM AlCl₃), Cd 1 (100 µM CdCl₂), Cd 2 (200 µM CdCl₂), WS 1 (moderate water stress) and WS 2 (severe water stress) respectively.

Statistical Analysis

The data were subjected to paired-sample T-tests, using SPSS 11.5 for Windows, with 95% (P < 0.05) significance of differences between means. Means are indicated with standard error (bars indicate s. e.).

Results and discussion

In this study, the diameters, perimeters and areas of stomata from the lower surfaces of leaves were measured using Image-Pro express version 6.0 scientific image processing and analysis software. In all cases, stomatal sizes were significantly decreased in stress-imposed replicates compared to control values (Fig. 2-3). The reduction in the values for first lateral branch leaves stomata for Al 1-2 and WS 1 treatments were slightly higher than the reduction in the main stem stomata for these treatments (Tab. 1). The reduction of the stomatal sizes in Al 1 and Al 2 showed similarities, indicating stability of effect, unlike Cd 1-2, and WS 1, which particularly reduced stomatal openings (Vitorello et al., 2005). The changes in Al treated plants suggest the inhibition of K⁺ in guard cells, which is correlated to stomatal opening (Schroeder, 1988; Schroeder et al., 1994). Al treatment of plants (9 h) induced stomatal closure (Sivaguru et al., 2003) and abscisic acid regulates potassium and chloride ion channels at the plasma membrane of guard cells, leading to stomatal closure by reducing transpiration (Leyman et al., 1999).

Comparison of the values between the main stem and first lateral branches, it was observed that the differences between decreasing values in all stomatal measurements fluctuated in WS 1, Cd 1 and Cd 2 (Tab. 1). The results of Cd 1 and Cd 2 treatments suggest that water absorption level was affected by Cd, as well as ABA changes, leading to stomatal closure and significant decrease in stomatal opening with increased Cd concentration. It has been suggested that Cd has a direct effect on the ion and water movement in the guard cells (Sayed, 1997); nitrogenase activity declined (30%) even at 18 µM, and photosynthesis was depressed by 60% by 300 μM Pb and Cd (Huang et al., 1974). It has also been reported that Cd reduces ATP and chlorophyll concentrations in many species, decreases oxygen production (Das et al., 1997), and that significantly reduced transpiration rates (Sayed, 1997) might be related to Cd-treated plants having smaller stomatal apertures (Huang et al., 1974; Sayed, 1997).

The various effects of water deficit seen on stomatal structure are clearly mechanisms to enable plants to survive in stress conditions. For instance, various strategies can be seen in wheat and other cereals in terms of turgor loss and stomatal closure at different relative water content (Richter and Wagner, 1982). In this study, Roman nettle seedlings responded differently to two levels of water stress. The first level of water stress (WS 1) caused the greatest reduction in main-stem leaf stomatal param-
more densely distributed stomata (no data obtained in the experiment) is seen as an adaptation in leaves growing under conditions of water deficiency, which allows a leaf to reduce transpiration by regulating stomatal mechanisms more rapidly (Hsiao, 1973; Larcher, 1995).

Conclusion

Environmental factors affect whole plants; however, it is the effects on the aerial parts that are most markedly visible. In leaves, guard cell regulation has become an important model system for understanding the regulatory signals that govern stomatal behavior (Comstock, 2002). Stomatal responses have been measured under stress factors such as salinity and drought however, investigations on the influence of heavy metals toxicity on stomatal regulation, especially the effects Al and Cd, were limited.

Al toxicity in molecular terms is still poorly understood (Rengel, 1992; Delhaize and Ryan, 1995). However,
common effects include: decrease in total leaf number and size, chlorosis and necrosis of leaves leading to decreased photosynthetic activity (Thornton et al., 1986; Kochian, 1995; Jones and Kochian, 1995) and reducing stomatal aperture (Rengel, 1992; Kochian, 1995; Delhaize and Ryan, 1995; Vardar and Ünal, 2007).

Cadmium toxicity on plants requires intensive studies, although some have been undertaken so far, providing some evidence that it affects water balance of plants by reducing root growth, thereby limiting water uptake, and causing partial stomatal closure (Barcelo and Poschenrieder, 1990; Prasad, 1995).

It is clearly known that plants exposed to short-term water deficit respond by reducing stomatal conductance and water loss (Jones and Famjul, 1982; Morgan 1980; Buckley, 2005) and field and greenhouse research indicate that races may respond differently to water stress (Rajkaruna et al., 2003). The stomata normally close to reduce water loss from the leaves under drought conditions (Van Iersel and Nemali, 2004). Compared with control values, the stomatal perimeter, diameter and area values were significantly reduced by all treatments of Al, Cd and water stress in both main stem and first lateral branch leaves. The reduction in area and diameter was conspicuous; however, the smaller reduction in perimeter suggests that stomatal closure is accompanied by decreasing stomatal sizes depending on a reduction in epidermal tissues. The data from first lateral branch leaves showed less sensitivity to the stress factors than those in main stems, compare to controls. However, the appliance of 100 and 200 μM Al and severe water stress (SS) showed a rather decrease in stomatal measurements in Roman nettle's first lateral branches, whereas 100 and 200 μM cadmium and moderate water stress (WS 1) caused fluctuations of the measured parameters in both

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**Fig. 3.** Stomatal guard cells of 2 months stress treated (Al, Cd and WS) Roman nettle (Urtica pilulifera L.). C= Control, S= Main Stem (Leaf), B= First Lateral Branch (Leaf) Al 1= 100µM AlCl3 Al 2= 200µM AlCl3 Cd 1= 100µM CdCl2 Cd 2= 200µM CdCl2 WS 1= Moderate water stress WS 2= Severe water stress. Bars = 5 µm.
main stems and first lateral branches. The results and those of similar studies prove that many stress factors effect hormonal changes (ABA). As a conclusion, stomatal pores are forced to close, as well as effects on different structural and ultrastructural mechanisms of the stomata (Tal and Imber, 1970; Grant and Vatnick, 1998; Buckley, 2005) and other factors, such as leaf position on the main stem or branches.

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