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COMPARISON OF CALCIUM DISTRIBUTIONIN LEAF CELLS OF *CARPINUS PUBESCENS* AND *CAMELLIA OLEIFERA* UNDER DROUGHT AND CALCIUM STRESS

XIMIN ZHANG[1,2], LUNXIAN LIU[1,2], JIYI GONG[1,2], JING TANG[1,2], MING TANG[1,2] AND YIN YI[1,2*]

[1]*Key Laboratory of Plant Physiology and Development, Guizhou Normal University, Guiyang 550001, P. R. China*
[2]*School of Life Science, Guizhou Normal University, Guiyang 550001, P. R. China*
[*]*Corresponding author, e-mail:gzklppdr@gznu.edu.cn*

Abstract

Potassium antimonite was used to locate calcium in the leaf cells of *Carpinus pubescens*and *Camellia oleifera* seedlings. The aim of this research was to reveal the difference of calcium distribution in cells of the leaves of both seedlings under culturing conditions of different external drought and calcium regimes. Abundant calcium precipitates accumulated mainly in the cell wall of *C. pubescens* and many precipitates were located in the inner plasma membrane of *C. oleifera* under excessive calcium as well as severe drought conditions. These results showed that *C. pubescens* and *C. oleifera* had diverse distributions of Ca^{2+} in the cells. *C. pubescens* could remove an excess of calcium from cytoplasm to cell wall and restore it. Therefore, the conceivable acclimation of *C. pubescens* to limestone soil might be related to its ability to transport calcium in cytoplasm caused by drought or calcium stimuli into the cell wall, thus maintaining normal physiological metabolism.

Key words: *Carpinus pubescens*, *Camellia oleifera*, Karst, Potassium antimonite.

Introduction

Carbonate bedrock (generally limestone or dolomite) is dissolved by water, thus forming the distinctive topography, known as Karst. The outcropping area of carbonate rocks is approximately 15 million km^2,some 11% of the land area on the globe (Ford &Williams, 2007; Xiao &Weng, 2007). In southwest China, karst areas are the largest in the world covering about 540,000 km^2 located mainly in seven provinces and one municipality (Li *et al.*, 2012). In the ecosystems of karst habitats, the soil is characteristically calcium-rich by virtue of bacterial and human activity or due to the processes of carbonate rocks dissolution (Wu *et al.*, 2011). Soil formation from the underlying limestone is found at an extremely slow rate. Moreover, this soil is shallow, patchy, and of high porosity with a low water storage capacity (Liu *et al.*, 2010; Culver & Pipan, 2011; Liu *et al.*, 2011; Liu *et al.*, 2012). Plants grown in these regions frequently suffer from water deficit and excessive calcium stress, especially in the dry season (Fan *et al.*, 2011; Fu *et al.*, 2012).

Plants can adapt to different environments. Different plant species have the optimum environmental conditions for their growth and development. Species cannot grow under harsh environmental conditions (Krasensky & Jonak, 2012; Li *et al.*, 2017). According to growth habitat, ecologists have classified plant species into calcifuges, which occur on acidic soils with low Ca^{2+} level, and calcicole, which occur on limestone soils. In their natural habitats, they exhibit varying strategies for acclimation to habitat (White & Broadley, 2003;Clarkson, 1965; Lee, 1999) including the regulationof Ca^{2+} in the stem tissue (Akram *et al.*, 2006; Kinzel & Lechner, 1992; Lee, 1999), the sequestration of calcium to form Ca-oxalatein trichomes (De Silva *et al.*, 1996), binding to calcium-specificproteins in the cytoplasm, preventing excessive calcium from reaching the stomata guard cells in leaves, and removing or sequestering calcium for delivery to the xylem (De Silva *et al.*, 1994).

Ca^{2+} is the most active divalent ion in the eukaryotic organisms. Ca^{2+} plays a role in plant growth and development. It participates in many processes of physiological and metabolic regulation(Hetherington &Brownlee, 2004; Reddy & Reddy, 2004; Bothwell & Ng, 2005; Hirschi, 2004). Studies of calcium distribution have indicated that calcium precipitates are located in different regions in cells, especially during the development of flowers, fertile and sterile anthers (Tian & Russell, 1997), and during fertilisation (Ge *et al.*, 2009). When a plant is subjected to various external stresses including cold, salinity, and drought, cytoplasmic calcium levels are regulated accurately by movements of Ca^{2+} into apoplasts or other intracellular organelles to maintain a normal physiological and metabolic calcium concentration (Mahajan & Tuteja, 2005). The change in calcium distribution is considered to be relative to the response of the plant to external environmental stress (Wang & Han, 2010; Huang *et al.*, 2013), such as arsenic (Li *et al.*, 2006), zinc (Wang *et al.*, 2009), heat shock (Huang *et al.*, 2013), or abscisic acid (Wang & Han, 2010; Huang *et al.*, 2013). It is therefore essential for the distribution of Ca^{2+} in the cells of a plant after a short-term external stress. Unfortunately, the worse external stress, or long-term unfavourable environment, the worse the ultrastructural damage, the plant might even die. For example, changes in chloroplasts, mitochondria, and the membrane systems have been revealed under drought stress (Xu *et al.*, 2009; Grigorova *et al.*, 2012). However, changes in the distribution of Ca^{2+}in the cells in a calcicole under drought and excessive calcium conditions have been poorly understood compared with a calcifuge, although a calcicole has some mechanisms to regulate acclimation to limestone soil (Lee, 1999; Kinzel & Lechner, 1992; De Silva, 1996).

The study was carried out to examine calcium distribution in the leaves of *C. pubescens* and *C. oleifera* under drought and excessive calcium supply conditions. It was expected that: 1) changes of calcium distribution in the leaf cells of *C. pubescens* and *C. oleifera* following drought and excessive calcium conditions were different, 2) a prolonged exposure to calcium and drought led to changes in the calcium distribution. To test these hypotheses, seedlings of *C. pubescens* and *C. oleifera* were subjected to different drought and calcium regimes, and the loosely bound calcium could be visualised by potassium antimonate precipitation.

Materials and Methods

Plant materials and growth conditions: Two species used for this study are shown in Table 1. Sterilised seeds of both were germinated in plasticpots filled with sands containing 0.5 × MS salt (Sigma) in an illumination incubator (SPX-150-GB) (25°C/20°C, 80 ± 5% RH, 1000 µmol $m^{-2}s^{-1}$PAR, and 14 h daylight)until experimental treatment. Then the seedlings (about 5 cm long) with plastic pots were moved into another plastic under-pan with 0.5 × MS salt hydroponics medium without calcium and placed into a plant growth incubator (HRR-1000K)(25°C/20°C, 80 ± 5% RH, 1000 µmol $m^{-2}s^{-1}$PAR, and 14 h daylight). After 14 days, the pH was adjusted to 5.5 for *C. oleifera* and 7.4 for *C. pubescens*.Calcium was then added to each pot.

Drought and Ca^{2+} treatment: After aspirating the solution in the under-pan, drought treatment was conducted by using 200 mL of polyethylene glycol 6000 solution containing 0.5 × MS salt solution at two different osmotic potentials, mild drought (MD) (–0.05 MPa), and severe drought (SD) (–1.0 MPa). The seedlings were dosed with 200 mL of H_2O in 0.5 × MS salt solution as a control. Calcium treatment was carried out using 200 mL of 0.5 × MS salt solution without calcium salt and the Ca^{2+} concentration was adjusted to 0.5 (control), 5, and 30 mmol/L by the addition of $CaCl_2$. Then these solutions were adjusted to pH 5.5 for *C. oleifera* and pH 7.4 for *C. pubescens* with 1 mol/L HCl or 1 mol/L NaOH. Cultural solutions were replaced after 7 days. Leaves were collected after being treated for 4, and 48 h, respectively. All experiments were conducted in four replicates (ten plants *per* treatment) (Li & Gabelman, 1990; Chan *et al.*, 2003).

Sample preparation for cytochemical localisation of Ca^{2+}: The treated leaves of *C. pubescens* and *C. oleifera* were collected after 4 and 48 h, respectively. The samples were fixed at 4°Cfor 4 h in 3% glutaraldehyde (v/v) in 0.1 mol/L phosphate buffer (pH 7.6) containing 2% potassium antimonate ($K_2H_2Sb_2O_7.4H_2O$). After pre-fixation, the samples were washed for 30 min in buffer with 2% antimonate and then post-fixed in 1% (w/v) osmium tetroxide (OsO_4) in 0.1 mol/L phosphate buffer (pH 7.6) at 4°C for 3 h. The samples were washed in a buffer, and then dehydrated in a graded acetone series in distilled water at pH 10 and embedded in Spurr resin 812. For each experiment, five embedded samples were sectioned and stained with 2% uranyl acetate (w/v) for 15 min. After being washed and aired, sections were observed and photographed under a Hitachi H-600 transmission electron microscope. To verify the Ca^{2+} distribution in the cells, an additional control trial was conducted by using EGTA to remove the calcium pyroantimonate precipitates. The previous grids were immersed in a 100 mmol/L EGTA solution (pH 8.0), and incubated at 60°C for 1 h. After treatment, the grids were rinsed and stained according to the aforementioned methods, then photographed under a transmission electron microscope (imaging any calcium loss was impossible, because the same visual field was difficult to find under transmission electron microscope conditions) (Tian & Russell, 1997; Wick & Hepler, 1982; Ma, 2011; Jian *et al.*, 1997).

Results

Subcellular localisation of Ca^{2+}under different calcium concentrations: The distribution of calcium precipitatesin the cells of *C. pubescens* exhibited different changes (Fig. 1). The precipitate of Ca^{2+} was not observed with treatment in 0.5 mmol/L Ca^{2+} for 4 h (Fig. 1a). However, some calcium precipitates were found in the cell wall when the treatment time was increased to 48 h (Fig. 1b). For seedlings treated with 5 mmol/L Ca^{2+}, a few calcium precipitates were located in the inner plasma membrane after 4 h treatment (Fig. 1c) and calcium precipitates were observed in the cell wall at 48 h treatment (Fig. 1d). For 30 mmol/L Ca^{2+} supply, calcium precipitates occurred mainly in the cytoplasm after 4 h (Fig. 1e), and some calcium precipitates appeared in the inner plasma membrane and reduced amount of calcium precipitate was observed in cytoplasm when the treatment duration was increased to 48 h (Fig. 1f).

Calcium distribution in the cells of *C. oleifera* are illustrated in Fig. 3. The calcium precipitates were not observed in the cells and cell walls when treated with 0.5 mmol/L Ca^{2+} for 4 and 48 h (Figs. 2a and 2b). At a calcium concentration of 5 mmol/L, there was no precipitation in the cells after 4 h treatment (Fig. 2c), but much evidence in the plasma membrane after 48 h treatment (Fig. 2d). No precipitates were observed in the cells when treated with 30 mmol/L Ca^{2+} for 4 h (Fig. 2e). However, after treatment for 48 h, some precipitates were found in the plasma membrane (Fig. 2f).

Subcellular localisation of Ca^{2+}under different drought regimes: *C. pubescens* were cultured in medium containing different concentrations of PEG6000 solution from 4 h to 48 h. In the control treatment (without PEG6000 solution), the Ca^{2+} precipitated in the cell could not be observed after 4 and 48 h (Figs. 3a and 3b). Similarly, no precipitated appeared in the cells when seedlings were cultured with –0.05 MPadroughts for 4 or 48 h (Figs. 3c and 3d). At –1.0 MPa, calcium precipitates appeared in the cell wall after 48 h treatment (Fig. 3f), but none were found in the cells after 4 h treatment (Fig. 3e).

Similar to *C. pubescens*, when *C. oleifera* was cultured with H_2O (without PEG6000 solution) there was no Ca^{2+} precipitation in the cells for 4 h or 48 h (Figs. 4a and 4b), but it appeared in the plasma membrane when –0.05 MPa drought was applied for 4 h (Fig. 4c) and 48 h (Fig. 4d). Calcium precipitates were observed in the plasma membrane after 4 h treatment (Fig. 4e) and appeared outside the cell wall after 48 h treatment (Fig. 4f) under –1.0 MPa drought supply, at the same time, some organelles (such as chloroplasts) were damaged under both –0.05 MPa and –1.0 MPa conditions.

Table 1. Plant species used in this study.

Species	Family	Habit and Habitat
Carpinus pubescens	Betulaceae	Deciduous tree up to 17 m, occurring in mountainous regions on limestone
*Camellia oleifera*Abel	Theaceae	Evergreen shrub up to 5 m, occurring on acidic soil.

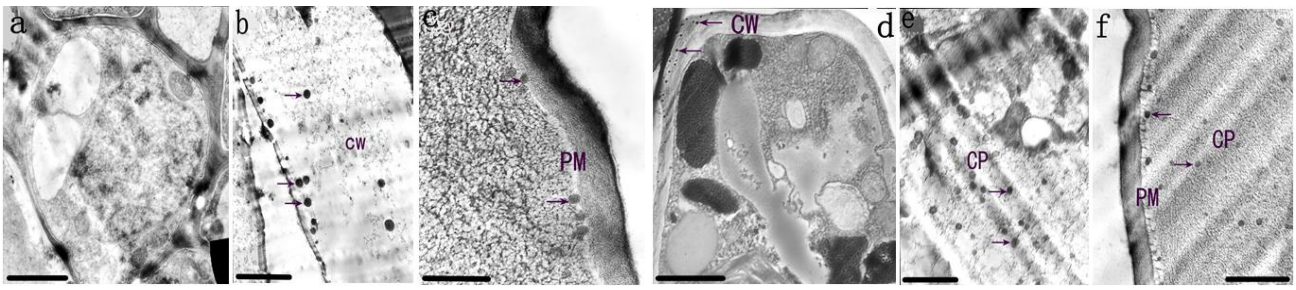

Fig. 1. The distribution of calcium precipitates in *Carpinus pubescens* under varying calcium supply conditions. a, b: treated with 0.5 mmol/L Ca2+ for 4 and 48 h, respectively. c, d: treated with 5 mmol/L Ca2+ for 4 and 48 h, respectively. e, f: treated with 30 mmol/L Ca2+ for 4 and 48 h, respectively. CW: cell wall; PM: plasma membrane; CP: cell plasma; a: No calcium precipitates are observed in the cells. b and d: Many calcium precipitates are distributed in the cell wall. c: Some precipitates appear in the plasma membrane. e: Many precipitates are distributed in the cytoplasm. f: Some precipitates appear in the cell plasma and many precipitates are distributed in the plasma membrane. Scale bars represent 1.7 μm (a, d), 2 μm (b), 4 μm (c), 1.5 μm (e), and 2.5 μm (f).

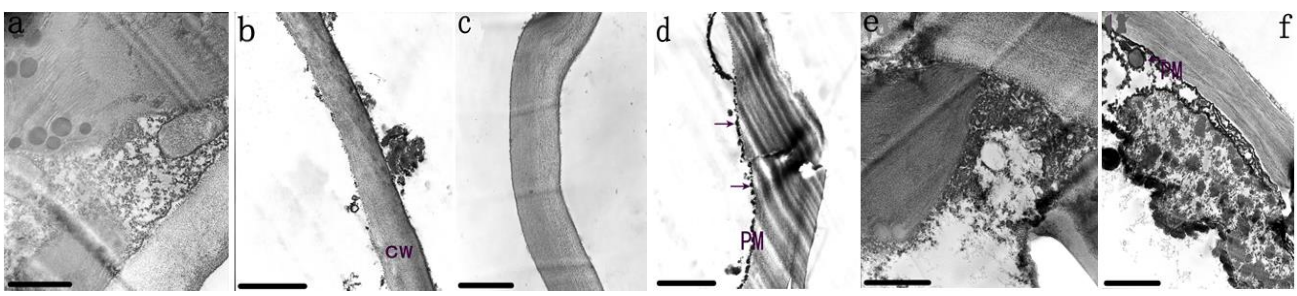

Fig. 2. The distribution of calcium precipitates in *Camellia oleifera* under diverse calcium supply conditions. a, b: treated with 0.5 mmol/L Ca^{2+} for 4 and 48 h, respectively. c, d: treated with 5 mmol/L Ca^{2+} for 4 and 48 h, respectively. e, f: treated with 30 mmol/L Ca^{2+} for 4 and 48 h, respectively. CW: cell wall, PM: plasma membrane. a, b, c, e: No calcium precipitates are observed in the cells. d and f: Many precipitates are distributed in the plasma membrane. Scale bars represent 3 μm (a), 1.5 μm (b and d), 2 μm (c), and 2.5 μm (e and f).

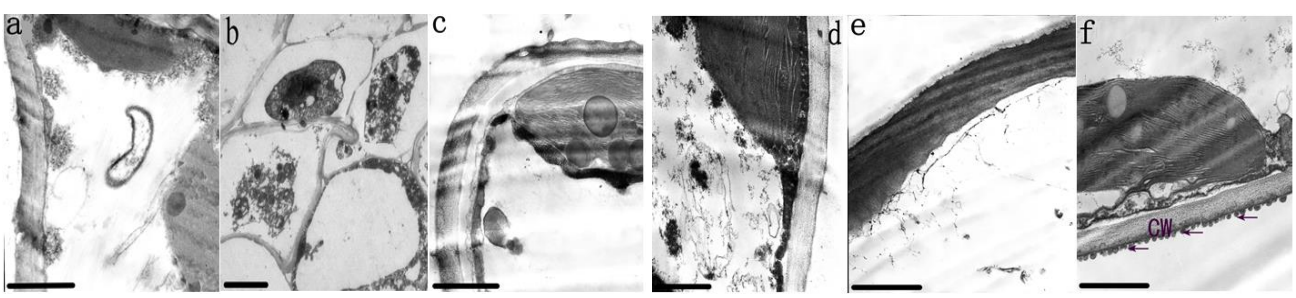

Fig. 3. The distribution of calcium precipitates in the cells of *Carpinus pubescens* under drought conditions. a, b: treated with H_2O for 4 and 48 h, respectively. c, d: treated with –0.05 MPa drought for 4 and 48 h, respectively. e, f: treated with –1.0 MPa drought for 4 and 48 h, respectively. CW: cell wall. a, b, c, d, e: No calcium precipitates are observed in the cells. f: Many calcium precipitates are distributed in the cell wall. Scale bars denote 2.5 μm (a and f), 2 μm (b, c and e), and 3 μm (d).

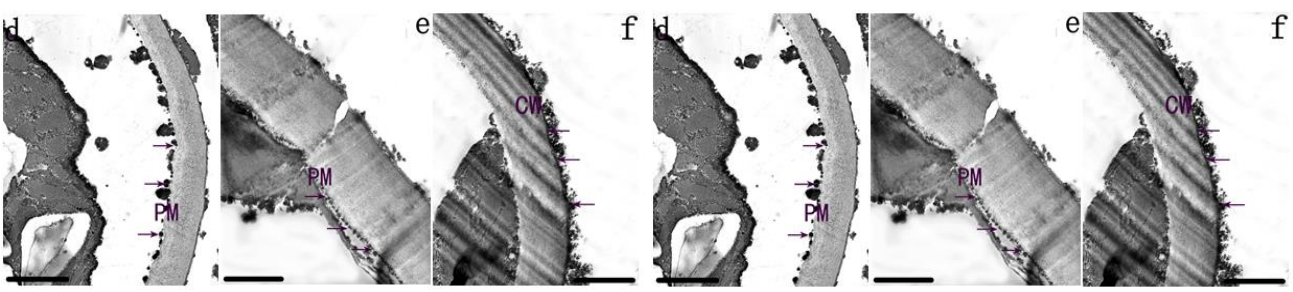

Fig. 4. The distribution of calcium precipitates in the cells of *Camellia oleifera* under drought conditions. a, b: treated with H_2O for 4 and 48 h, respectively. c, d: treated with –0.05 MPa drought for 4 and 48 h, respectively. e, f: treated with –1.0 MPa drought for 4 and 48 h, respectively. CW: cell wall, PM: plasma membrane. a, b: No calcium precipitates are observed in the cells. c, d, e: Many calcium precipitates are distributed in the plasma membrane. f: Some calcium precipitates were located outside cell wall. Scale bars represent 1.5 μm (a and f), 1 μm (b, c and e), and 1.2 μm (d).

Discussion

Cytochemical techniques for potassium pyroantimonite precipitates could explore calcium localisation in cells. This method was firstly used in plants by Slocum and Roux in 1982 and its application was extended later (Dauwalder *et al.*, 1985; Moore, 1986). We have employed this technique to examine the Ca^{2+} distribution in the leaf cells of *C. pubescens* and *C. oleifera* during plant culture. The precipitates were mainly found in the cell wall and plasma membrane.

Calcium distribution in the cells of *C. pubescens* was examined under diverse drought and calcium supply conditions, because *C. pubescens* grows well on limestone soil with a high concentration of calcium and frequent drought conditions, compared with *C. olerfera*, which grows on acid soil. Precipitates were found in the cell wall, plasma membrane, and cytoplasm of *C. pubescens* and seem to be transported from cytoplasm to plasma membrane, and finally reserved in cell wall (Fig. 2). The previous study indicated that calcium played the role in the rigidity of the cell wall (Wyn & Lunt, 1967; Burstrom, 1968). The cell wall was more pliable and easily ruptured when the Ca^{2+}in the cell wall was present at a low concentration; however, high concentrations of Ca^{2+} in the cell improved the rigidity of the cell wall(Hepler& Winship, 2010). For calcicole, some of the Ca^{2+} entered the cell wall might participate in controlling the structural integrity of the cell walls and increase their rigidity. Other Ca^{2+} in the cell wall might be provided as a secondary message in signal transduction.

Plants should grow in their natural environment with adequate calcium concentration as a nutrient (Marschner, 1995). When excessive calcium is present, in solution, in the rhizosphere, plants may suffer from its toxicity. This may prevent the germination of seeds, decrease plant growth rate, and disturb the process of metabolism (White & Broadley, 2003). Although calcium precipitates in the inner plasma membrane of *C. oleifera* were observed, the subcellular structures were destroyed, when the seedling of *C. oleifera* were cultured in an environment with –1.0 MPa drought and 30 mmol/L Ca^{2+} for 4 to48 h (Fig. 2). In contrast, subcellular structures of *C. pubescens* showed normal development under –1.0 MPa drought and 30 mmol/L Ca^{2+} supply conditions (Fig. 1). So, *C. oleifera* may suffer from the toxicity of calcium, affecting their normal growth and development.

Ca^{2+}, as a secondary message, also plays an important role in signal transduction in plants. Many biotic or abiotic stimuli such as low temperature, drought, high salinity, light, and ABA have been shown to induce a transient increase of cytoplasm free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$), which indicates that this process results from plant acclimation to environments and production of a response to various external stresses (Knight *et al.*, 1992; Knight, 2000; Wayne & Hepler, 1985; Baum *et al.*, 1999; Jian *et al.*, 1997). Because the increased calcium in the cytoplasm needs to return to its normal physiological concentration, the removal of Ca^{2+} from the cytoplasm against its electrochemical gradient to either the apoplast or intracellular organelles such as endoplasmic reticulum, vacuole, and cell wall was catalysed mainly by Ca^{2+}-ATPase and H^+/Ca^{2+}-antiporter in plasma membrane (White & Broadley, 2003). In the current study, in *C. pubescens*, calcium precipitates during external excessive calcium and drought states and as a result of sufficient culturing time (indirectly): precipitation was mainly observed in cell walls and restored therein (Figs. 1 and 3). Contrarily, in *C. oleifera*, calcium precipitates at external excessive calcium and drought states were found mainly in the inner plasma membrane (Figs. 2 and 4). These results showed that Ca^{2+}-ATPase activity located on the plasma membrane of *C. oleifera* might be interrupted, or inactivated, so that it could not transfer calcium from cytoplasm into the cell wall. Therefore, calcium precipitates were formed in the plasma membrane in *C. oleifera*.

Conclusion

In conclusion, *C. pubescens* and *C. oleifera*indicated different distributions of Ca^{2+} in cells. *C. pubescens* could remove calcium from cytoplasm to cell wall. Therefore, the acclimation of *C. pubescens* to limestone soil might be because *C. pubescens* was able to transfer excessive calcium from cytoplasm (when subjected to drought or calcium stimuli) into cell wall for storage. This enabled cells to maintain normal physiological metabolism. The Ca^{2+}-ATPase activity of *C. oleifera* might be interrupted, or inactivated, under excessive calcium and drought conditions.

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