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Impact of exogenous caffeine on morphological, biochemical, and ultrastructural characteristics of *Nicotiana tabacum*

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Abstract

Caffeine (1,3,7-trimethylxanthine) is purine alkaloid, and it is secondary metabolite produced naturally in plants. It plays a crucial role in defense and stress tolerance. A hydroponic experiment was carried out to study the morphological, biochemical, and ultrastructural effects of caffeine treatment on seedlings of tobacco (*Nicotiana tabacum* L. cv. Turkish). The plants were grown in a growth chamber for 14 d in Hoagland's nutrient solution supplemented with 0 (control), 25, 50, 100, 1000, and 5000 μM caffeine. Shoot heights as well as root lengths significantly decreased in the plants treated with 1000 and 5000 μM caffeine. Total protein and sugar content in leaves increased significantly in the 5000 μM caffeine-treated plants. Moreover, electron microscopic analysis shows that in plants exposed to 1000 and 5000 μM caffeine, mesophyll cells possessed enlarged chloroplasts with disrupted thylakoid membranes associated with large starch grains and plastoglobules. Scanning electron micrographs exhibit that the vast majority of stomata in the plants treated with 1000 and 5000 μM were closed. In contrast, the 25 and 50 μM caffeine-treated plants showed an enhanced growth and no signs of injury.

Additional key words: plastoglobules, scanning electron microscopy, stomata, starch, tobacco, transmission electron microscopy, trichomes.

Introduction

Abiotic stresses are major causes to the agriculture crop loss all over the world. However, plants have developed a variety of defense strategies to cope up with these stresses such as change in gene expression, change in protein content and composition, accumulation of osmolytes (*e.g.*, proline, sugars, and proteins), and production of secondary metabolites (Gilbert *et al*. 1997, Dhanapackiam and Iiyas 2010). Caffeine (1,3,7-trimethylxanthine) is purine alkaloid and secondary metabolite that is produced naturally in young leaves and immature fruits of more than 80 plant species including tea, coffee, cacao, and cola (Ashihara and Crozier 1999, 2001, Ashihara *et al*. 2008). The mechanisms of effects of purine alkaloids in plants are not fully elucidated. In coffee and tea plants, caffeine might have an allelopathic effect (Smyth 1992, Anaya *et al*. 2006) or an antiherbivory effect (Nathanson 1984). In addition, caffeine and its derivatives have been reported to be induced in plants exposed to various stresses such as pathogen infection, wounding, and high salinity.

Plants exposed to exogenous caffeine at a relatively high concentration (0.01 - 0.1 %, m/v) can be more resistant to herbivores and pathogenic microbes. For example, caffeine directly inhibits growth of *Aspergillus ochraceus* and *Pseudomonas syringae* (Tsubouchi *et al*. 2001, Kim and Sano 2008). Repulsion of slugs was observed in cabbage leaves sprayed with 1 - 2 % caffeine, and of orchid snails by spraying with 0.5 or 2 % caffeine (Hollingsworth *et al*. 2002). Moreover, in tobacco plants, applied caffeine was effective in repelling tobacco

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Abbreviations: EC_{50} half maximum effective caffeine concentration; PBS - phosphate buffered saline; ROS - reactive oxygen species; r - Pearson correlation coefficient; rs - Spearmen correlation coefficient; SEM - scanning electron microscope; TEM - transmission electron microscope.
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hornworms and in disturbing the reproductive ability of some species of moths (Nathanson 1984, Mathavan *et al*. 1985). In addition, caffeine-producing plants acquire pathogen and herbivore resistance (Uefuji *et al*. 2005). For example, transgenic chrysanthemum plants that produce caffeine at a level of about 3 μg g^{-1}(f.m.) show resistance against *Botrytis cinerea* (Kim *et al*. 2001), and transgenic tobacco plants are more resistant to *tobacco mosaic virus* and to microbial pathogens *Pseudomonas syringae* and *pv. glycinea*. Several studies identified the characteristics of these transgenic tobacco plants, and all results show that the transcripts of defense related genes encoding pathogenesis related protein 1a and inhibitor II constitutively express even under non stressed conditions. In contrast, control plants show no accumulation of these transcripts (Kim and Sano 2008).

In contrast, Mohanpuria and Yadav (2009) reported that *Arabidopsis* and tobacco seeds that are grown for 17 d in media containing 1 and 5 mM caffeine exhibit an obvious decrease in shoot and root lengths. Moreover, many other morphological alterations were observed such as yellowing of leaves, decrease in chlorophyll content, and diminished branching system. These symptoms are more severe in seedlings exposed to 5 mM caffeine than in those exposed to 1 mM caffeine. Similar morphological changes were also observed in many plants upon exposure to heavy metals and salt stress (Yadav *et al*. 2005, Singla-Pareek *et al*. 2006). In *Arabidopsis* and tobacco seedlings, Rubisco transcript expression and activity decrease after caffeine exposure. This decline elevates by increasing exposure time and caffeine concentration suggesting that retardation in seedling growth could be correlated to decreasing Rubisco activity. However, it is still not fully known whether caffeine treatments downregulates Rubisco expression, which leads to senescence in plants, or caffeine induces early senescence in plants, which leads to downregulation of Rubisco expression and its activity (Mohanpuria and Yadav 2009).

The overall goal of this study was to examine the impact of caffeine in different concentrations on morphological, biochemical, and ultrastructural parameters of *Nicotiana tabacum,* bearing in mind that tobacco was used as model plant which naturally does not synthesize caffeine.

Materials and methods

Plants and treatments: Tobacco seeds (*Nicotiana tabacum* L. cv. Turkish) were germinated in potting soil (*Thimar Chemical and Agricultural Manure*, Amman, Jordan) for four weeks. The seedlings were carefully removed from the soil and their roots were thoroughly washed with tap water. Plants with the same number of leaves and the same height were placed in Hoagland's No. 2 nutrient solution (*Caisson Laboratories*, North Logan, UT, USA) and grown hydroponically in a growth chamber (model *VS-3DM*, *Bionex*, Bucheon, Korea) at a 16-h photoperiod, a photosynthetic photon flux density of 250 to 300 μmol m^{-2} s^{-1}, day/night temperatures of 30/23 °C, and relative humidities of 60/30 %.

In order to target caffeine concentrations used in this experiment, eight concentrations of caffeine were used for screening purposes. By the end of the screening experiment, six different concentrations of caffeine were selected and their impacts were examined. Each plant was grown for 3 d in Hoagland's solution in 250 cm^3 Erlenmeyer flasks with 0 (control), 25, 50, 100, 1000, and 5000 μM caffeine (*AZ Chem*, Jacksonville, FL, USA). The hydroponic solution was changed every three days. The experimental design was completely randomized with three replicates per treatment and lasted 14 d.

At the end of day 14, all control and caffeine-treated plants were harvested. Then, roots were detached from shoots and measured separately. Root length and shoot height in the control and caffeine-treated plants were measured using a ruler. Then, roots and shoots put in paper bags were placed in an oven (*HI 950D*, *Thermolab Industries*, Codogno, Italy) at 60 °C for 2 d. Then, dry masses were recorded.

Sugar content was estimated by the anthron method (Yemm and Willis 1945, Parthiban *et al.* 2012). Briefly, 100 mg of a leaf sample was ground in liquid nitrogen, extracted in 5 cm^3 of 80 % (v/v) ethanol, and centrifuged at 1 500 rpm for 10 min (a low speed centrifuge *TD6*, *Pingfan Instruments*, Changsha, China). The supernatant was placed in 5 cm^3 *COD* vials and evaporated using a water bath at 80 ºC. To dissolve sugar, 1 cm^3 of distilled water was added and after 3 min, 4 cm^3 of a freshly prepared 0.2 % (m/v) anthron solution [0.2 gram of anthron dissolved in 100 cm^3 of chilled 75 % (m/m) H_2SO_4] was added and stirred well by a vortex. The mixture was heated in a water bath at 95 °C for 15 min and then rapidly cooled. The absorbance of the solution was measured at 630 nm using a spectrophotometer (*250 UV-VIS*, *Shimadzu*, Tokyo, Japan). Distilled water was used as blank, and as reference, D-glucose solutions were prepared for a calibration curve.

Protein content was determined by the method described by Bradford (1976), and bovine serum albumin was used as standard.

Microscopic analysis of caffeine accumulation: Leaves from the control and caffeine-treated plants were cut in small pieces (0.5 - 1.0 cm) and fixed in 2.5 % (m/v) glutaraldehyde in a 0.1 M cacodylate buffer, pH 7.4 (*EMS*, Hatfield, PA, USA) at 4 ºC overnight. The samples were postfixed in cacodylate-buffered (pH 7.4) 1 % (m/v) osmium tetroxide, dehydrated in a graded series of ethanol (50, 70, 80, 95, and 100 %, v/v) for 10 - 15 min each, and embedded in a freshly prepared Araldite resin

(6500, *EMS*).

For scanning electron microscope (SEM) analysis, fresh leaf samples (0.5 × 1 cm) were placed on SEM stubs with a carbon sticky tape (*EMS*). Then, leaves were dusted with 2 nm of gold using a sputter coater (*Q 150R ES*, *Quorum*, Laughton, UK) and examined using the *FEIQ 450* SEM running at 5 kV (*FEI Company*, Hillsboro, USA).

For a transmission electron microscope (TEM), thin sections (70 nm) of leaf samples were cut with a diamond knife (ultra 45º, *Diatome*, Biel/Bienne, Switzerland) using an ultramicrotome (*Leica* ultracut *R-GA- D/E-1/100*, Wien, Austria) with a typical cutting speed of about 1 mm s^{-1}, stained with uranyl acetate (Epstein and Holt 1963) followed by lead citrate (Reynolds 1963) and examined using the TEM running at 60 kV (*Zeiss EM 10 OCR*, Oberkochen, Germany).

Statistical analyses: Intra-experimental reproducibility was assessed for each morphological and biochemical parameter. The Pearson and Spearman correlation coefficients (r and r_s, respectively) between pairs of replicate plants for each experiment were used to assess intra-experimental reproducibility. For this analysis, all replicate measures were randomly selected for each concentration and experiment pair (4 - 11 experiments × 6 concentrations × 3 replicates = 132 - 198 total replicate pairs sampled). Computations and graphs used the software for statistical computing and graphics (*2.13.1*; *R Development Core Team*, Vienna, Austria). One-way *ANOVA* was used to assess significance of concentration effect *vs*. experimental effect for each physiological and biochemical parameter with *SAS 9.2* (Cary, NC, USA) at $\alpha = 0.05$. Tukey's post hoc tests were used to assess significant differences between different treatments.

Concentrations were log transformed and responses were normalized to the smallest (0 % of the control, BOTTOM) and highest (100% of the highest concentration, TOP) values. A sigmoidal four-parameter logistic regression was fitted for all four morphological parameters. Half maximum effective caffeine concentration (EC_{50}) and Hill slope were estimated for each fit:
$Y = BOTTOM + (TOP - BOTTOM)/(1 + 10^{(logEC50 - x) + Hill\ slope})$
For each concentration, the mean response and the standard error of the mean were plotted for all morphological and biochemical parameters using *Graph Pad Prism 5*.

Results

To validate the robustness of our data, intra-experimental reproducibility for replicate plants were thoroughly assessed (Fig. 1 Suppl.) using r and r_s between pairs of replicate plants. The values of r and r_s were highly significant ($P < 0.001$) for all parameters assessed. Strong correlation coefficients were observed for shoot height ($r = 0.8$, $r_s = 0.8$), shoot dry mass ($r = 0.7$, $r_s = 0.7$), root dry mass ($r = 0.8$, $r_s = 0.8$), sugar content ($r = 0.9$, $r_s = 0.7$), and protein content ($r = 0.98$, $r_s = 0.72$), however, the correlation for root length was moderately strong ($r = 0.6$, $r_s = 0.6$, Fig. 1 Suppl.).

The plants treated with 25 and 50 µM caffeine did not show any visible reductions in their shoot height and root length, and the overall appearance of shoots and roots was very similar to that of the control plants. In contrast, shoots and roots treated with 100, 1000, and 5000 µM caffeine showed severe reductions in shoot height and root length, and these reductions increased as caffeine concentration increased (Fig. 1*A,B*). Additionally, the 100, 1000 and 5000 µM caffeine-treated plants had a severe reduction in leaf area, noticeably in the 1000 and 5000 µM treatments. Also, severe chlorosis and necrosis symptoms were shown on the surface of leaves in the 1000 and 5000 µM caffeine treated plants (Fig. 1*G,H*). The plants treated with 100, 1000, and 5000 µM caffeine showed changes in root-branching pattern and some discoloration (brownish) as compared to the control plants. Further, shoot height of the plants exposed to 1000 and 5000 µM caffeine significantly decreased up to 44.3 and 77 %, respectively ($P < 0.0001$), as compared to the control plants (Fig. 2*A*). Similarly, root system length in these plants significantly decreased up to 29 and 42 %, respectively ($P < 0.0001$), as compared to the control plants (Fig. 2*B*). A significant decrease in dry mass of plant parts was observed under the caffeine treatment. Dry mass of shoots significantly decreased up to 63.4 % in the plants exposed to 5000 µM as compared to the control plants ($P < 0.0001$, Fig. 2*C*). Dry mass of roots significantly decreased up to 76.3 % in the plants exposed to 5000 µM as compared to the control plants ($P < 0.0001$, Fig. 2*D*).

A standard concentration response curve was conducted, and EC_{50} was estimated. In Fig. 2 Suppl., it is illustrated the concentration of caffeine required to provoke a response halfway between the baseline (0 %) and maximum responses (100 %) after a specified exposure time (14 d). The EC_{50} concentrations for root length, shoot dry mass, and root dry mass were: 335, 302, and 348 µM, respectively. However, shoot length was less sensitive, where EC_{50} was reached at 747.1 µM.

Accumulation of proteins and sugars in leaves showed no significant differences among the control and the plants treated with 25, 50, 100, and 1000 µM caffeine (Fig. 2*E,F*), but they dramatically increased in the plants exposed to 5000 µM caffeine ($P < 0.001$).

The plants treated with 25 and 50 µM caffeine-showed no changes in stomatal size and opening (Fig. 3). However, differences were observed in the plants treated with 100, 1000, and 5000 µM caffeine. The plants exposed to 1000 and 5000 µM caffeine showed distinct changes not only in size and opening of stomata but also in delaying their maturation, which was manifested in the

occurrence of many not-fully developed stomata (Fig. 3*C,D*). Furthermore, the 1000 and 5000 µM caffeine treated plants exhibited large glandular trichomes with a unicellular base and a spherical multicellular head (Fig. 3*E,F*).

The chloroplast shape of the plants treated with 25, 50, and 100 µM caffeine was normal (elliptical) as in the control plants as well as the integrity and the density of thylakoid membranes showed no differences from the control plants (Fig. 4*B,C,D*). The plants treated with 25, 50, and 100 µM caffeine exhibited small-size plasto-globules (Fig. 4*B,C,D*). In contrast, the plants treated with 1000 and 5000 µM caffeine showed enlarged, bulgy-shaped chloroplasts (Fig. 4*E,F*). Also, grana stacks and stroma were severely deformed, and their integrity was lost. Further, the plants treated with 1000 and 5000 µM caffeine showed larger and more numerous starch grains as compared to the control plants (Fig. 4*E,F*).

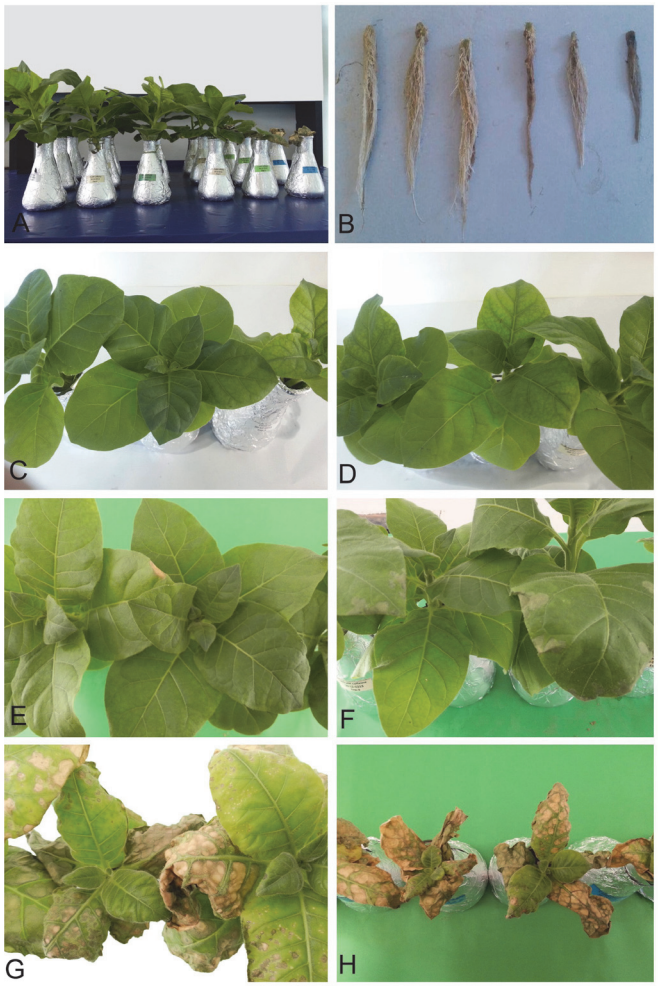

Fig. 1. Effects of caffeine on *Nicotiana tabacum* growth. *A* - shoot growth (*from left to right*; control, 25, 50, 100, 1000, and 5000 µM caffeine). *B* - root growth (*from left to right*; control, 25, 50, 100, 1000, and 5000 µM caffeine). *C* to *F* - an upper view of *Nicotiana tabacum* leaves (control, 25, 50, 100, 1000, and 5000 µM caffeine). *E* and *F* show severe necrosis.

Discussion

Our data show that there was a strong relationship between caffeine concentration and plant growth reduction. Negative effects of caffeine on tobacco growth could be related to inhibition of cell division, cell elongation, activities of some enzymes, various photosynthetic functions, *etc*. However, these alterations depend on concentration, treatment duration, plant species and/or plant age. Although caffeine is toxic at high concentrations and causes growth retardation, it was reported that suitable caffeine concentrations can activate the host self-defense system that normally occurs, so the host becomes ready to cope with biotic attackers in a way similar to mammalian vaccination (Kim *et al.* 2010).

High caffeine concentrations (1 000 and 5 000 µM) show a significant reduction in shoot height accompanied by alterations in photosynthesis, transpiration, respiration,

and water relations (Greger *et al.* 1992, Yamamoto *et al.* 2002). Kosobrukhov *et al*. (2004) showed that photosynthetic activity is controlled by many factors such as stoma numbers, stoma cell size, leaf area, and stomatal conductance. In this study, it is suggested that the reduction in growth of plants treated with high caffeine concentrations was due to a decline in photosynthetic rate, which resulted from stomatal closure (Fig. 3) rather than from a direct effect of caffeine on the photosynthetic apparatus. However, detailed measurements of further parameters such as stomatal conductance, transpiration

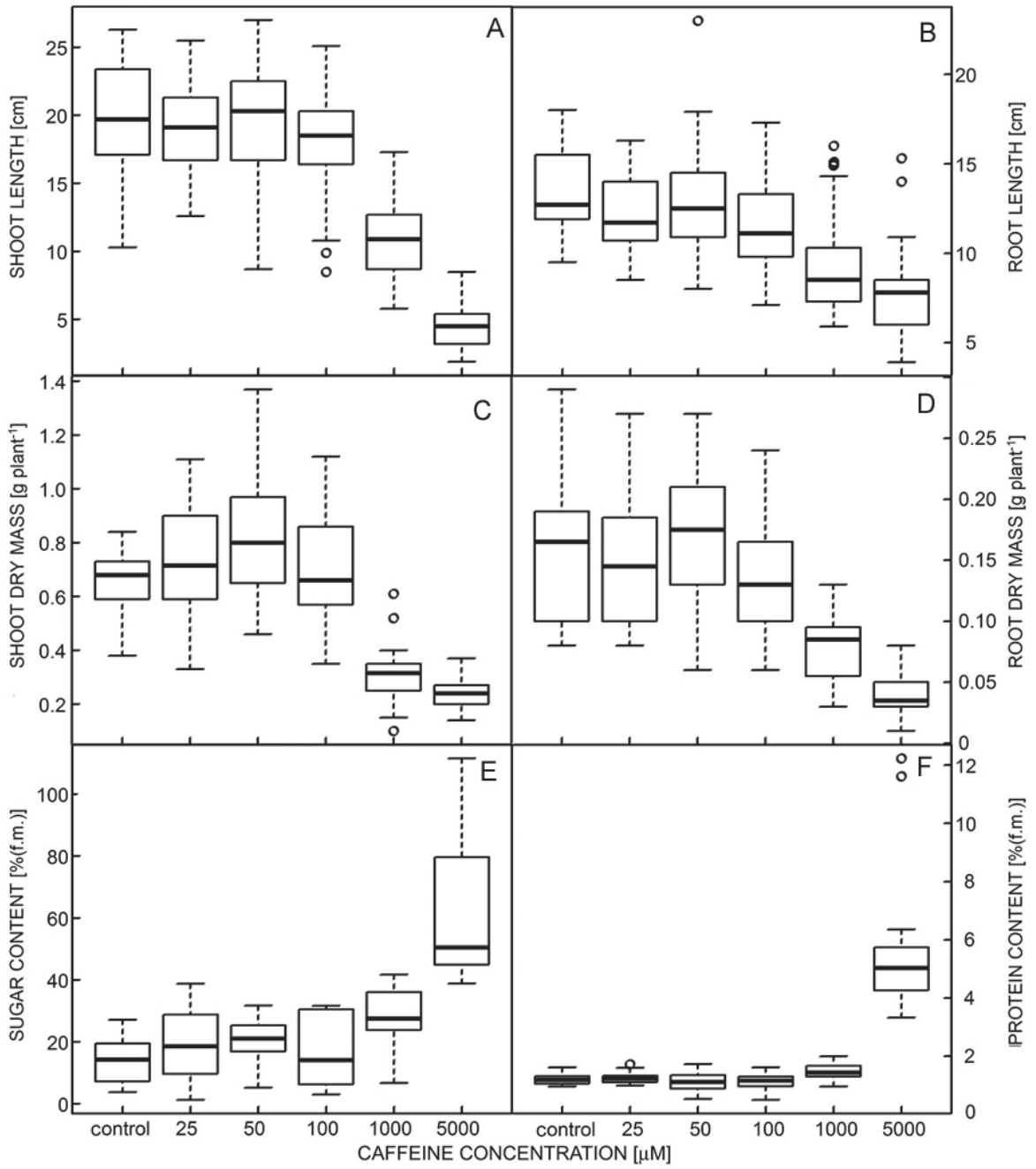

Fig. 2. Side-by-side boxplots: *A* - Shoot height, *B* - root length, *C* - shoot dry mass, *D* - root dry mass ($P < 0.0001$ for all parameters), *E* - sugar content, *F* - and protein content ($P < 0.001$) in response to each of six caffeine concentrations. Medians (*straight lines*) for 11 experiments with 3 replicates for each treatment are shown inside the box for morphological parameters and 4 experiments with 3 replicates for each treatment for biochemical parameters. *P*-values at $\alpha = 0.05$. *Boxes* represent the first and third quartiles: *horizontal lines* inside the boxes are medians: *whiskers* are 1.5 inter-quartile ranges; *circles* are outliners with > 1.5 inter-quartile ranges above minima or maxima.

rate, internal CO_2 concentration, and chlorophyll content are needed to determine how caffeine affects plant growth. Root length of the plants treated with 1000 and 5000 μM caffeine significantly decreased as compared to the control plants and plants treated with 25 and 50 μM caffeine (Fig. 2*B*). A similar reduction in root system was observed in *Arabidopsis* and tobacco seedlings after caffeine exposure (Mohanpuria and Yadav 2009). A similar reduction of root growth was detected in *Allium cepa* treated with different concentrations of lead nitrate (Wierzbicka 1994). In general, heavy metals alter uptake of nutritional elements, such as Ca, Mn, Fe, and N, by roots, which in turn reduces cell division or cell elongation in root tips (Haussling *et al.* 1988, Godbold and Kettner 1991, Eun *et al.* 2000).

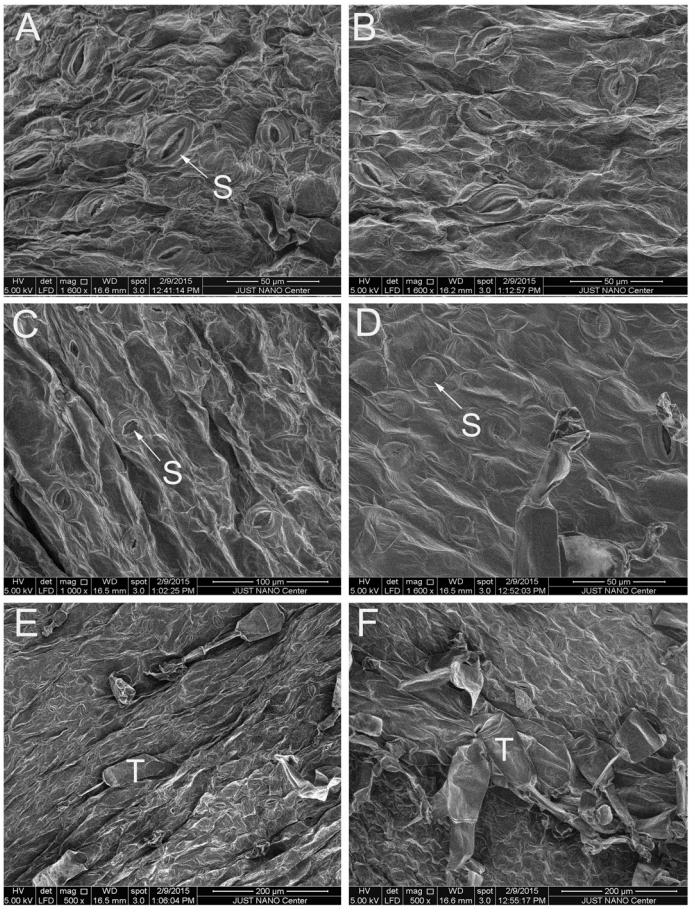

Fig. 3. *A* to *D* - Scanning electron micrographs of the surface of tobacco leaf epidermis after 14 d of caffeine treatment showing stomatal distribution; *A* - control; *B* - 50 μM; *C* - 1000 μM; *D* - 5000 μM. (*E* to *F*): Scanning electron micrographs of the surface of tobacco leaf epidermis after 14 d of caffeine treatment showing trichomes; *E* - 1000 μM; *F* - 5000 μM. S - stomata, T - trichomes.

In general, it has been shown that in abiotic stresses, a considerable reduction in growth and subsequent decrease in dry mass of different plant parts was observed (Ghoshroy *et al.* 1998, Kosobrukhov 2004, Moussa and El-Gamal 2010, Alkhatib *et al.* 2011). This is in full agreement with our findings.

Changes in protein content and composition are crucial to elucidate physiological processes in plants underlying several abiotic stresses. In this study, a significant increase in total protein content was observed in plant leaves treated with 5000 μM caffeine (Fig. 2*F*). This suggests that amino acids deriving from protein catabolism may be reallocated in plants *via* the phloem and used for protein synthesis in other plant organs and may accumulated in high amounts in leaves (Desimone *et al.* 1996, Crafts-Brandner *et al.* 1998, Herrmann and Feller 1998, 1999, Demirevska-Kepova *et al*. 2004, 2005, Thoenen *et al*. 2007, Feller *et al*. 2008).

Furthermore, several studies reported that synthesis of osmolytes in plants undergoing abiotic stress is increased significantly. Our data show that a high caffeine concentration (5000 μM) induced accumulation of sugars in tobacco leaves (Fig. 2*E*). Several studies reported that these phenomena occurr in most plant species under different abiotic stresses (Gilbert *et al*. 1997, Balibrea *et al.* 2000, Pattanagul and Thitisak 2008). The accumulation of these organic solutes plays a vital role in increasing internal osmotic pressure causing the plant to cope up with the abiotic stress (Dhanapackiam and Ilyas 2010).

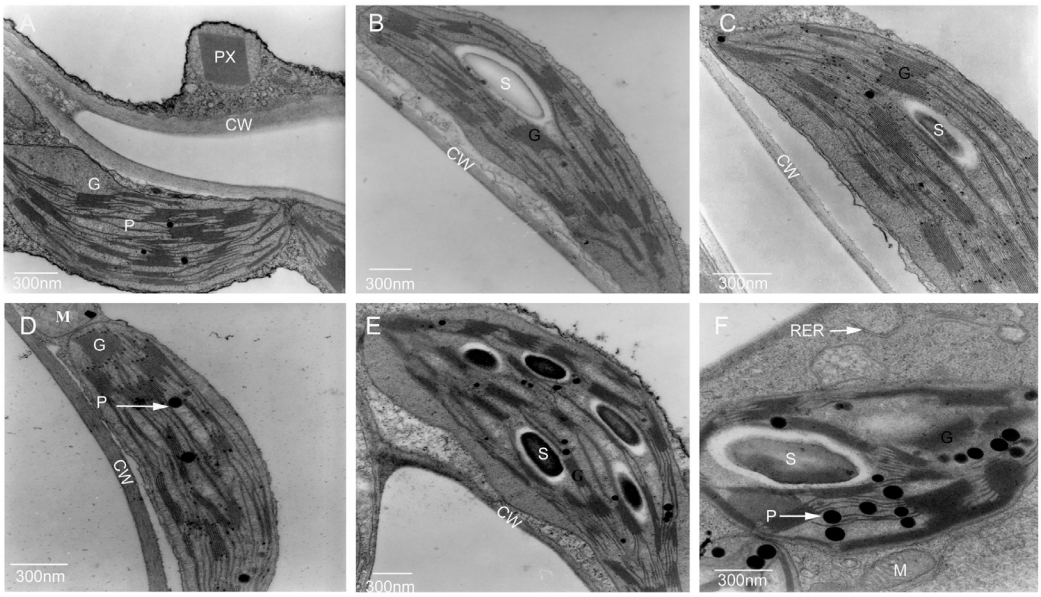

Fig. 4. Transmission electron micrographs of chloroplasts in tobacco leaves. *A* - A control leaf showing regular arrangement of grana (G) formed from several thylakoid membranes with regular starch grains (S). *B* to *D* - chloroplasts of plants treated with 25, 50, and 100 μM caffeine with the regular cell wall (CW) similar to the control. *E* to *F* - chloroplasts of plants treated with 1000 and 5000 μM caffeine exhibited deformed thylakoid membranes associated with large starch grains and large-sized plastoglobules. *Scale bar* = 300 nm. Peroxisomes (Px), plastoglobules (P), mitochondria (M), rough endoplasmic reticulum (RER), and starch grains (S) are also shown.

In the plants treated with 1000 and 5000 μM caffeine (Fig. 3), the majority of stomata were closed and their shape was also deformed. This could be related to an alteration of K^+ fluxes (Bazzaz *et al.* 1974). Thus, closure of stomata could be a keystone factor for growth retardation as it plays a major role in photosynthesis.

In the plants treated with 25, 50 ,and 100 μM caffeine, the ultrastructural organization of chloroplasts was similar to that in the control plants (Fig. 4*B,C,D*). In contrast, chloroplasts of a different shape were observed in the plants treated with 1000 and 5000 μM caffeine (Fig. 4*E,F*). A remarkable accumulation of large starch grains in chloroplasts of the plants treated with 1000 and 5000 μM caffeine could result from a less efficient transport of photosynthates from leaves to non-photosynthetic organs causing sugar accumulation in the leaves (McQuattie and Schier 1993).

Mesophyll chloroplasts from the plants treated with 1000 and 5000 μM caffeine were characterized by the presence of larger and more numerous plastoglobules than those from the control plants (Fig. 4*E,F*). Recent studies have reported the presence of larger and numerous plastoglobules in chloroplasts from plants exposed to different stresses (Gillet *et al.* 1998, Langenkamper *et al*. 2001, Yang *et al.* 2006). Moreover, expression of plastoglobule genes is modulated under such stresses (Manac'h and Kuntz 1999). Increased levels of plastoglobulin transcripts were observed in *Arabidopsis* and tobacco under light stress (Rey *et al*. 2000, Yang *et al.* 2006). It was suggested that enlargement of plastoglobules associates with high amounts of plastoglobulin proteins and antioxidants such as tocopherols (Claire et *al*. 2007).

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