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EXPERIMENTAL ARTICLES

Germination, Duplication Cycle and Septum Formation Are Altered by Caffeine, Caffeic Acid and Cinnamic Acid in *Aspergillus nidulans*

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Abstract—Phytogenous Phenolic and benzene compounds have been described as being responsible for many biological activities including antifungal effects. The effect of caffeine and cinnamic and caffeic acids on a model fungus, *Aspergillus nidulans*, was investigated at its initial stage of germination. Conidia did not germinate in the presence of cinnamic acid (1 mM). Caffeine and caffeic acid exerted a negative effect on germination, on the nuclear duplication cycle and on first septum formation. The effects of caffeine were dose-dependent; effects of caffeic acid (1 mM) were more intense than those of caffeine (10 mM).

Key words: *Aspergillus nidulans*, caffeic acid, caffeine, cinnamic acid.

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Many compounds present in whole grains have been shown to be useful for human health due to their antimicrobial and antioxidant effects; however their mechanisms of action are not clear [1]. Some are benzene and phenol derived compounds; among the latter, hydroxycinnamic acid derivatives are predominantly present in cereal bran [2, 3]. Nevertheless, the role of these compounds in phytopathogenicity evoked by microorganisms, including fungi, remains unknown.

Caffeic acid is the most abundant hydroxycinnamic acid derivative in foodstuffs like coffee, apples, pears and certain vegetables; antioxidant and antimutagenic activities of these products have been attributed to its action [4]. Previous studies in our laboratory have shown that the presence of caffeic acid (250 μg/ml) in a *Neurospora crassa* culture did not inhibit fungal growth [5], while cinnamic acid (200 μg/ml) delayed such growth; after 48 h of cultivation, however, cinnamic acid was converted into acetophenone [6]. Cinnamic acid, the deaminated derivative of phenylalanine, is an aromatic fatty acid present in plant tissues; it exhibits many pharmacological activities like delaying the growth of human tumors including melanoma, prostate adenocarcinoma, and lung cancer cells in vitro; inhibition of *Plasmodium falciparum* the agent of malaria, by this compound has also been reported [7–9].

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Another natural compound, caffeine, is an alkaloid that modulates cell signaling by increasing intracellular cAMP levels [10, 11]. There are many reports about caffeine degradation by bacteria, but studies of this alkaloid in relation to fungi are few [12].

In the present work, the effects of cinnamic acid, caffeic acid, and caffeine on germination, nuclear division, and septation in *Aspergillus nidulans* were studied.

MATERIALS AND METHODS

Strain and growth conditions. *Aspergillus nidulans* strain FGSC26 (biA1, veA1), maintained as previously described [13], was used throughout this study. In the time course of experiments, liquid minimal medium [14] (pH 6.0) was supplemented with 1% glucose and 0.02 μg/ml of biotin, inoculated with 10^6 conidia/ml, poured on a petri dishes containing sterile glass cover slides, and incubated at 30°C. Cinnamic acid, caffeic acid, and caffeine were dissolved in dimethylsulphoxide [DMSO 99.5% (vol/vol)] and introduced into the media to yield a final concentration of 1 mM (cinnamic or caffeic acids) or of 5 or 10 mM (caffeine). DMSO concentration in the final experimental and control media was 0.01%. After 6, 8, and 24 h of incubation, the cover slides with adhered conidia were removed and processed for microscopy. The results of at least three independent experiments are reported as means ± SD.

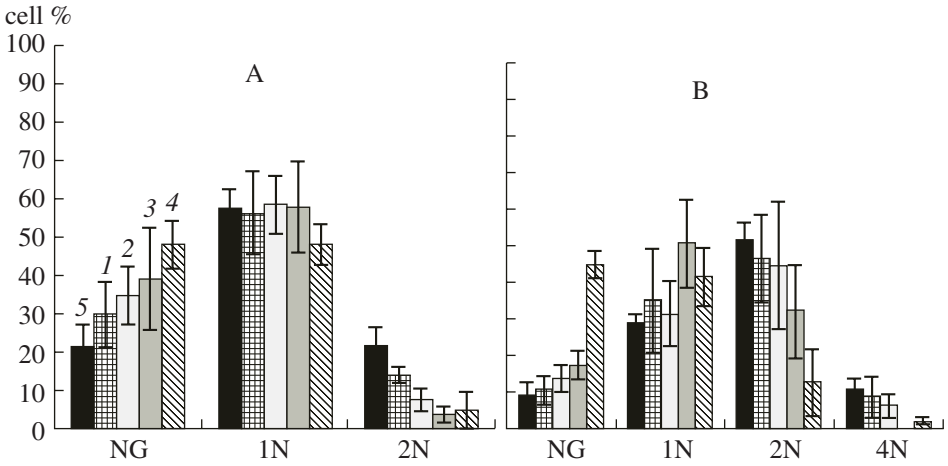

Fig. 1. Effect of caffeine and caffeic acid on nuclear division. Culture media were supplemented with 1, 5, or 10 mM of caffeine (*1, 2, 3*) or 1 mM of caffeic acid (*4*). Control cultures (*5*). Samples were collected after 6 (A) and 8 h (B) of cultivation and the cells were stained with DAPI. No germinated cells (NG); nucleus number (N).

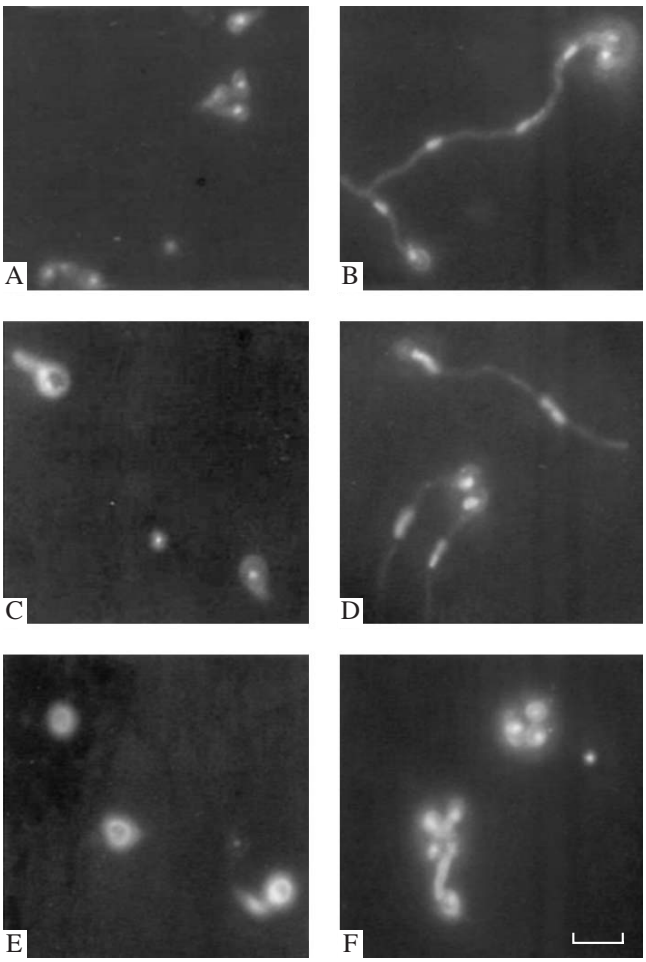

Fig. 2. Effect of caffeine and caffeic acid on nuclear division. Conidia were inoculated in media containing 1 mM caffeine (C, D) or caffeic acid (E, F) and incubated for 6 (A, C, E) and 8 hours (B, D, F). (A, B) were control cultures. The cells were stained with DAPI. Bar: 10.5 μm.

Microscopy and staining. Cover slides were removed from the media with tweezers; the adhered germlings were fixed and stained with DAPI or Calcofluor White to visualize nuclei or septae respectively, as previously described [13]. Photographs were taken with a TMAX 400 film under a fluorescence microscope (Zeiss Axioskop) using a Zeiss MC 80 microscope camera system and a ×100 oil objective with filters for DAPI (359–441 nm) or Calcofluor White (395–440 nm).

RESULTS AND DISCUSSION

Even after 24 h of incubation, no conidial germination was detected in the presence of cinnamic acid (1 mM). On the other hand, 70% of the conidia had germinated and 80% of them contained one nucleus after 6 h in the caffeine-containing medium (1 mM). The effect of the same concentration of caffeic acid after this incubation period was more pronounced than that of caffeine, since germination was detected in 52% of the conidia and 92% of them showed one nucleus. After the same incubation period, 80% of the conidia had germinated in the control cultures; of these germlings, 58% had still one nucleus, while in 22% two nuclei could be detected, indicating that first mitosis had already occurred. The delay in germination and first nuclear division depended also on the dose of caffeine (Fig. 1A). After 8 h, the results were similar to those observed after 6 h. In the presence of 1 mM caffeic acid they were more pronounced than in the presence of 10 mM caffeine; at this time, 45% of the conidia had not yet germinated in the cultures containing caffeic acid (1 mM), while in the control cultures only 9% of conidia had not germinated (Fig. 1B; Figs. 2B and 2F). After 8 hours, long germ tubes were detected in the cul-

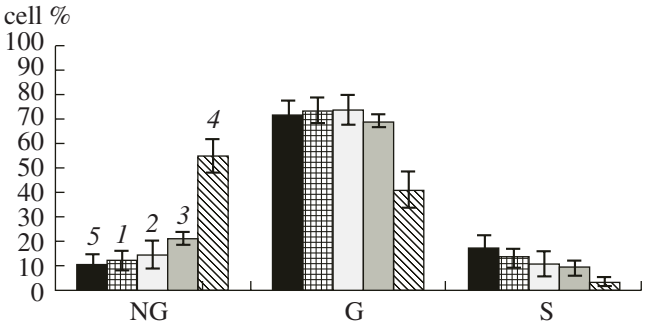

Fig. 3. Effect of caffeine and caffeic acid on the first septum formation. Culture media were supplemented with 1, 5, and 10 mM of caffeine (*1, 2, 3*) or caffeic acid 1 mM (*4*). Control cultures (*5*). Samples were collected after 8 hours of cultivation and the cell walls were stained with Calcofluor. No germinated cells (NG); germinated cells (G); septum (S).

tures containing 1 mM of caffeine but none were found in the presence of 1 mM caffeic acid (Figs. 2D and 2F).

A delay was also observed in septum formation in the presence of caffeic acid. Usually, after 8 h of incubation, the first septum was present in 15–19% of the germlings while in the presence of 1 mM caffeic acid it was detected only in 3.5% of the cultures (Fig. 3).

These results show that caffeine and especially caffeic acid exert a negative effect on germination, the nuclear duplication cycle, and the formation of the first septum in *A. nidulans*.

In contrast, caffeic acid (1 mM) stimulated the germination of *Plasmodiophoro brassicae* the phytopathogenic agent of clubroot disease in cruciferous plants [15]. Fungi respond to compounds present in the environment in different ways; for example, cinnamic acid was transformed by *N. crassa* after 24 hours [6], but, at the same concentration, prevented the germination of *A. nidulans*.

It is known that caffeine is an inhibitor of the phosphodiesterase activity on cAMP [16]; its presence increases the levels of cAMP, an activator of PKA [17]. PKA activated delayed nuclear division and septum formation in *A. nidulans* cultures, especially when glucose was the carbon source [13]. However, cAMP levels may also be increased when adenylyl cyclase, which synthesizes cAMP, is stimulated. This enzyme has been shown to be regulated by α-subunits of G proteins (G_{α}), which either stimulate ($G_{(s)\alpha}$) or inhibit ($G_{(i)\alpha}$) it in various organisms including the fungi *N. crassa* [18]; caffeic acid could be acting on subunits of G proteins that, through the adenylyl cyclase/cAMP/cAMP-dependent protein kinase (PKA) cascade, activate PKA.

The effect on fungi of phytogenous benzene or phenolic derivatives are probably efficient ways for plants to protect themselves from soil fungi.

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