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Cytokinin-like effects of caffeine in bioassays

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Abstract

Cytokinin-like effects of pure caffeine were tested in bioassays specific for this hormonal activity [radish cotyledon growth and chlorophyll (Chl) biosynthesis in cucumber cotyledon and tobacco cell suspension] and in cell elongation bioassays [elongation of segments from soybean internode and internode elongation in dwarf cultivars of guandu (*Cajanus cajan*) and mucuna (*Mucuna deeringiana*)]. 6-Benzylaminopurine and kinetin (KIN) were used for comparison with caffeine. Although weaker than those given by cytokinins, positive responses were observed in all specific bioassays and in elongation of soybean internodes. A remarkable synergistic effect between caffeine and KIN was observed for the synthesis of Chl in the tobacco cell suspension bioassay, in which different concentrations of the alkaloid were combined with a single concentration of KIN. The hormone-like effect of caffeine might be related to the resemblance between caffeine and adenine derivatives.

Additional key words: 6-benzylaminopurine, *Cajanus cajan*, chlorophyll biosynthesis, *Cucumis sativus*, *Glycine max*, kinetin, *Mucuna deeringiana*, *Nicotiana tabacum*, *Raphanus sativus*.

Introduction

As an intact purine ring is essential for cytokinin activity in plants (Matsubara 1990), most of the investigations on cytokinin activity have been done with N^6-substitutes adenines. Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid, and therefore resembles cytokinin structure. This could explain the induction of stomatal opening by caffeine in detached epidermis of *Vicia faba* (Curvetto and Delmastro 1990, Morsucci *et al.* 1991), where it might elicit the stimulation of adenylate cyclase present in the nuclear membrane and chloroplasts of guard cells. In addition, there was an enhancement of stomatal aperture when caffeine was combined with adenosine, KIN and KIN riboside. This indicates that cyclic AMP is involved in the hormonal action.

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Abbreviations: BAP - 6-benzylaminopurine; Chl - chlorophyll; KIN - kinetin.
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The synergistic effect with caffeine, a phosphodiesterase inhibitor, is a good evidence that the hormones stimulate adenylate cyclase. Indeed, the synergistic effect between cyclic AMP and caffeine had been previously observed in the promotion of tuber tissue growth of Jerusalem artichoke (Kamisaka *et al.* 1973) and germination of seeds of *Lactuca sativa* (Hall and Galsky 1973) and *Phacelia tanacetifolia* (Safran and Galsky 1974).

Tao *et al.* (1991) presented evidence that caffeine could inhibit cytokinin N-glucosylation (deactivation) in radish cotyledons, increasing the pool of the active hormone. Adenine, which is structurally related to caffeine, exogenously supplied to coffee suspension cells was readily glucosylated (Baumann *et al.* 1994). The authors suggested that the benefitial effect of adenine addition in various shoot multiplication media might be due to reduced deactivation of cytokinins. However, both works did not show typical biological response of cytokinins, such as increase of Chl biosynthesis and growth.

Here, we present results of cytokinin-like effect of caffeine when applied in bioassays that have traditionally been used to test cytokinin activity, such as radish cotyledon growth (Letham 1971) and Chl biosynthesis in cucumber cotyledons (Fletcher and McCullag 1971). In addition, we tested the elongation of etiolated soybean internode segments (Ross 1974), Chl synthesis in cell suspensions of tobacco, and internode elongation in dwarf cultivars of guandu (*Cajanus cajan*) and mucuna (*Mucuna deeringiana*).

Materials and methods

The bioassays involving radish cotyledon growth, elongation of etiolated soybean internode segments, Chl synthesis of cucumber cotyledons and internode elongation of dwarf mucuna and guandu were carried out as described by Ross (1974). In the radish (*Raphanus sativus* L.) cotyledon bioassay, seeds were germinated in the dark and detached internal cotyledons were transferred to Petri dishes (diameter 5 cm) with filter paper wet with 1 cm^3 of caffeine or BAP solutions. Concentrations ranged from 0.051 to 51 μM and 0.044 to 44 μM, respectively, with 10 μM intervals. After 3 d at room temperature (about 25 °C) and under continuous irradiation (180 μmol m^{-2} s^{-1}) fresh mass of the cotyledons was determined. As control, cotyledons were incubated with water. Each concentration had three replicates, with ten cotyledons per replicate.

In the bioassay of elongation of etiolated soybean [*Glycine max* (L.) Merr.] internode segments, seeds were germinated in vermiculite in the dark for 15 d. Segments (11 mm) of the apical internode were transferred to Petri dishes with 4 cm^3 of 10 mM potassium phosphate buffer, pH 6.5, containing 8.8 mM sucrose. Caffeine and BAP concentrations ranged from 10^{-7} to 10^{-4} M, with 10^{-1} M intervals. The Petri dishes were maintained in the dark for 48 h at room temperature (about 25 °C) when the length of the segments were determined. As control, segments were incubated with water. Each concentration had three replicates, with ten segments per replicate.

In the bioassay of Chl synthesis in cucumber (*Cucumis sativus* L.) cotyledons, seeds were germinated in the dark, and under green radiation the cotyledons were transferred

to Petri dishes containing 3 cm^3 of caffeine (0.051 to 51 μM) or BAP (0.044 to 44 μM) solutions, with 10 μM intervals. After 16 h at room temperature (about 25 °C) and under continuous irradiation (300 μmol m^{-2} s^{-1}) the cotyledons were extracted with ethanol (96 %) in a *Politron* homogenizer, centrifuged, and Chl determined in the supernatant by measuring the absorbances at 649 and 665 nm (Arnon 1949) with a spectrophotometer (*Model B390, Micronal,* Brazil). As control, cotyledons were incubated with water. Each concentration had three replicates, with eight cotyledons per replicate.

In the bioassay of internode elongation of dwarf plants, mucuna (*Mucuna deeringiana* Merrill) and guandu [*Cajanus cajan* (L.) Huth] were used instead of soybean, as recomended by Ross (1974). The seeds of dwarf mucuna and guandu were kindly supplied by the Legume Division of the Agronomy Institute, Campinas, São Paulo, Brazil. Seeds were germinated in vermiculite in greenhouse and after 20 d from the sowing, when the plants had one expanded leaf, the first apical internodes (already formed) were measured and the plants sprayed with 10^{-6} and 10^{-4} M solutions of caffeine or BAP. Controls plants were sprayed with water. Eight replicates were used for each treatment. Seven days later the previous first internodes and the new internodes formed were measured.

The assay of Chl synthesis in tobacco (*Nicotiana tabaccum* L.) cell suspension was an adaptation of that described by Skoog *et al.* (1967) for tobacco pith callus. Friable cali were obtained in solid nutrient medium (Kao *et al.* 1974) from tobacco leaves. Weighed portions were transferred to liquid medium with the same composition and maintained in a rotatory shaker (2 rps) under continuous irradiation (300 μmol m^{-2} s^{-1}). Caffeine was combined with KIN (0.93 μM or absent) in the liquid medium at concentrations of 0, 0.77, 1.54 and 3.08 μM. Naphthaleneacetic acid was present in the medium at 2.15 μM. After 30 d the cells were recovered by filtration and weighed. Part of the material was taken for determination of Chl after extraction with 96 % ethanol in homogenizer (Arnon 1949). Each treatment was composed of five replicates.

Values were analyzed statistically by analysis of variance and differences between means by the Duncan test (5 %).

Results

Cytokinin-like effects of caffeine were observed in the bioassays dependent on Chl synthesis in tobacco cell suspensions and cucumber cotyledons, radish cotyledon growth and elongation of etiolated soybean internode segments.

Chl biosynthesis was enhanced in cucumber cotyledons with increasing concentrations of BAP in the solutions (Fig. 1*A*), ranging from 0.044 to 44 μM. Caffeine, ranging from 0.051 to 51 μM, also caused an increase of Chl content in the cotyledons. The highest concentration of caffeine was equivalent in response to the lowest BAP concentration.

The highest increase in radish cotyledon growth with caffeine was observed at 0.51 μM, being equivalent to 0.44 μM BAP (Fig. 1*B*). However, statistically this caffeine concentration did not differ from 5.1 and 51 μM.

In the tobacco cell suspension bioassay, caffeine also induced Chl biosynthesis (Fig. 2*A*) and a remarkable synergistic effect was observed when caffeine was added at 0.77 and 1.54 μM together with KIN (0.93 μM) in the culture media. At 3.08 μM caffeine, Chl content was reduced, probably due to a toxic effect as demonstrated by the ratio between final and initial fresh mass (Fig. 2*B*). Caffeine at 1.54 μM also caused a reduction in growth, but the Chl synthesis was not affected.

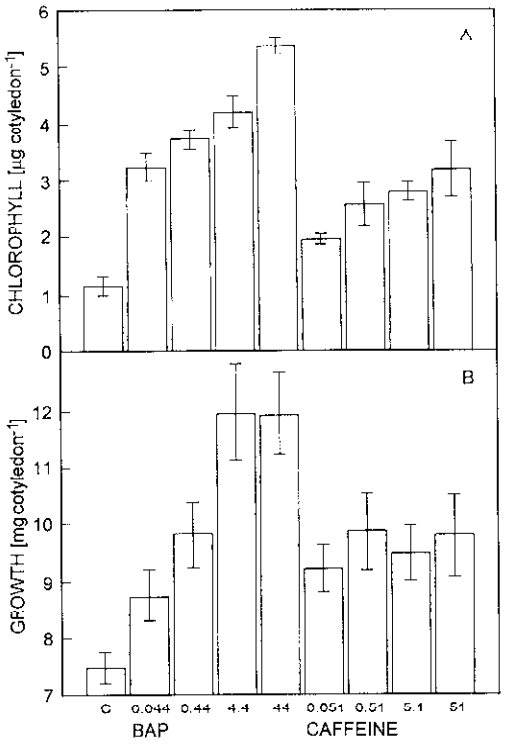

Fig. 1. Effect of different concentrations [μM] of BAP and caffeine on chlorophyll accumulation in cucumber cotyledons (*A*) and on radish cotyledon growth (*B*) in comparison with control (*C*). Bars represent S.E.

The elongation of internode segments from etiolated soybean also increased in the presence of caffeine (Fig. 3). A response to caffeine was observed at concentrations between 10^{-4} and 10^{-6} M, producing values similar to those obtained with 10^{-6} and 10^{-7} M BAP.

No differences were observed between dwarf mucuna and guandu seedlings sprayed with BA or caffeine and control plants sprayed with water (values not shown).

Discussion

Besides their chemical definition as adenine derivatives, cytokinins are physiologically defined as those that induce cell division in the presence of optimal auxin concentrations (Horgan 1985). Among the common bioassays used for determining cytokinin activity are radish cotyledon growth, Chl synthesis in tobacco cells and cucumber cotyledons (Matsubara 1990). Cell elongation of sections from young wheat cotyledons (Wright 1966) and dwarf cultivars (Loy 1980) have been also used, however, in these cases care should be always taken when an unknown substance is being characterized for cytokinin activity (Horgan 1985).

We used both types of bioassays: in those specific for cytokinin activity, caffeine induced similar responses, however, always with less efficiency than BAP or KIN. Activity was observed for elongation of etiolated soybean internode segments but not for dwarf cultivars of mucuna and guandu, both non-specific bioassays.

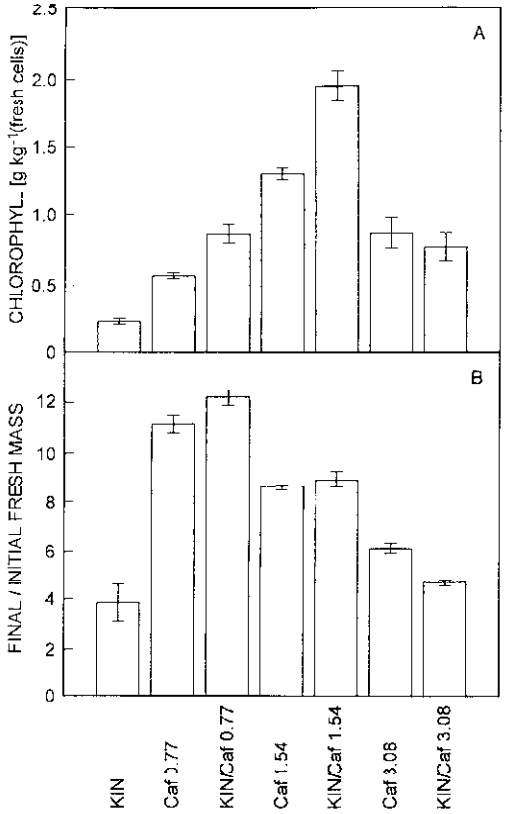

Fig. 2. Effect of different concentrations of caffeine [μM] on chlorophyll biosynthesis (*A*) and growth (*B*) of tobacco cell suspension. Kinetin (KIN) was added at 0.93 μM in the medium.

Of special interest was the synergism between KIN and caffeine in the growth and biosynthesis of Chl in tobacco cell suspension. This might be explained by the effect of caffeine in the cyclic AMP pool (Curvetto and Delmastro 1990, Morsucci *et al.* 1991)

or cytokinin N-glucosylation (Tao *et al.* 1991). In addition, cytokinin may stimulate Chl biosynthesis by affecting the amount of mRNAs related to Chl *a/b* binding protein and the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Cotton *et al.* 1990).

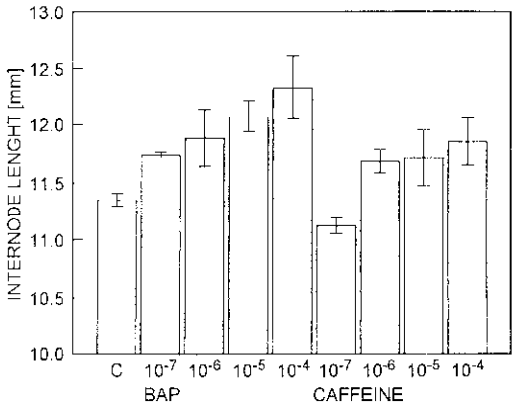

Fig. 3. Effect of different concentrations [M] of BAP and caffeine on etiolated soybean internode segments elongation.

Cytokinesis is the typical response for cytokinin. This effect is the basis of the bioassay with tobacco cell suspension. According to Houssa *et al.* (1990), cytokinins cause a significant decrease of the S phase of the cell cycle, promoting cell division. However, the increase in biomass in the radish cotyledon bioassay is almost entirely dependent of cell expansion, caused by cell-wall loosening accompanied by water uptake (Rayle *et al.* 1982, Ross and Rayle 1982). The growth in length of segments and intact stems due to cytokinin might also be a consequence of cell enlargement since very few divisions occur.

Despite the well known physiological effects of cytokinins in plants, very little knowledge of caffeine action has accumulated in the last few years. Therefore, the positive response for caffeine in cytokinin-specific bioassays cannot be explained except in terms of their similar structure. Variability for caffeine content has been found in coffee (Mazzafera and Carvalho 1992, Mazzafera *et al.* 1997) and Chinese tea (Suzuki *et al.* 1992) and it would be of interest to investigate whether caffeine has some hormonal effect in such plants. However, for this purpose, it would be necessary to first develop a bioassay responsive to cytokinin with tissues of these plants.

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