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Iron plays a critical role in stomatal closure in cauliflower

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ABSTRACT

Studies have been made of the effects of iron deficiency on water status and transpiration parameters in iron-deficient plants. Cauliflower (*Brassica oleracea* L. var *Botrytis* cv *Pusa*) plants were grown in sand culture with 0.1 mM (control) and 0.001 mM (deficient) iron supply. Young leaves of Fe-deficient plants showing mild interveinal chlorosis were thin and soft with lesser epicuticular wax than the green leaves of Fe-sufficient plants. Fe deficiency induced increases in specific water content, water potential, stomatal aperture and transpiration and a decrease in diffusive resistance in the young leaves. Although Fe deficiency enhanced stomatal and cuticular transpiration, the greater stomatal opening was mainly responsible for increased transpiration. Fe deficiency stifled diurnal changes in diffusive resistance, ABA accumulation and guard cell K^+ content in the leaves. Though Fe deficiency did not affect maximum stomatal opening under favourable conditions, it retarded stomatal closure under conditions of developing water deficit or decreasing light intensity. Higher rates of transpirational water loss in Fe-deficient leaves are appearing to be related to the slow and incomplete stomatal closure, which are attributable to the putative involvement of Fe in the biosyntheses of wax and ABA in Fe-deficient leaves.

1. Introduction

There are diverse reports in the literature about the effects of Fe deficiency on stomatal opening and transpiration in plants. Shimshi (1967) reported decreased stomatal opening in groundnut plants showing chlorosis which he presumed to be due to flooding-induced Fe deficiency in the fields. Hutchinson (1970a), on the other hand, observed increase in transpirational water loss (measured gravimetrically, E_g) in the leaves of 30 different species showing lime-induced chlorosis under field as well as pot culture conditions. He found that chlorotic leaves continued to lose water faster than the green leaves even after they had developed water deficit greater than the normal green leaves. He also observed alleviation of chlorosis and reduction in E_g on the supply of Fe to the chlorotic plants. He attributed increased E_g in the chlorotic leaves to the increases in the stomatal as well as cuticular E as a result of decreased ability of chlorotic leaves to retain its water, implying thereby a decreased partitioning of leaf tissue water into bound form (Hutchinson, 1970a). Similar effects of lime-induced 'iron-chlorosis' on stomatal opening and E_g have also been described in *Eucalyptus* (Anderson, 1984). He observed that a combination of low moisture availability, high temperatures and severe chlorosis increased water stress to the extent that the seedlings of susceptible genotypes failed to establish under these conditions (Anderson, 1984). Sharma and Sharma (1987) attributed increased stomatal opening and transpiration (E) in Fe-deficient cauliflower plants grown under sand culture conditions, to increased water potential (Ψ) and relative water content (RWC) of leaves. Contrary to these observations, Fe deficiency is reported to decrease stomatal conductance in sugar beet plants grown hydroponically (Terry, 1983), and in silver maple trees in experiments on recovery from Fe chlorosis (Hurley et al., 1986). Kleinkopf et al. (1976) and Davis et al. (1986) on the other hand, failed to observe any significant effects of lime-induced 'Fe chlorosis' on stomatal conductance and E in soybean. According to Kleinkopf

Abbreviations: DR, diffusive resistance; E, transpiration; Eg, transpirational water loss measured gravimetrically; EC-wax, epicuticular wax; PPFD, photosynthetic photon flux density; RWC, relative water content; Ψ, water potential.

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et al. (1976) Fe deficiency *per se* may be expected to lower the energy budget of the leaves by decreasing the photochemical activity and ATP synthesis, and thus decreased stomatal aperture, if at all.

Iron deficient plants generally generate and accumulate large quantities of superoxide anion radicals and H_2O_2 due to depletion of various antioxidant proteins such as superoxide dismutase, catalase, ascorbate peroxidase and component of the electron transport system of mitochondria and chloroplasts (Tewari et al., 2015; Tewari et al., 2013; Tewari et al., 2005). The ABA-mediated accumulation of H_2O_2 in the guard cells has already been implicated in stomatal closure in the *Arabidopsis thaliana* (Bright et al., 2006; Xiao Zhang et al., 2001). Moreover, H_2O_2 disproportionating catalase, CAT3, interact with a calcium dependent protein kinase, CPK8, and induce stomatal closure in the presence of ABA or H_2O_2 or Ca^{2+}(Zou et al., 2015). The *cat3* and *cpk8* mutants (plants deficient in CAT3 and CPK8 protein), did not close stomata under drought due to abolishment of ABA, H_2O_2 and Ca^{2+} mediated inhibition of inward K^+ current in the guard cells (Zou et al., 2015). Moreover, ABA promotes stomatal closure indirectly through a decrease in water permeability within leaf vascular tissues (leaf hydraulic conductance) (Pantin et al., 2013). Despite of great significance stomatal closure in plant-water-relation, the mechanism of its retarded closure under Fe-deficient condition is still not fully deciferred. We present a solution to the problem and proposed a new role for Fe in plants. The present studies on stomatal responses to photosynthetic photon flux density (PPFD), plant water status, and diurnal changes in Fe-deficient cauliflower plants reveal that Fe deficiency enhances E by restricting stomatal closure under conditions conducive of stomatal closure in normal and healthy plants.

2. Material and methods

2.1. Plant material

Cauliflower (*Brassica oleracea* L. var *Botrytis* cv *Pusa*) plants were grown in sand culture in a glass house with 0.1 mM (control) and 0.001 mM (deficient) iron supply. White silica sand was purified by churning in a KEEBUSH sand digester (A.P.V.-Kestner Ltd., Kent, UK) with steam-heated mixture of HCl (17% v/v) and oxalic acid (1% w/v) as recommended by Hewitt (1966). Seeds of cauliflower were sown in mid-October in purified sand in polyethylene trays. The seedlings were transferred three weeks after sowing, after emergence of first true leaf, into 5 l 'Corning' glass containers filled with purified silica sand. There were ten pots, each with two plants, for each of the two treatments. The composition of the nutrient solution provided to the plants daily around 8:00 AM was (Hewitt, 1966): KNO_3, 4 mM; Ca $(NO_3)_2$, 4 mM; $MgSO_4$, 2 mM; NaH_2PO_4, 1.33 mM; NaCl, 0.1 mM; $MnSO_4$, 10 μM; $CuSO_4$, 1 μM; $ZnSO_4$, 2 μM; H_3BO_3, 33 μM; Na_2MoO_4, 0.2 μM; $CoSO_4$, 0.1 μM; $NiSO_4$, 0.1 μM. Iron was supplied as Fe-EDTA according to the treatment. The experiments were performed at least six-times with similar observations. Studies were made in the fully expanded young leaves of the plants after 50 days, except for those stated otherwise, of initiating differential Fe supply. The average glasshouse conditions during the experiment were: PPFD (12:00 noon), 1025–1310 μmol m^{-2} s^{-1}; daily maximum and minimum temperatures, 22.8–30.7 °C and 9.3–16.3 °C, respectively; RH (9: AM), 39–54%. Average day length during the period was 10:50 ± 0.22 h.

2.2. Iron, chlorophyll, carotenoids, epicuticular wax, cuticular studies and guard cell K^+

Iron (Fe) was estimated in HNO_3: $HClO_4$ (10:1, v/v) digest of young leaves atomic absorption spectrophotometrically. Leaf chlorophyll and carotenoids content was measured spectrophotometrically in the acetone extracts (Lichtenthaler, 1987). Epicuticular (EC)-wax of the sixth-youngest expanded leaves was determined gravimetrically after extraction in petroleum ether (Sharma et al., 1995). Stomatal frequency and aperture were measured using micro-relief impressions of the abaxial surface of the fifth-youngest expanded leaves made in QUICKFIX adhesive (Wembley laboratories, Bombay, India). K^+ content of guard cells in the epidermal peels was assessed histochemically by Macallum's Na-cobaltinitrite test (Sharma et al., 1995). Epidermal peels from the abaxial surface of the fifth-youngest expanded leaf were cleared of EC-wax by elution in petroleum ether for 5 s, treated with Na-cobaltinitrite solution chilled in an ice bath, washed in glass distilled water and then treated with ammonium sulphide solution. Microscope images were analysed by selecting a region of interest of the distribution of K^+ in the guard cells and subsidiary cells using Image J software (http://imagej.nih.gov/ij/) and data was normalized against the background. The ratio [K^+_{GC}/K^+_{SC}] was presented along with the representative images as a figure.

2.3. Leaf water status

Measurements of Ψ were made hygrometrically on five discs (11 mm diameter) cut from five fifth youngest expanded leaves. The measurements were taken on a Wescor (Logan, UT) microvoltmeter (model HR33T) using C-52 leaf chambers. Determination of RWC was made by measuring fresh, re-hydrated (for 3 h at 11 °C in dark on glass-distilled water), and oven-dry weight of 45 leaf discs (3 replicates of 15 discs each) from the leaves used for Ψ measurements. Leaf area was measured on a Li-cor's portable area meter model Li-300A.

2.4. Diffusive resistance and transpiration

Measurements of diffusive resistance (DR) and E were made, unless otherwise stated, under glasshouse conditions between 9:00 and 9:30AM on the abaxial surface of the fifth-youngest expanded leaves using Li-cor steady-state porometer (model Li-1600).

Measurement of E_g from excised leaves was carried out in an improvised growth chamber fitted with an exhaust system to provide a mild draft of air, and a bank of Philips TL 40 W cool fluorescent tubes and 100 W halogen lamps providing PPFD of *Ca* 170 μmol m^{-2} s^{-1} on the leaf surface. After 30 min of acclimation of the plants to the growth chamber conditions, fifth-youngest expanded leaves were excised and weighed on and electrical 'Sartorius' balance immediately after excision, and every 2 min thereafter, for 120 min. In between the weighings, the leaves were rested on a thin cotton gauge stretched 30 cm above the bench level. Water-loss (E_g) curves (Slavík, 1974) were plotted as the relationship of the log of water content at zero time as 1000, with time. Stomatal and cuticular E_g were separately calculated from the water loss (Stomatal E_g = total E_g during stomatal phase − cuticular E_g).

2.5. Abscisic acid

Abscisic acid in the tissue was extracted following procedure described by Zhou et al. (2003). Fresh tissue (1 g) was homogenised in liquid nitrogen and extracted in 3 ml solvent (acetone:water: acetic acid 80:19:1). The homogenate was centrifuged in cold (<4 °C) at 15,000 × *g* for 10 min. The residue was re-extracted in 3 ml solvent and centrifuged as above and the supernatant was pooled. The extract was dried under stream of nitrogen and redissolved in minimum amount of solvent and cleared by centrifugation in cold. ABA in the extract was determined by