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Exogenous Ca_2SiO_4 enrichment reduces the leaf apoplastic Na^+ and increases the growth of okra (*Abelmoschus esculentus* L.) under salt stress

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ABSTRACT

Salt-sensitive plants have been hypothesized to deposit excessive Na^+ in their leaf apoplast, which can mainly disturb shoot growth of crop plants. Knowledge of the subcellular location of Na^+ is important to ascertain its toxicity level under salt stress. However, this hypothesis of extracellular salt deposition under salinity stress is still not fully understood. This research mainly focuses on the influence of 1 mM calcium silicate (Ca_2SiO_4) on growth and Na^+ accumulation in the leaf apoplast of hydroponically grown salt sensitive okra plants under short-term stress of 100 mM NaCl. It has been found that Na^+ concentration not only increases in total shoot and root of okra, but also in the leaf apoplast of salt-stressed plants. Na^+ concentration was significantly reduced in total shoot (52%) and leaf apoplast (30%), when 1 mM Ca_2SiO_4 was applied as a foliar plus pot under salt stress. Moreover, calcium silicate treated plants showed significant improvement in shoot fresh weight, shoot length, leaf area and leaf length under 100 mM NaCl stress. Together, these results suggest that calcium silicate enrichment in pot and as a foliar spray increases the growth of *Abelmoschus esculentus* by reducing the Na^+ concentration in the leaf apoplast, which could thus ameliorate the negative impacts of salt stress on plants.

1. Introduction

Increasing soil salinity poses a major threat to growth and production of agricultural crops throughout the world, especially in the regions of arid and semi-arid climate. Increasing population requires increased quantity and quality of food, whereas salinity is the major hindrance for both the growth and yield of crop plants (FAO, 2010). Okra (*Abelmoschus esculentus* L.) has been suggested to be severely affected and can result in huge reduction in per hectare yield when subjected to salt stress (Khan et al., 2001). Earlier growth stages of okra plants are more susceptible to salt stress (Cerda et al., 1995), as it alters water relations and nutrient uptake of plants. Moreover, it is also well known that at later growth stages of okra, ionic stress reduces leaf expansion (Shahid and Rahman, 2011). Therefore, Na^+ toxicity may alter nutrient uptake pattern and results in plant growth reduction, as negative impacts of salinity on crop plants includes low osmotic potential, specific ion toxicity, disturbances in nutrient balance, and more likely a combination of these factors (Diao et al., 2014).

Shoot growth reduction is rapid and is considerably affected as a consequence of osmotic stress in the first stage of salt stress (Munns and Tester, 2008). According to Marschner and White (2012), shoot growth is more susceptible to osmotic stress caused by salinity as compared to root growth. When exposed to a highly saline environment, plants can face a wide range of metabolic and osmotic challenges for its normal functioning through the increase in Na^+ contents of shoots. On the other hand, this is also reported that leaves are more susceptible to sodium ions since sodium ions accumulate more in shoots as compared to roots (Tester and Davenport, 2003). Therefore, we aim to investigate the apoplastic Na^+ concentration in the *Abelmoschus esculentus* L. leaf in the first phase of salinity stress. Reduced life of salt-stressed leaves results in the declined growth and development, which ultimately reduces the crop yield and net productivity. Partitioning of Na^+ ion inside the leaf cells and tissues of the plants along with the storage rate of Na^+

Abbreviations: AWF, apoplastic washing fluid; FW, fresh weight; DW, dry weight; CRD, completely randomized design.

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ions determine the stage where the adversity caused by Na^+ ions is evident (Munns, 2002). At this stage Na^+ may results in direct effects of salt on the rate of cell division, a slower rate of expansion or a decrease in the duration of expansion (Volkmar et al., 1998). Flowers et al. (1991) suggested that osmotic imbalance as a result of absolute Na^+ concentration in the leaf apoplast can cause dehydration of leaf cells and turgor loss.

Earlier, Oertli (1968) hypothesized that death of the leaves by the dehydration of leaf cells and turgor loss can be a consequence of excessive salt build-up in the leaf apoplast under salinity stress. Limited investigations dealing with apoplastic Na^+ concentration under salinity stress revealed inconsistent results in different crop plants. Possible variances in Na^+ concentration in the leaf apoplast could be due to differences in leaf anatomy of the selected monocot and dicot plants as well as salinity exposure duration. Earlier, high Na^+ concentration in the leaf apoplast of salt sensitive field bean was found under short term salt stress (Shahzad et al., 2013). Less Na^+ accumulation in the leaf apoplast has been found under salt stress in studies conducted using monocots (maize and wheat) (Lohaus et al., 2000; Mühling and Läuchli, 2002; Wimmer et al., 2003; Masood et al., 2012). Other distinctive differences in previous studies include; (i) application of 100 mM NaCl at once rather than in splits (Speer and Kaiser, 1991) with salt-sensitive pea plants, hence, not letting the plants to adjust to amended conditions, especially by osmosis and (ii) the use of X-ray microanalysis technique in Flowers et al. (1991) study for the investigation of Na^+ in rice leaves, which poses problems in the accuracy of detecting Na^+ and determining the differences between Na^+ and K^+. In the current study, *Abelmoschus esculentus* a dicot plant was exposed to high levels of salt (NaCl) in a short-term experiment to investigate the Na^+ deposition in the leaf apoplast.

Beneficial impacts of both calcium and silicon on plants have been widely investigated under different abiotic stresses including salinity stress (Saqib et al., 2008; Pei et al., 2010; Abdel Latef, 2011). Despite plenty of research dealing with calcium and silicon application in monocots and dicots which have shown improvements in plant growth under salt stress, its role at subcellular level is still not clear. Free calcium in apoplast can influence plant growth (Lawlor and Cornic, 2002) while, silicon has been suggested to deposit largely in the apoplast (Rogalla and Römheld, 2002). Hence, the current study reported for the first time the use of calcium silicate in pot, as a foliar spray and a combination of foliar plus pot, to relieve the accumulation of Na^+ in the leaf apoplast of okra plant under salt stress.

2. Materials and methods

2.1. Plant cultivation

Salt sensitive *Abelmoschus esculentus* L. plants were grown hydroponically in the greenhouse of COMSATS Institute of Information Technology, Abbottabad. Initially, seeds of *Abelmoschus esculentus* L. were soaked in aerated 0.5 mM $CaSO_4$ solution for eight hours. The seeds were germinated in quartz sand which was placed under the sunlight during the daytime and indoor during night at room temperature. After five days, seedlings were shifted to continually aerated pots with 4.0 L of one-fourth concentration of nutrients. After two days of acclimatization, nutrient concentrations were raised to half, whereas the full strength of the nutrient medium was achieved after four days. The nutrients culture comprised of the following elements: 2.0 mM $Ca(NO_3)_2$, 1.0 mM K_2SO_4, 0.1 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.2 mM KCl, 1.0 mM NaCl, 10.0 μM H_3BO_3, 2.0 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 0.05 μM $(NH_4)_6Mo_7O_{24}$, 60 μM Fe-EDTA. Nutrient solution was later changed twice a week to avoid any nutrient scarcity. After exposure of 100 mM NaCl stress for seven days, third and fourth-paired leaves from the bottom of the plants showing no salt injury were excised for the collection of apoplastic washing fluid (AWF).

2.1.1. NaCl and Ca_2SiO_4 treatments

NaCl treatment was initiated when full strength of nutrient solution was attained or at the appearance of the fourth leaf stage. The examined levels of NaCl doses were 1 mM for controls and 100 mM for saline treatments unless otherwise illustrated. Plants were gradually acclimatized to salt stress via daily additions of 25 mM NaCl, till the final concentration of 100 mM NaCl was achieved. At the time of salt application, calcium silicate (Ca_2SiO_4) with different treatment types (*i.e.* in nutrient solution, as a foliar application and both in nutrient solution and foliar spray together) was added with at least four replicates using completely randomized design (CRD). The treatments were as: control (1 mM NaCl), salt (100 mM NaCl), pot + salt (1 mM Ca_2SiO_4 [pot] + 100 mM NaCl), foliar + salt (1 mM Ca_2SiO_4 [foliar] + 100 mM NaCl), and foliar + pot + salt (1 mM Ca_2SiO_4 [foliar + pot] + 100 mM NaCl).

2.1.2. Spray treatments

Calcium silicate (Ca_2SiO_4) in the concentration of 1 mM was prepared in distilled water comprising of 0.02% polyoxyethylenesorbitan monolaurate (Tween 20 Sigma Chemicals, UK). Foliar treatments were initiated in respective pots when the seedlings were started having NaCl treatment. Lower as well as upper leaf surfaces were sprayed until wet according to Hull et al. (1975) protocol.

2.2. Apoplastic washing fluid extractions (AWF)

Infiltration–centrifugation technique was employed for the collection of AWF as described by Mühling and Sattelmacher, (1995) and Lohaus et al. (2001). Initially, leaves were detached with a razor blade and were later carefully washed using deionized water. Leaves were thoroughly dried with tissue paper and were weighed prior to infiltration. Intact leaves were kept in a plastic syringe (60 mL) that was later filled with deionized water (40 mL) for infiltration. Infiltration of the leaves was accomplished by pulling the plunger, which produced a reduced pressure of about 20 kPa (Lohaus et al., 2001). Afterwards, the upper and lower surface of each leaf was thoroughly blotted dried with tissue paper and leaves were weighed again to get an estimate of an infiltrated volume of water. Intact leaves were positioned with the xylem wound facing upwards between two plastic funnels fitted into a 10 mL vessel located in a centrifugation tube and centrifuged with a relative speed of 94*g* at 5 °C for 5 min. Extracted apoplastic washing fluid was pipetted into 1.5 mL eppendorf tube and was stored at −80 °C until further analyses. The calculation of the ion concentration in the apoplastic water space of *Abelmoschus esculentus* L. leaves were performed after a multiplication by the dilution factor of 9, as given by Lohaus et al. (2001).

2.3. Growth parameters

Fresh weight of the plants was obtained soon after the harvest with the help of an analytical balance having precision of 0.1 mg. Roots and shoots were weighed separately. Later, dry weight of the plant root and shoot was obtained by placing them in an oven at 60 °C for three days. Digital images of the plants and leaves were taken after harvesting. Later on, area and length of each leaf and shoot length of the harvested plants were calculated with the help of ImageJ 1.49 software by setting a scale against a specific number of pixels. Leaf area and leaf length were demarcated, thereby obtaining leaf area and leaf length measurements.