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CHANGES IN *CONYZA CANADENSIS* (L.) CRONQUIST LEAF ANATOMY UNDER CAPRYLIC ACID STRESS

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Abstract

The deleterious influence of caprylic aicd stress on several weeds have been reported, mainly in the context of the biochemical, physiological and growth parameters of weeds. However, few studies have examined the anatomical and ultrastructural changes in response to caprylic acid. Anatomical injures were observed in *Conyza canadensis* (L.) Cronquist leaves at 0, 2, 4, 8, 12, and 24 h after 625 μM caprylic acid application in the present study. The initial damage was observed in the mesophyll percentage area and then marginal leaf regions, mid-leaf areas, and the midvein. The accumulation of caprylic acid in the cells, resulted in palisade parenchyma collapse and reduce, cell wall deform, and veins punctual necrosis, was evident in the leaf sprayed with caprylic acid. Chloroplasts and mitochondria in mesophyll cells were disturbed, and followed by markedly reduced photosynthetic activity during caprylic acid application. The leaf anatomy of leaves of *C. canadensis* treated with caprylic acid displayed time-dependent depletion and disintegration. The degree of changes in the anatomical and ultrastructural leaves of the *C. canadensis* were studied, suggesting the mechanisms by which caprylic acid act as an effective herbicidal substance.

Key words: Caprylic acid, *Conyza canadensis* (L.) Cronquist, Anatomy, Transmission elections microscopy, Light microscopy.

Introduction

The last half-century has been an exciting time for plant biology research on the whole and for plant anatomy research in particular (Berlyn & Miksche, 1979; Albrechtova & Kubinova, 1991; Kubinova, 1993). Advances in natural sciences and in technologies together with their applications created an increasing demand for experimental plant biology as a means of more accurate and more detailed study of plant structure and function (Williams *et al.*, 2016; Su *et al.*, 2017). This invigorated research started to prioritize complex, integral studies, thereby enabling the study of plants on different hierarchical levels involving the spectral, physiological, biochemical, structural and molecular approaches and providing a complex insight into the problems under study (Kwanghun *et al.*, 2013; Chang & Katherine, 2017; Christian *et al.*, 2017). In the current period of rapid development of molecular methods and comparative genome analysis of living organisms, the apparent limit of understanding of their functions is a matter of obtaining detailed knowledge of cellular and subcellular structures, dynamic complexity of tissues, and organ structure (Cole *et al.*, 2000; Zamore, 2017). Ecological, environmental, and stress physiology are concerned fundamentally with the physiology of plants as modified by fluctuating external factors (Terzi *et al.*, 2010; Makbul *et al.*, 2011; Avenot *et al.*, 2017; Yendrek *et al.*, 2017). According to Vannier (1994) ecophysiology involves both the descriptive study of the responses of organisms to ambient conditions and the casual analysis of the corresponding ecologically dependent physiological mechanisms, at every level of organization. The ecophysiological approach must take into account structural and functional diversity (Larcher, 1995).

Weeds absorded many herbicides by roots and through the xylem throughout the plant when applied before emergence; and weeds leaves also absorbed herbicides substance when applied after emergence (Simpson *et al.*, 2005; Demily *et al.*, 2017). Weeds suffer several harmfull effects from herbicides, such as inter vein alchlorosis, necrosis, and leaf wilting (Bell & Duke, 2005), damage to epicuticular waxes (Sadler *et al.*, 2016) and the photosynthetic organ (Goltsev *et al.*, 2001), and morpho-anatomical changes in leaves (Moskova *et al.*, 2011). According to Ferreira et al. (2002) and Procópio *et al.*, (2003), the anatomical study of leaves can improve the understanding of the barriers that each weed imposes on herbicide penetration and contribute to find the strategies that overcome these obstacles. Thus, leaf anatomical changes can be successfully applied to the identification of weeds that are susceptible, tolerant or resistant to a given chemical, as well as in the description of phytotoxic symptoms, contributing to the current understanding of herbicide selectivity in annual and perennial weeds.

Caprylic acid belongs to the eight-carbon saturated fatty acids, and its systematic name is octanoic acid. It is found in coconut oil, lemongrass, and hops. Caprylic acid is an oily liquid that is marginally soluble in water with a slightly unpleasant rancid-like smell and taste. Caprylic acid has been used as an antimicrobial pesticide, a disinfectant, and an algaecide. In our preliminary study, we found caprylic acid can be saftey used for cane crops by using protective shielding during spraying as nonselective herbicide and can eradicate many weeds including *Conyza canadensis* (L.) Cronquist. *C. canadensis* commonly could be found in dryland as a weed, such as grain fields, orchards,

vineyards, pastures, uncultivated land, roadsides, railroads, stream banks, and even urban areas (Koger *et al.*, 2004). This weedy species is difficultly controlled by glyphosated and developed resistance to herbicides (Heap, 2012). Accordingly, this present study we investigated the anatomical changes in the leaf tissue of *C. canadensis* by caprylic acid, aimed to characterize foliar effect caused by this herbicidal substance and view whether the observed structural damage occured prior to visible damage.

Materials and Methods

Horseweed (*C. canadensis*) seeds were collected from the premises of 2 Yuanda Street, Changsha, China. Seed were directly sown into potted compost, and the plants were raised in an artificial climate box with a 16h photoperiod (100-120 $\mu molm^{-2}s^{-1}$) maintained at 22/18°C during the light/dark cycle, respectively. Plants were used for experiments when they reached the 10-15 leaf growth stage. Plants were treated with 625 µM caprylic acid. Leaves were harvested 0, 2, 4, 8, 12, and 24h later.

For the light microscopy analysis, fresh leaves were cut middle part and fixed immediately in FAA (formalin: acetic acid: 70% alcohol= 5: 5: 90 volume/volume) for at least 48 h. Samples were then dehydrated from 30% alcohol to100% alcohol, embedded in paraffin, sectioned to a thickness of 10µm by rotary microtome, stained with 1% safranin and 0.5% methylene blue, and deposited in Canadian balm (Li *et al.*, 2016). Picture of cross sections were taken by using an Olympus BX51 (Olympus, Tokyo, Japan).

For scanning electrons microscopy (SEM) analysis, fresh leaves were fixed in PBS, and then dehydrated by alcohol-isoamyl acetate, and dried to a critical point using CO_2 (Luan *et al.*, 2017). The aluminum stubs and gold-palladium were coated on the surface of leaves, and then they were observed under the S-4000SEM (Hitachi, Ltd, Tokyo, Japan).

For the transmission electrons microscopy (TEM) analysis, fresh leaves were fixed in 0.05 M cacodylate buffer (pH 7.2) for 2 h, which was an aqueous solution containing 2.5% glutaral dehyde and 4% paraform aldehyde. Samples were post-fixed with 1.0% OsO4 in the same buffer for 1 h, and then dehydrated by acetone and embedded by Epoxiresin. The 70 nm of ultrathin sections were cut by Reichert Ultracutsul tramicrotome (Leica, Mark Solms, Germany). Uranyl acetate was stained on the ultrathin sections followed by lead citrate, and photographed with a TEM 900 ZEISS microscope (Zeiss, Oberkochen, Germany).

Results

Light microscropy observations: The untreated *C. canadensis* leaves exhibited a normal leaf anatomy, with dorsoventral mesophyll tissue consisting of a palisade layer immediately beneath spongy parenchyma followed by the adaxial epidermis. Cross-sections of the leaves midrib region displayed a uniseriate epidermis, a vascular system and fundamental parenchyma. The vascular system consisted of xylem on the adaxial side and phloem on the abaxial side (Fig. 1a).The differentiation in the leaf tissue became apparent 2 h after caprylic acid application (Fig. 1b). Palisade layer cells and spongy parenchyma cells were disoriented and the leaf-blade had decreased in thickness in plants subjected to caprylic acid stress. However, the midvein was not influenced (Fig. 1c).The damage was more severe in the plants after4 and 8 h, as effects had spread throughout the mesophyll and reached the two leaf sides as well as the midvein (Fig. 1d). At 12 and 24 h later, the caprylic acid had almost completely destroyed the leaf tissue (Fig. 1e, f). Notably, the mesophyll percentage area was first damaged and then the margin leaf regions, middle leaf, and midvein occured. The accumulation of caprylic acid into the cells, resulted in palisade parenchyma collapse and reduce, cell wall deform, and veins punctual necrosis, was evident in the leaf sprayed with caprylic acid.

No abnormalities on both palisade and spongy mesophyll cells were observed in control plants in semi-thin sections when viewed under light microscopy (Fig. 2a). Palisade mesophyll cells were a typical, oblong shape, slightly wider at the end adjacent to the abaxial epidermis. Chloroplasts were clearly visible near the abaxial epidermis. Spongy mesophyll cells were oval or longish with slight weavings (Fig. 2b). By 2 h after caprylic acid application, mesophyll cells were firstly affected, though the midvein was not affected (Fig. 2c-d).Then (after 4 and 8 h), vascular bundles became deformed and more seriously affected (Fig. 2e-h). By 12 and 24 h after treatment, the leaf mesophyll tissue was tally degraded (Fig. 2i- l).

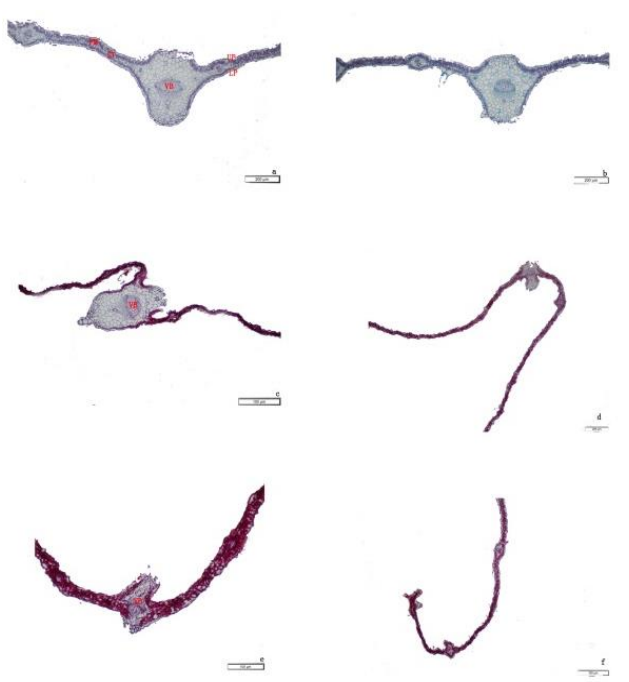

Fig. 1. Transverse paraffin section of leaf blades showing leaf anatomical changes in *Conyza canadensis* with control treatment (a) and 2 h (b), 4h (c), 8h (d), 12h (e), and 24 h (f) after caprylic aicdtreatment (Bars=100 or 200 µm). LE, lowerepidermis; UP, upper epidermis; PM, Palisade mesophyll; ST, spongytissue; VB, vascular bundle.

Scanning electron microscopy observations: The *C. canadensis* foliar surfaces were covered with many vertical shape epicuticular waxes (Fig. 3a). Few tector trichomes and glandular trichomes were presented on the adaxial and the abaxial surface (Fig. 3b, c). The amphihypo-stomatic stomata were distributed on the same level as the other epidermal cells (Fig. 3b,d). The leaves epidermal waxes on both sides were disrupted 2 h after the caprylic acid application (Fig. 3e-h); this effect was enhanced with the passage of time. The epidermis with more severely rupture was detected in parts of the leaf blade 4 h after the caprylic acid-treatment (Fig. 3i-l). As times progressed, turgor was gradual loss of and the external periclinal wall was flattening on the abaxial epidermal cells of treated leaves after caprylic aicd-treatment (Fig. 3 m-p). There were concavities in the leaf surfaces under plants exposed to caprylic acid 12 and 24 h later. The loss of turgor result that larger wrinkled regions occurred, which progressed to fully plasmolysed areas (Fig. 3q, r). The adaxial epidermal surface had a similar damage, whereas more severe damage was prevalent in plants exposed to caprylic acid, resulting in discontinuities on the leaf epidermis (Fig. 3s, t). The stomata were not damaged on both side of leaf epidermis (Fig. 3t). However, the trichomes cells had collapsed, lost their turgor, and showed a twisted appearance in those plants which were sprayed higher (Fig. 3r, s). The *C. canadensis* leaf surfaces and structure were impacted on caprylic acid, indicating progressive damage that increased with time.

Transmission electron microscopy observations: TEM micrographs of leaves with intermediate symptoms are presented in Figure 4 to illustrate the ultrastructural characteristics of caprylic acid treatment. Leaf cells from samples without caprylic acid had a high cytoplasmic content and limited vacuolar space. Cells exhibited well-delimited nuclei, and mitochondria were prevalent (Fig. 4a, b). Chloroplasts were elongated and contained grana consisting of several normaly arranged thylakoids (Fig. 4c, d). Starch was observed occasionally in the chloroplasts. By 2 h after treatment, the cell walls remained regularly shaped. Cells had more extensive intercellular spaces and exhibited large vacuoles with the cytoplasm restricted to parietal areas (Fig. 4e). Chloroplasts were still prevalent and internal membranes maintained their integrity. Mitochondria were less numerous in treated leaves than in control leaves (Fig. 4f). By 4 h after treatment, there was a separation of plasma membrane from the cell wall in some areas (Fig. 4g).The thylakoids became wrinkly and were destroyed, while the chloroplasts and starch granules disintegrated (Fig. 4h). By 8 h after treatment, mitochondria were few and far between (Fig. 4k), and the chloroplasts and thylakoids were completly disintegrated (Fig. 4l). Cytolysis and overall tissue damages in the leaf anatomy of samples both 12 and 24 h after treatment were observed (Fig. 4n-p). These alterations corresponded to damages observed in palisade and spongy cells.

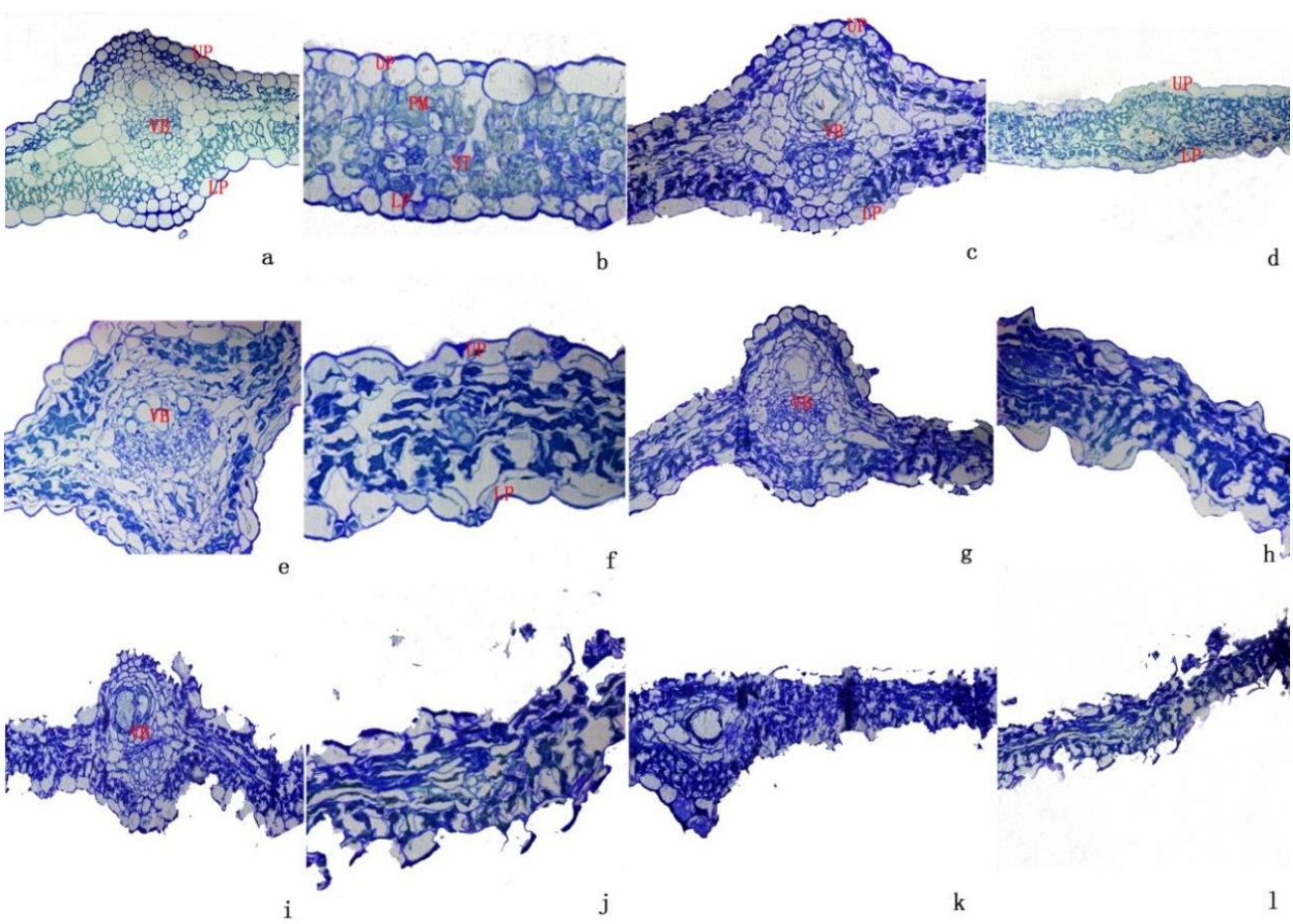

Fig. 2. Semi-thin transversesections of leaf blade-sections showing leaf anatomical changes in *Conyza canadensis*, withControltreatment (a, b) and 2 h (c, d), 4h (e, f), 8h (g, h),12h (i, j), 24 h (k, l) after caprylicaicdtreatment (Bars=100 or 200 µm).LE, lower epidermis; UP, upper epidermis; PM, Palisade mesophyll; ST, sponytissue; VB, vascular bundle.

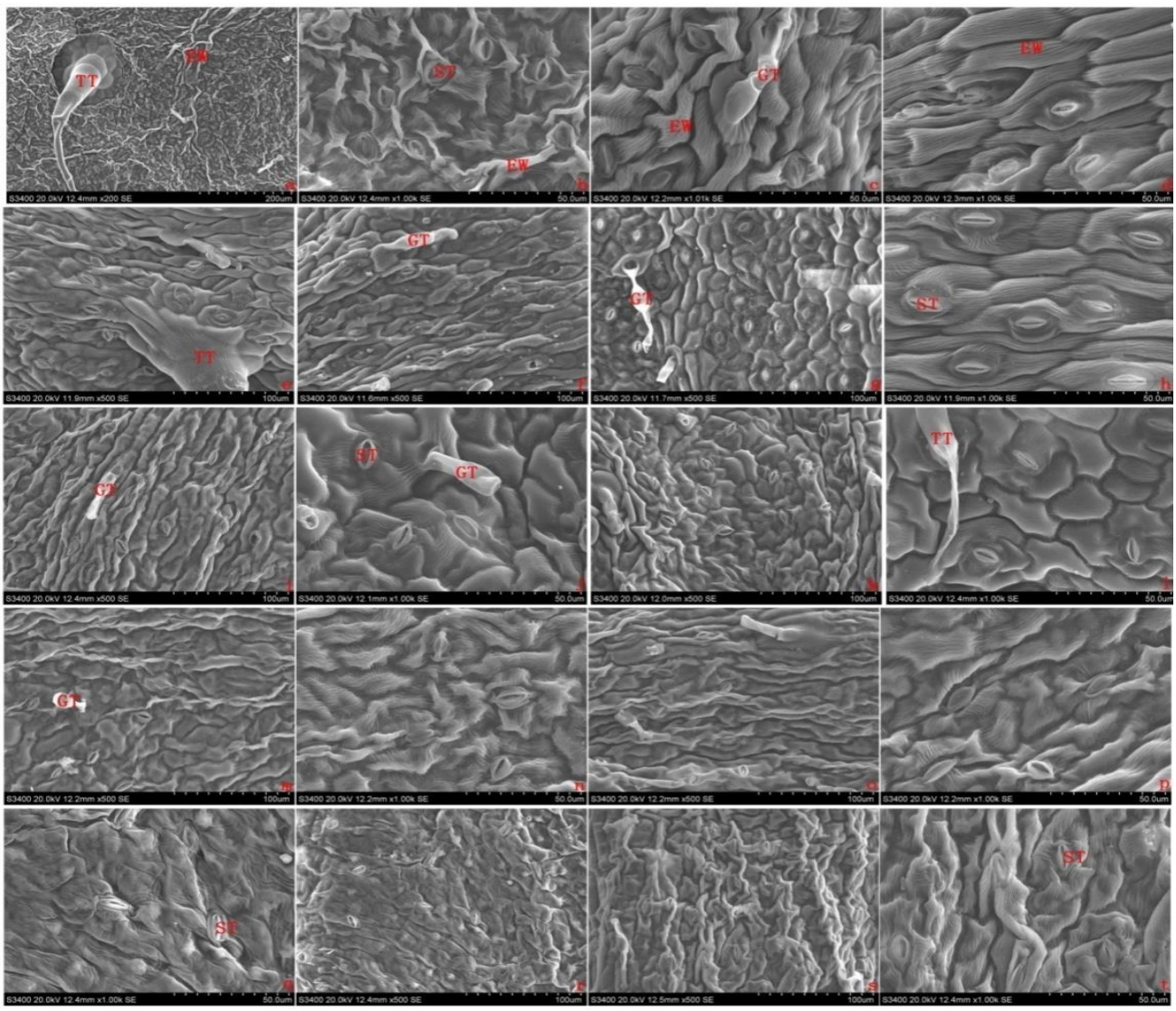

Fig. 3. Scanning electron micrograph of the leaf surface of *Conyza canadensis*: adaxial surface (a, b, e, f, i, j, m, n, q, r); abaxial surface (c, d, g, h, k, l, o, p, s, t); control treatment (a, b, c, d); and 2 h(e, f, g, h); 4h(i, j, k, l), 12 h(m, n, o, p), 24 h(q, r, s, t) post-applicationcaprylicacid treatments. EW,epicuticular waxes; GT, glandular trichomes; ST, stomata; TT, tectortrichomes.

Discussion

Anatomical differences were more marked with passage of time after the leaves had been exposed to the herbicides. Leaves exhibiting intermediate versus severe poisoning symptoms showed key differences in anatomical characteristics. Various anatomical injury followed by herbicides infect depended on adaptive characteristics expressed through several mechanisms. Thus, the affected responses of plant organs depend on the anatomic characteristics that regulate the transmission of herbicide stress effects to the cell. Anatomic analyses are necessary to aid the early diagnosis of injury, helping to clarify the mechanisms of molecules action (Sant'Anna-Santos *et al.*, 2006).

C. canadensis leaf anatomy was greatly affected by caprylic acid stress. Under light microscopy, cells of the palisade and spongy parenchyma layers were disoriented, thereby reducing the leaf-blade thickness at the initial phase, and leaf tissues were almost completely destroyed by the last observation time point. The damage effect is manifested from the mesophyll at the margin of leaf, and then middle part of leaf and midvein. The accumulation of caprylic acid in the cells, resulted in palisade parenchyma collapse and reduce, cell wall deform, and veins punctual necrosis, was evident in the leaf sprayed with caprylic acid. The SEM observation revealed the disruption of waxes, rupture of the epidermis, and larger wrinkled regions. After a moderate period of time post treatments (i.e. 2,4, and 8h), the TEM observations revealed affected and non-affected organelles together, demonstrating gradual damage. In longer treatments (i.e. 12 and 24 h), chloroplasts, mitochondria, and other organelles were completly disintegrated. These anatomical changes showed that the leaf anatomy of caprylic acid-treated *C. canadensis* displayed a time-dependent depletion and disintegration. Variable palisade parenchyma was first negatively impacted on the presence of the caprylic acid, which was an important apparatus linked to leaf protection against high light intensity (Tuffi *et al.*, 2008). Caprylic acid affects directly leaf photosynthetic process, causing a decrease in the availability of metabolites and in the growth rate of plant (Pasqualini *et al.*, 2002; Andosch *et al.*, 2015).

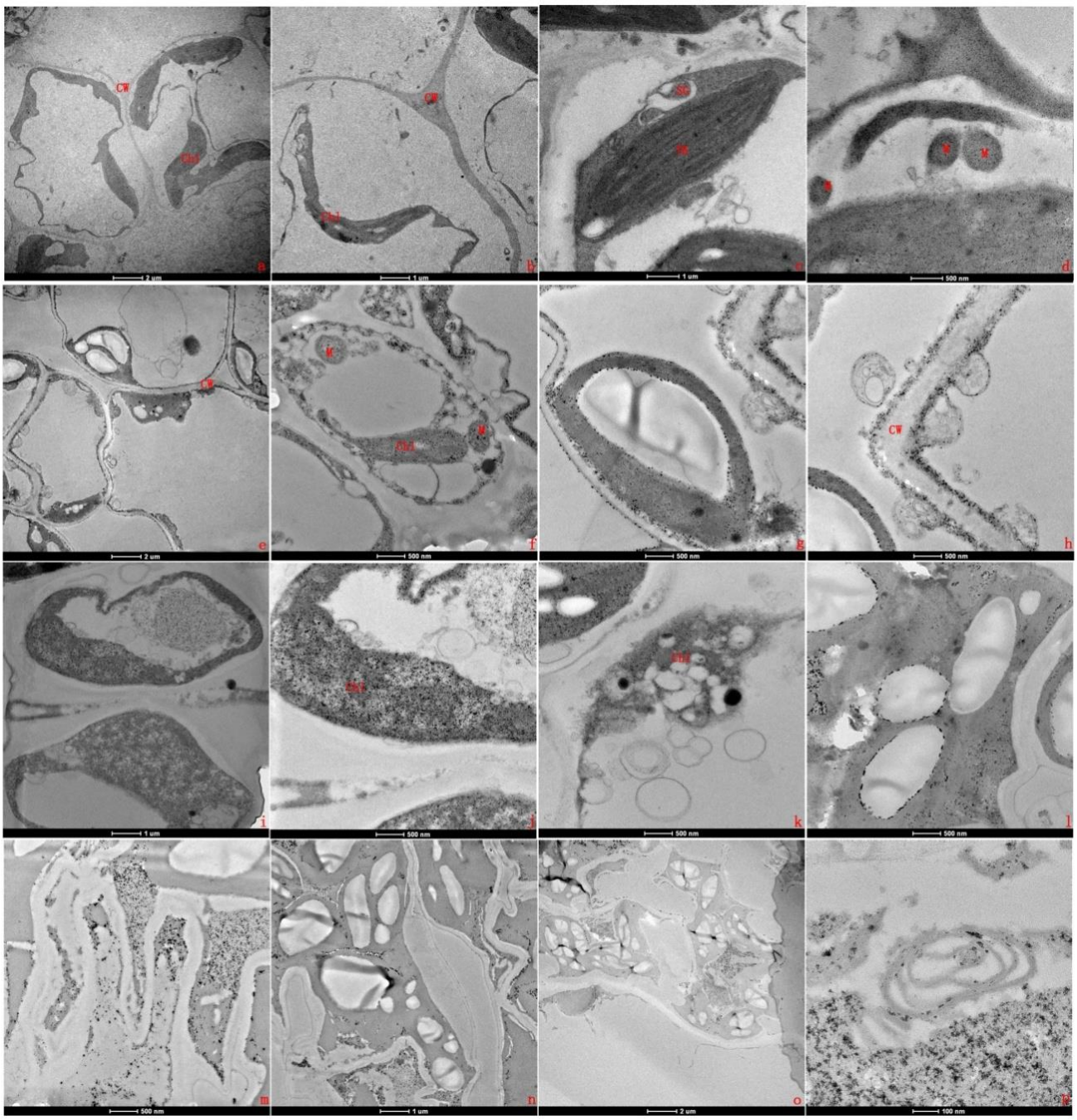

Fig. 4.Transmission elections micrograph of *Conyza canadensis* leaf. Untreatment (a, b, c, d) and 2 h (e, f, g, h), 4h (i, j), 8h (k, l), 12h (m, n), and 24 h (o, p) post-application caprylic acid treatment.Chl, Chloroplast; CW, cell wall; M, mitochondria; SG, starch granules; TK, thylakoids.

The stress response is first recognizable at the metabolic level; if the exposure to stress factors persists, then changes are recognizable at the microscopic level and finally are expressed as visible, morphological changes. These unobservable stages of damage can be recognized as alterations of different metabolic pathways or by the occurrence of new ones, often connected with plant avoidance or resistance to the specific stress factor. An altered metabolism can cause further changes in different physiological processes and often causes changes in plant structural (i.e., cellular and/or morphological) organization. Some of these changes can be irreversible and are recognized as a macroscopic damage. Visible structural changes in response to chemical stimuli can be recognized as caused by some of these metabolism alterations. Baur *et al.*, (1969) investigated the gross morphological symptoms and observed the disruption of membrane integrity on the ultrastructure of mesophyll cells of honey mesquite leaves after gramoxone application, which followed by wilting, necrosis and the ultimate death of leaves. Crowley & Prendeville (1980) choosed different action modes of herbicides on leaf-cell membranes, which included 'slow-acting' herbicides. The leaf-cell permeability alteration is one of the initial effects responsible for phototoxicity, which can occur before visible injury appears as result in

a fast acting herbicide. The Crowley & Prendeville's findings showed that visible injury need not precede increased permeability as in *Phaseolus*. In the present study, the chloroplasts in mesophyll cells were severely affected by caprylic acid toxicity. Photons can be utilized by chloroplasts from solar radiation to synthesize energy-rich molecules such as ATPs and NADPHs, which are further utilized in active cellular processes. Various stressors might be directly or indirectly interacted with photosynthetic components and processes such as heavy metals (Bashir, 2015; Muhammad *et al.*, 2016) and drought (Keshav & Vanlerberghe, 2017). Mitochondria in mesophyll cells also suffered injury under caprylic acid treatments. Various cellular activities need the chemical energy from mitochondria. Poor health and disease can be from defects in mitochondrial structures or in the gene organization in mitochondria (Rampelt *et al.*, 2017; Raven & Beardall, 2017). Chloroplasts and mitochondria were degradation and photosynthetic activity was markedly reduced during caprylic acid application as a consequence.

This work represents the first detailed microscopic evaluations of caprylic acid in leaves and provides insight on how caprylic acid affects leaf structure and function. The results show the poisoning effects and the highly reduced effectiveness of photosynthetic processes caused by caprylic acid in *C. canadensis*. The futher studies may be necessary to understand the mechanism exerted by the inhibiting effect.

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