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Effect of Methylxanthine Treatment on Rice Seedling Growth

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Abstract. Methylxanthine treatment of rice seeds (*Oryza sativa* L. cv. Lemont) was used to determine the relative efficiencies of caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine) as growth regulators in a plant not producing these compounds. Caffeine inhibited growth more effectively than the dimethylxanthines. Treatment with 2.5 mM caffeine inhibited shoot elongation by half after 6 days of growth, and inhibited root elongation by 90% compared to control plants germinated in water. Although caffeine treatment inhibited growth of roots more 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.

Caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine) are synthesized by a group of plants including coffee (*Coffea arabica*), cacao (*Theobroma cacao*), and tea (*Camellia sinensis*). The methylxanthine content in young leaves and seeds of producing plants may be 2% or more of the organ dry weight (Baumann and Frischknecht 1988, Frischknecht et al. 1986, Suzuki and Waller 1985, Timbie et al. 1978). The pathway for synthesis of methylxanthines probably starts with purine bases in nucleic acids or nucleotides and proceeds from 7-methylxanthine to theobromine to caffeine (Suzuki and Waller 1984a). Although caffeine is the predominant methylxanthine accumulated in many plants (*C. arabica, Coffea robusta,* and *C. sinensis*), some plants, such as *T. cacao,* accumulate fivefold more theobromine than caffeine in the seed (Timbie et al. 1978).

Caffeine appears to function as an allelopathic compound, or part of the chemical defense of the producing plant. Caffeine inhibits insect phosphodiesterase activity (Nathanson 1984). The resulting imbalance in cAMP is thought to disrupt insect metabolism and discourage sensitive insects from feeding on caffeine-accumulating plants (Nathanson 1984). Another allelopathic role for caffeine may be to inhibit the growth of competing plants once caffeine leaches into the soil with plant debris (Waller et al. 1986). The growth of both caffeine-producing plants and nonproducers can be inhibited by millimolar amounts of caffeine in vitro (Rizvi et al. 1981, Waller et al. 1986). The concentration of caffeine in the soil around caffeine-producing plants is not known due to difficulties in extracting caffeine from the soil matrix (Waller et al. 1986). Furthermore, the mechanism for caffeine inhibition of plant growth has not been established.

Here the early development of rice seedlings was used as a model system to compare the effectiveness of caffeine, theobromine, and theophylline as growth regulators in plants which are not normally exposed to methylxanthines. Both caffeine and theobromine could be candidates for allelopathic compounds in the soil because these compounds are produced in substantial amounts by certain plants.

Materials and Methods

Growth of Rice Seedlings

Rice grain (*Oryza sativa* L. cv. Lemont) was harvested in Beaumont, Texas. Prior to use, rough grain was soaked in 95% (vol/vol) ethanol for 5 min, and then dehulled. The caryopses were surface-sterilized by soaking in 1% (wt/wt) NaOCl for 5 min, and then thoroughly rinsed in tap H_2O.

Rice grain was grown at 18–26°C in darkness, except for nominal amounts of light used during watering and growth measurements. Ten to 20 seeds were placed on 9-cm filter paper discs (Whatman no. 1, Van Waters & Rogers Inc., Seattle, WA, USA) in polystrene Petri dishes (100 mm diameter × 20 mm height). Seed germination was started by adding 2.5 ml of deionized H_2O (Millipore Milli-Q Water Purification System, Bedford, MA,

USA), or the appropriate methylxanthine treatment. Methylxanthines were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Additional H_2O was added to the Petri dishes at days 2 and 4. In some experiments, the appropriate methylxanthine treatment was added at later times as indicated. The length of the shoot or primary root was measured with a ruler. Fresh weights were measured using a Mettler model AE240 balance. All measurements were taken from plants without obvious contamination.

Tissue Extraction and Caffeine Analysis

Shoots and roots were washed twice in 25 ml of deionized H_2O, blotted on paper towels, cut into segments where appropriate, weighed, and then stored at −20°C in 1.5-ml microcentrifuge tubes. Tissue samples were frozen in liquid N_2 and then crushed during the course of the hot H_2O extraction with either a glass Pasteur pipet, or a Teflon pestle. The tissue samples were extracted twice at 100°C for 20 min with 1-ml volumes of deionized H_2O, and a third time with 1 ml of H_2O at 25°C. The H_2O extracts were pooled. If necessary, extracts were further diluted with deionized H_2O so that the final caffeine concentration was 5 μM or less.

Tissue extracts were filtered (Millipore Millex-HV, 0.45 μm) and a 20 μl aliquot injected onto a Hewlett-Packard ODS-Hypersil column (Palo Alto, CA, USA; 2.1 × 100 mm, 5 μm particle size). The injection volume accounted for extract from 50–100 μg fresh weight of tissue. The mobile phase was 15% (vt/vt) methanol in H_2O. The flow rate was maintained at 0.3 ml min^{-1} by a Waters model 510 pump (Milford, MA, USA). Methylxanthines were detected by their absorbance at 280 nm. Absorbance peak height was proportional to the amount of caffeine standard injected in the range from 0–100 pmol; and this measurement was used to calculate the caffeine content of tissue extracts. The retention time for caffeine with this chromatography system was 8.9 min, whereas the dimethylxanthines, theophylline, 1,7-dimethylxanthine, and theobromine eluted at 4.65, 4.41, and 3.04 min, respectively.

The efficiency of caffeine recovery was tested by adding 10 μl of 1 mM caffeine to roots and shoots just prior to freezing the tissue samples for extraction. Recovery of the added caffeine was 104 and 108%, respectively, for root and shoot extracts.

Rice Grain Respiration

Grain respiration was measured 1 day after the start of imbibition with H_2O control or methylxanthine treatments using an O_2 electrode (Rank Brothers, Bottisham, Cambridge, UK) as described previously (Smyth et al. 1986). Ten rice seeds were preincubated in 2 ml of deionized H_2O in the electrode chamber for 3 min. The respiratory rate was measured for the next 10 min at 25°C.

Results

The growth of rice seedlings was measured for 6 days after the start of seed imbibition (Fig. 1). The embryos were swollen after 1 day of imbibition, but shoot and root length were not measurable until day 2.

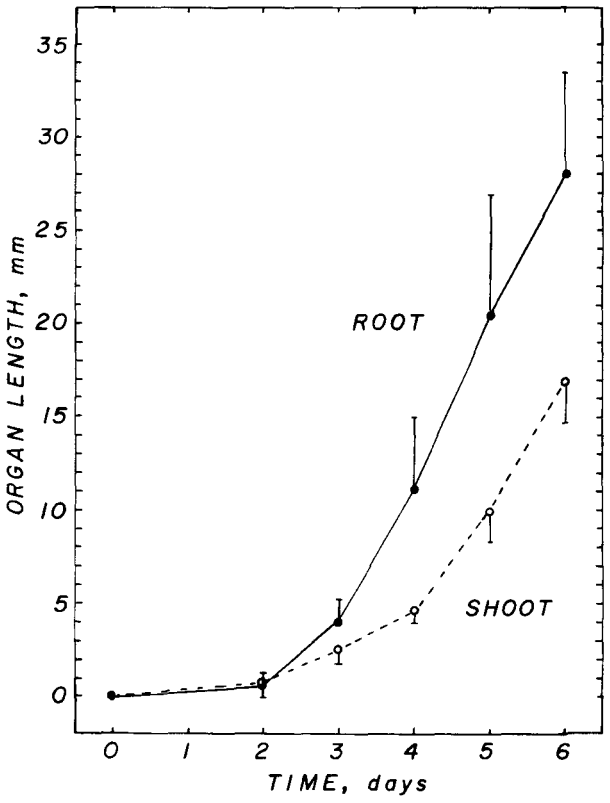

Fig. 1. Elongation of rice shoots and roots during the first 6 days after the start of germination. Each data point represents the mean for 27–60 rice plants. Bars represent the SD.

Caffeine treatment was inhibitory over the concentration range of 0.5–10 mM (Fig. 2) during the first 5 days of growth, relative to control plants which were only watered. Root elongation was more sensitive to added caffeine than shoot elongation. Shoot elongation was inhibited by 50% with 2.5 mM caffeine (Figs. 2 and 3), whereas root elongation was reduced by 80% with one treatment at the same concentration (Fig. 2), or by 90% with several treatments (Fig. 3). The relationship between caffeine concentration and shoot and root fresh weights was similar to that shown for organ length (data not shown).

The relative effects of caffeine, theobromine, and theophylline on rice seedling growth were tested at 2.5 mM. This concentration approaches the H_2O solubility of theobromine. After 5 or 6 days of growth, root length was reduced by 33, 67, and 91%, respectively, for theobromine, theophylline, and caffeine treatments relative to H_2O controls (Fig. 3). Caffeine treatment at this concentration inhibited shoot elongation by half, whereas the dimethylxanthines were less inhibitory.

Grain respiration was measured after 22–25 h of

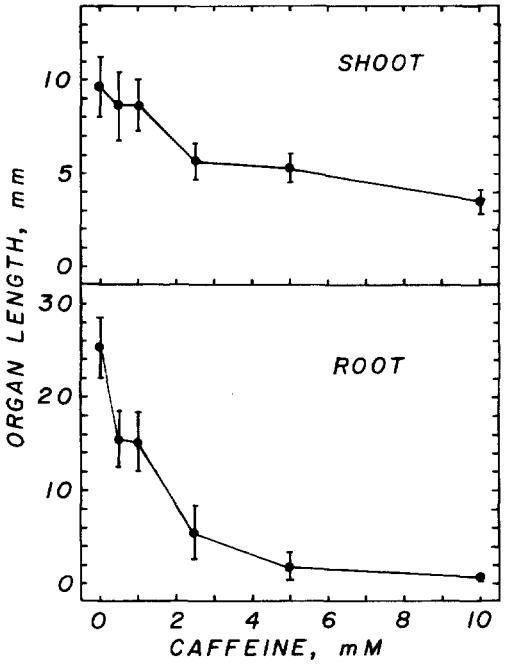

Fig. 2. Concentration curve for the effect of caffeine treatment on rice shoot and root length after 5 days of growth. The data points represent the mean lengths for 14–21 seedlings ± SD.

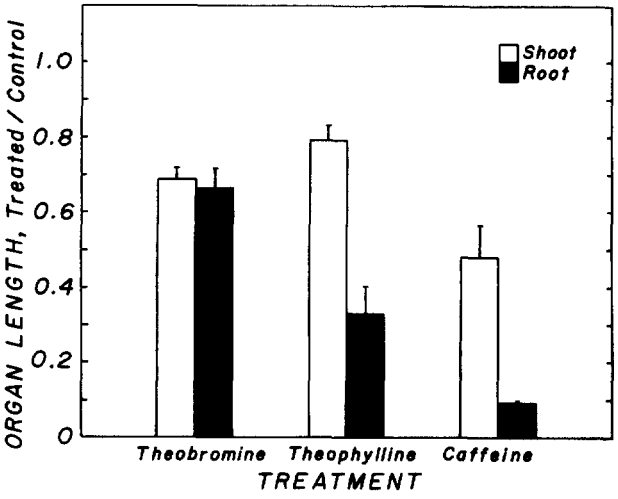

Fig. 3. Comparison of methylxanthine treatments on rice shoot and root length after 5 or 6 days of growth. Data are the mean of three experiments ± SD. From 12–28 seedlings were used in each experiment, with 5-day-old plants used once and 6-day-old plants used twice. Treatments were as follow: 2.5 ml of H_2O (control), 2.5 mM theobromine, 2.5 mM theophylline, or 2.5 mM caffeine at time 0; 2 ml of each respective treatment on day 2; and, in one experiment, 2 ml of each treatment on day 4.

imbibition to test for possible early treatment effects on metabolism. Respiration of H_2O-imbibed grain was 63.4 nmol O_2 h^{-1} $seed^{-1}$, or 2.3 μmol O_2 h^{-1} (g fresh weight)$^{-1}$ based on 275 mg fresh weight per 10 seeds. Other seeds were treated with 2.5 mM caffeine, theobromine, or theophylline at the start of imbibition. Respiratory rates after 1 day of germination for theophylline, theobromine, and caffeine treatments were 67.5, 69.7, and 65.9 nmol O_2 h^{-1} $seed^{-1}$, respectively. There were no statistically significant differences between respiratory rates of control and methylxanthine-treated seeds after two experiments.

Rice seedlings accumulated considerable amounts of caffeine after caffeine treatment at the start of imbibition (Fig. 4). Tissue caffeine concentration increased with increasing the concentration of caffeine in the medium. The internal caffeine concentration was calculated (using 1 g fresh weight = 1 ml solution) to exceed 1 mM for both the shoot and roots of plants treated with 2.5 mM caffeine (Fig. 4). The apparent difference in caffeine content between shoots and roots after 2.5 mM caffeine treatment (Fig. 4) was not statistically significant when analyzed by the paired *t*-test.

Shoot and root extracts from caffeine-treated plants contained caffeine, but no detectable dimethylxanthine breakdown products, such as theophylline, 1,7-dimethylxanthine, or theobromine (data not shown). Other potential breakdown products, such as monomethylxanthines, eluted from the column at the same approximate time as other extract components, and, consequently, could not be measured using this chromatographic system.

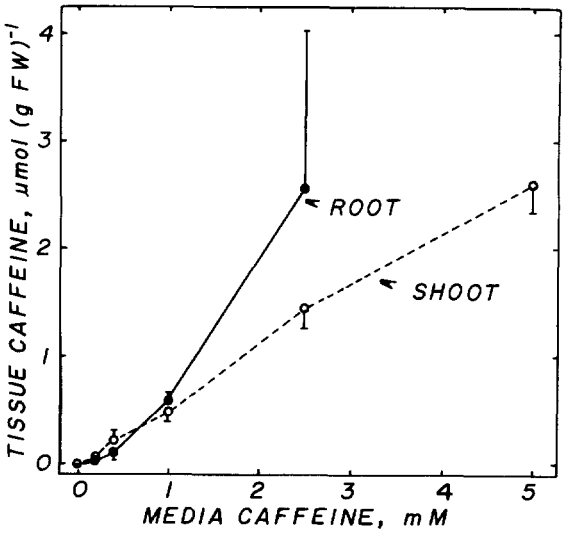

Fig. 4. Effect of media caffeine concentration on rice shoot and root caffeine content after 6 days of growth. The shoots or roots of four to six seedlings were pooled and extracted with hot H_2O. The data points represent the mean of three experiments ± SD (bars represent SD) where it is large enough to be seen.

Discussion

Rice seedling growth is inhibited by methylxanthine treatment. The inhibitory effect observed with 2–10 mM caffeine is similar to that observed for caffeine-metabolizing *C. arabica* seedlings (Waller et al. 1986). Theophylline was a less effective inhibitor of *C. arabica* seedling growth than caffeine (Waller et al. 1986); likewise, the dimethylxanthines, theophylline and theobromine, inhibited rice seedling growth much less than caffeine (Fig. 3). It may be a general principle that trimethylxanthine is a more potent growth inhibitor than dimethylxanthines. For example, the bacterium *Listeria monocytogenes* grew slower with added caffeine, but was little affected by theobromine (Pearson and Marth 1990). Additionally, insects such as *Manduca sexta* are likewise more strongly inhibited by caffeine than theophylline (Nathanson 1984).

Complexation of caffeine with chlorogenic acid may be one mechanism for reducing the toxic effects of methylxanthine accumulation in *C. arabica* cells (Baumann and Röhrig 1989). The similarities in *C. arabica* (Waller et al. 1986) and rice growth inhibition to a given caffeine treatment suggest that the presence or absence of caffeine synthesis in the plant tissue is not an important factor in the response to high external caffeine concentration. It is unknown whether rice tissues synthesize additional complexing agents to counter an influx of inhibitory compounds like caffeine. Rice shoots do appear to be more effective than roots at countering caffeine influx because shoots continue to grow at internal caffeine concentrations which severely inhibit root elongation (Figs. 2 and 4).

The leaves (Kalberer 1965) and beans (Suzuki and Waller 1984a) of *C. arabica* slowly metabolize radioisotope-labeled caffeine to methylxanthines, allantoin, allantoic acid, urea, and carbon dioxide. Theophylline and theobromine normally accumulate during caffeine catabolism in methylxanthine-producing plants. In this study, there was no evidence for dimethylxanthine accumulation in caffeine-treated rice seedlings. Nonmethylxanthine-accumulating plants may not be able to metabolize significant amounts of exogenously supplied caffeine. The metabolites produced in *C. arabica* fed caffeine (Kalberer 1965, Suzuki and Waller 1984a, b) are consistent with N-demethylation by a mixed-function oxidase and oxidation by a xanthine oxidase. The plant oxidases involved in caffeine catabolism remain uncharacterized so it is not known whether there are enzymatic differences between caffeine-producing plants and nonproducers.

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