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Retardation in seedling growth and induction of early senescence in plants upon caffeine exposure is related to its negative effect on Rubisco

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Abstract

Adverse effect of caffeine consumption has been well documented in animals and in human beings. However, here we studied the influence of caffeine exposure on seedling growth of *Arabidopsis* and tobacco plants. Retardation in the seedling growth of these plants was observed when grown on MS medium plates containing 1 mM caffeine and their growth retarded further upon increasing the concentration of caffeine to 5 mM. Retardation in seedling size including both root and shoot size, yellowing and decrease in chlorophyll content of seedlings upon caffeine treatment indicated that caffeine exposure induced early senescence in plants. Therefore, the influence of caffeine exposure on transcript expression and activity of Rubisco in tobacco and *Arabidopsis* seedlings was monitored. Caffeine exposure has been found to decrease the expression and activity of Rubisco in both the plants. Hence, this study documents that caffeine exposure retarded seedling growth and one reason for this could be its negative effect on Rubisco.

Additional key words: *Arabidopsis*; caffeine; Rubisco; seedling growth; tobacco.

Introduction

Caffeine, a purine alkaloid, is naturally produced in the leaves and seeds of many plants like tea, coffee, cocoa *etc*. It is also produced artificially and added to certain foods as an additive. High levels of caffeine intake causes several problems, at least in sensitive people, such as anxiety, elevated blood pressure, increased cholesterol, nutritional deficiencies, fatigue *etc*. The adverse effects of caffeine on human health have long been studied. Since caffeine is produced by the above mentioned plants, their processing generates a lot of waste pulp. This pulp contains carbohydrate, proteins and appreciable quantity of caffeine (Bresanni 1979). During tea preparation, a lot of tea remnant is left after filtration of sup. This all is thrown in soil for decomposition. Caffeine has also been obtained from the tea fibers (1.16 %) and stalk (0.92 %) wastes of tea indicating its presence in tea waste (Guru and Icen 2004). Caffeine in the waste might affect other plantation crops grown in such caffeine enriched areas.

Towards evaluating the toxicity of purine and pyrimidine, 6-methyluracil, 5-aminouracil and methyl-xanthines (*e.g.* caffeine, theophylline, theobromine, uric acid and their analogues) have been found to be inhibiting seed germination in *Cicer arietinum* (Dwivedi *et al.* 1981). Stallwood and Davidson (1977) also reported the inhibitory influence of methylxanthine on proliferating cells, however, that was reversed on colchicine applications. A mitotic delay, and a potentiation of the chromosome damage by caffeine post-treatment has been shown in proliferating plant cells (Gonzalez-Fernandez *et al.* 1985). Similarly, chromosome damage at mitotic stage of plant cell division upon caffeine treatment has been reported in various studies (Ahnstrom 1974, Swietlinska and Zuk 1974, Timson 1997, O'Connell *et al.* 2000, Samuel *et al.* 2002).

Coffee and tea plants accumulate caffeine in seeds, cotyledons, and young leaves. Caffeine has biological

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Abbreviations: cDNA – complementary deoxyribonucleic acid; Chl – chlorophyll; DM – dry mass; dNTP – deoxynucleotide triphosphate; MS medium – Murashige and Skoog medium; PCR – polymerase chain reaction; RNA – ribonucleic acid; RuBP – ribulose 1,5-bisphosphate; Rubisco – ribulose 1,5-bisphosphate carboxylase/oxygenase.
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roles in these plants serving as chemical defense, an antiherbivory (Nathanson 1984, Glendinning *et al.* 2000) and allelopathic compound (Smyth 1992). The role of caffeine synthesizing enzymes has been indicated during growth and ripening of *Coffea arabica* and *Coffea canephora* fruits (Koshiro *et al.* 2006). Recently, transgenic tobacco producing caffeine has been reported to be showing potential as a pest repellant (Uefuji *et al.* 2005, Kim *et al.* 2006).

Rubisco has been reported to be a main target protein affected in plants during exposure to various compounds and many stress conditions. Decrease in Rubisco activity has been observed due to high irradiance, high CO_2, ozone, drought, high temperature or heavy metal influence. Inhibition of Rubisco activity has a great impact on the whole photosynthesis (Siedlecka and Krupa 2004) and hence on the survival of plants. However, the mechanism underlying the retardation in seedling growth of plants, which are generally not producing caffeine *in vivo*, during exposure to caffeine is not known. In this study we investigated whether caffeine exposure could affect Rubisco in *Arabidopsis* and tobacco.

Materials and methods

Plant materials, growth conditions and caffeine treatments: Seeds of *Arabidopsis thaliana* and *Nicotiana tabacum* were surface sterilized with 70 % alcohol for 30 s and 5 % bleach (prepared by dissolving 5 ml Tween-20 in 95 ml water) for 10 min. Subsequently the seeds were washed 4–5 times with autoclaved distilled water in a laminar flow chamber. These surface sterilized seeds were germinated on MS medium plates containing no caffeine (control), 1, and 5 mM caffeine. Plates were kept in tissue culture room at 25±2 °C with 16 h light photoperiod. Light intensity of tissue culture room was 70 µmol(photons) m^{-2} s^{-1} and air humidity was 55±5 %. After 17 days, seedlings were photographed and used for shoot and root length measurement.

Caffeine from *Arabidopsis* and tobacco seedlings was extracted and its content measured employing the pro–cedure developed in our laboratory earlier (Sharma *et al.* 2005). After 17-d exposure of *Arabidopsis* and tobacco seedlings to 1 and 5 mM caffeine on MS plates, 3 g of tissues were dried at 80 °C and used for caffeine extraction with 70 % methanol. The caffeine content was estimated by *Merck Hitachi HPLC* (Darmstad Germany) using *C18 Lichrocart column* (250 × 4 mm × 5 µm) and the absorbance was read at 210 nm. The caffeine content was calculated from standard curve prepared from pure caffeine (*Sigma*).

Total chlorophyll (Chl): To check the influence of caffeine on total Chl, seeds of *Arabidopsis* and tobacco were germinated on MS medium for 17 d and then treated with 1 and 5 mM caffeine for 96 h. Seedlings kept in water alone for 96 h were used as a control. 100 mg of fresh seedlings were ground with 80 % acetone for total Chl measurement as described earlier (Mohanpuria *et al.* 2007).

Expression analysis of Rubisco by Reverse Transcriptase-polymerase chain reaction (PCR): For expression analysis, seeds of *Arabidopsis* and tobacco were germinated on MS medium for 17-d and then treated with 1 and 5 mM caffeine. Seedlings kept in water were used as a control. After 48, 72 and 96 h of caffeine treatment, total RNA was isolated from the seedlings. cDNA was synthesized using 4 µg of RNA in the presence of 200 U reverse transcriptase *Superscript*™ III (*Invitrogen*), 0.001 cm^3 of 10 mM dNTPs and 250 ng oligo $(dT)_{12–18}$. Resulting cDNA was used to carry out PCR with a Rubisco (large subunit) gene specific internal primers; 5'-CACAATGATAGGAAGAGCCGAC-3' and 5'-CAAGGGAACGGGCTTGGCAGAATC-3'. After standardizing the optimal amplification at exponential phase, PCR was carried out under the following conditions: 94 °C, 4 min for 1 cycle; 94 °C, 30 s; 60 °C, 40 s; 72 °C, 1 min for 25 cycles. α-tubulin based gene primers; 5'-GAGAGTTCATTTCGATCCAC-3' and 5'-CTGAGA GACGAGCCTGTTG-3' were used as the internal control for expression studies.

Rubisco activity was determined following the earlier described methods with some modifications (Rice and Pon 1978, Yokota *et al.* 1996). 100 mg caffeine treated and untreated seedlings of *Arabidopsis* and tobacco were ground in ice cold activating mixture and after centrifugation at 4 °C, clear supernatant was activated at 25 °C for 30 min. 1 ml of the activating mixture contained 25 mM Hepes-KOH buffer (pH 8.3), 20 mM $NaHCO_3$ and 20 mM $MgCl_2$. 1 ml of the Rubisco assay mixture contained 25 mM Hepes-KOH buffer (pH 8.3), 1 mM RuBP, 10 mM $MgCl_2$, 20 mM $NaHCO_3$, 3 mM DPNH, 200 units of carbonic anhydrase (Sigma) and 100 µl of activated solution. The change in absorbance was read at 280 nm and activity was calculated using the extinction factor of 50 M^{-1} cm^{-1}.

Results and discussion

Retardation in seedling growth upon caffeine exposure: To assess whether caffeine exposure has any influence on seedling growth of plants, *Arabidopsis* and tobacco seeds were grown on MS medium containing no caffeine (control), 1 mM and 5 mM caffeine continuously for 17-d and photographed (Fig. 1*A–C* for *Arabidopsis*

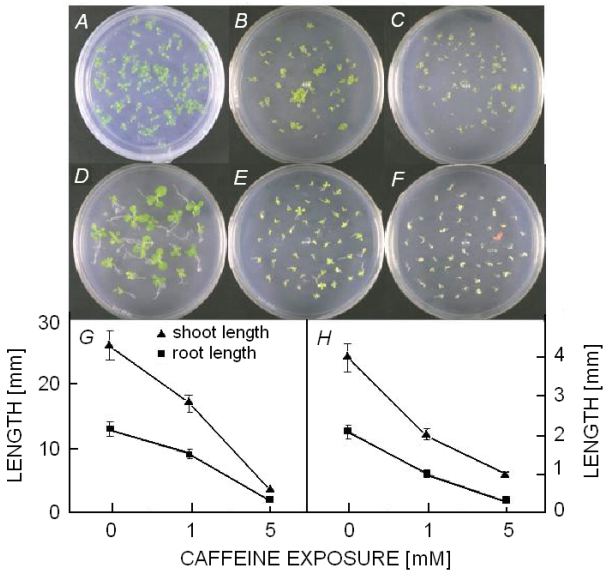

Fig. 1. Effect of caffeine on seedling growth. Phenotype of 17-d old *Arabidopsis* (*A–C*) and tobacco (*D–F*) seedlings grown on MS media plates containing no caffeine (*A*,*D*), 1 mM (*B*,*E*), and 5 mM caffeine (*C*,*F*). After 17 d of caffeine treatment shoot and root length of tobacco (*G*) and *Arabidopsis* (*H*) was measured. Values are the mean length ± SD (*n*=9).

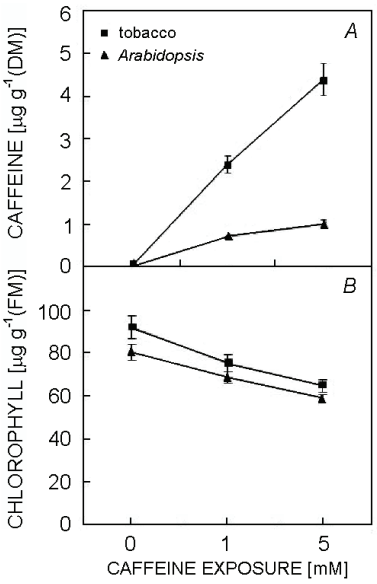

Fig. 2. Caffeine (*A*) and total chlorophyll (Chl) content (*B*) in tobacco and *Arabidopsis* seedlings. Caffeine content was measured in 17-d old seedlings grown on MS plates containing 0 (control), 1 and 5 mM caffeine. For Chl estimation, 17-d old seedlings grown on MS plates were treated with 0 (control), 1, and 5 mM caffeine for 96 h. Values are the mean of three independent experiments ± SD.

and Fig. 1*D–F* for tobacco). Seeds of both the plants grew well on MS plates containing no caffeine, while the growth was inhibited in presence of 1 mM caffeine and decreased further upon increasing the caffeine exposure concentration to 5 mM. Seedlings were smaller and pale yellowish in color growing in 1 mM caffeine containing MS medium plates. Size of seedlings decreased and yellowness increased further upon increasing the concentration of caffeine exposure to 5 mM. The difference in the growth pattern of *Arabidopsis* and tobacco was clearly visible upon caffeine treatment. Caffeine effect on seedling length was also monitored (Fig. 1*G*,*H*). Decrease in shoot and root length was observed upon caffeine treatment in both plants. The inhibition in shoot elongation by half and root elongation by 90 % after 6-d of 2.5 mM caffeine treatment has been documented in a previous study (Smyth 1992). Although caffeine treatment inhibited growth of roots to a greater extent than shoots, caffeine accumulation was similar in both organs. Apparently, shoots have a more effective mechanism than roots for maintaining growth in the presence of caffeine (Smyth 1992). Morphological alterations upon caffeine exposure such as reduction in plant height, yellowing of leaf, diminished branching and reduction in rooting were some of the common features observed in this study as well as reported in previous study (Smyth 1992). Similar morphological changes in plants have also been observed upon exposure to various other compounds. Heavy metals and salt stress exposure of plants resulted in a similar kind of symptoms (Yadav *et al.* 2005, Singla-Pareek *et al.* 2006). Results of the present study document that caffeine exposure leads to retardation in seedling growth.

Caffeine content: To check whether plants absorb caffeine during the exposure, caffeine content was estimated in *Arabidopsis* and tobacco seedlings grown on MS medium containing no caffeine (control), 1 mM and 5 mM caffeine for 17 d. *Arabidopsis* seedlings exposed to 1 mM caffeine were found to contain 0.7 µg caffeine g^{-1}(DM), while seedlings exposed to 5 mM caffeine contained 1 µg caffeine g^{-1}(DM). Caffeine was not detected in control *Arabidopsis* seedlings grown on caffeine minus MS plates (Fig. 2*A*). Similarly, tobacco seedlings exposed to 1 mM caffeine were found to contain 2.4 µg caffeine g^{-1}(DM) and seedlings exposed to 5 mM caffeine were contained 4.4 µg caffeine g^{-1}(DM). Caffeine was not detected in control tobacco seedlings grown on caffeine minus MS plates (Fig. 2*A*). These results document that both *Arabidopsis* and tobacco seedlings absorb caffeine to an appreciable level causing the phenotypic differences in their growth pattern as shown in Fig. 1*A–F*.

Caffeine exposure decreased Chl content: The ability of plants to maintain Chl under any kind of environmental stresses is taken as an index for determining the stress induced effect or injury (Singla-Pareek *et al.* 2006). In view of this, influence of caffeine was monitored on total Chl content of *Arabidopsis* and tobacco seedlings (Fig. 2*B*). *Arabidopsis* and tobacco exhibited 14.3 and

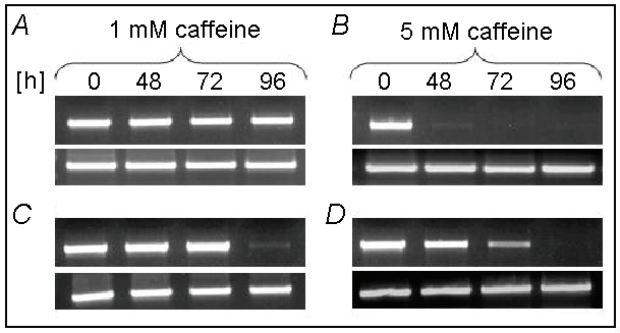

Fig. 3. Reverse transcriptase-PCR analysis of Rubisco gene expression in *Arabidopsis* (*A*,*B*) and tobacco (*C*,*D*). Total RNA was isolated from 1 and 5 mM caffeine treated 17-d old seedlings after 0 (,control), 48, 72 and 96 h. cDNA was synthesized and used in polymerase chain reaction (PCR) with Rubisco gene specific primers (*upper panels*). The α-tubulin gene specific primers were used as the internal control in PCR (*lower panels*).

18.6 % decrease in their Chl content upon 1 mM caffeine exposure compared to their respective controls (Fig. 2*B*). Chl levels decreased further to 26.2 and 29.6 %, respectively in *Arabidopsis* and tobacco, upon increasing the caffeine exposure concentration to 5 mM (Fig. 2*B*). Loss in chlorophyll content upon increasing the caffeine treatment concentration suggests that caffeine promoted early senescence in plants. It has earlier been reported that the amount of Chl decreased substantially during senescence of rice (Inada *et al.* 1999) and wheat (Ono *et al.* 1995) leaves. During senescence, one of the most obvious enzymatic events of leaves has been identified as proteolysis (Yoshida 2003, Donnison *et al.* 2007, Imai *et al.* 2008).

Caffeine exposure down-regulated Rubisco expression and activity levels: Since caffeine induces early senescence in plants and Rubisco is one of the major proteins (Makino *et al.* 2003), Rubisco gene (encoding larger subunit) expression as well as activity was monitored in response to caffeine exposure in *Arabidopsis* and tobacco seedlings. There was no change in Rubisco transcript expression of *Arabidopsis* upon 1 mM caffeine exposure (Fig. 3*A*). However, a significant down-regulation in the expression of Rubisco was observed upon 5 mM caffeine treatment for 48 h and expression was almost completely inhibited after 96 h (Fig. 3*B*). A similar decrease in transcript level of Rubisco was observed in tobacco upon caffeine exposure. Interestingly, tobacco seedlings showed a significant decrease in expression even during 1 mM caffeine exposure. However, this decrease was observed only after 96 h of caffeine exposure (Fig. 3*C*). Exposure of tobacco to 5 mM caffeine exhibited gradual decrease in Rubisco transcript expression levels (Fig. 3*D*). These results document that the expression of Rubisco was down-regulated upon caffeine exposure in plants.

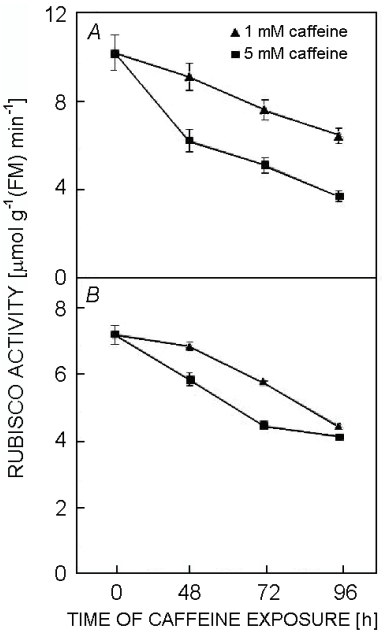

Fig. 4. Decrease in Rubisco activity of *Arabidopsis* (*A*) and tobacco (*B*) upon caffeine exposure. 17-d old seedlings treated with 1 and 5 mM caffeine for 0 (control), 48, 72 and 96 h were used for the activity measurement. Values are the mean of three independent experiments ± SD.

Rubisco activity was also decreased upon caffeine treatment. The decrease in Rubisco activity was higher with the increase in time and concentration of caffeine exposure. In *Arabidopsis*, Rubisco activity was decreased by 32 and 62 % after 96 h of 1 mM and 5 mM caffeine treatment, respectively (Fig. 4*A*). In tobacco, Rubisco activity was decreased by 5 and 19 % after 48 h of 1 and 5 mM caffeine treatment, respectively. After 96 h of 1 and 5 mM caffeine treatment, the decrease in Rubisco activity of tobacco was 38 and 43 %, respectively (Fig. 4*B*). These results indicate that decrease in Rubisco activity upon caffeine exposure could be responsible for retardation in seedling growth.

Photosynthesis-related genes showed higher expression levels in young, expanding leaves in contrast to senescent leaves (Mullet 1993), and hence promote photosynthesis (Nakano *et al.* 1997). When a leaf is senescent, photosynthesis-related proteins are actively degraded and are hardly synthesized again (Yoshida 2003, Krupinska and Humbeck 2004). Thus, a senescent leaf appears not to have the ability to synthesize Rubisco protein. It is still unknown whether caffeine induces senescence that leads to down-regulation of Rubisco or if it is the caffeine down-regulated Rubisco that leads to senescence. In conclusion, for the first time this study evidenced that caffeine had inhibitory effect on seedling growth and this could be due to its senescent and down-regulatory effect on Rubisco.

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