Folia Microbiol. 39 (4), 269–275 (1994)

Growth and Morphogenesis of *Botrytis cinerea*. Effects of Exogenous Calcium Ions, Calcium Channel Blockers and Cyclosporin A

D. HUDECOVÁ, Ľ. VAREČKA, V. VOLLEK, and V. BETINA

Department of Microbiology, Biochemistry and Biology, Faculty of Chemical Technology, Slovak Technical University, 812 37 Bratislava, Slovakia

Received December 22, 1993
Revised version February 25, 1994

ABSTRACT. Calcium channel blockers, verapamil, nitrendipin and nifedipin, and cyclosporin A inhibited growth of colonies of *Botrytis cinerea* in a concentration-dependent manner and simultaneously induced morphological changes of its hyphal tips. Exogenous calcium at the concentration of 100 mmol/L decreased the growth-inhibitory effects of channel blockers and cyclosporin A; however, at the concentration of 500 mmol/L Ca^{2+} their inhibitory effects were increased. At the latter concentration, calcium partly reversed the morphogenic effects of the blockers but not of cyclosporin A.

Growth and morphology of mycelial fungi can be affected by a variety of external factors including metabolic activators and inhibitors. In the case of *Botrytis cinerea* growth inhibition accompanied by morphological changes can be substantially influenced by the fungicide benomyl (Richmond and Pring 1971), the antibiotic griseofulvin (Brian *et al.* 1946) and a series of other antibiotics (Baráthová *et al.* 1975). Profound alterations of *B. cinerea* morphology have been induced by cytochalasins (Betina *et al.* 1972) and other macrocyclic antibiotics, such as cyanein (brefeldin A) or monorden (Betina and Mičeková 1973). Introduction of the so-called ramification test into the primary screening of antifungal antibiotics resulted in the discovery of ramihyphins (Baráth *et al.* 1974). Most recently, ramihyphin A has been found to be identical with cyclosporin A (Proksa *et al.* 1991), an immunosuppressive agent isolated independently by a Swiss group (Dreyfuss *et al.* 1976). In many filamentous fungi including *Saprolegnia*, *Fusarium* and *Aspergillus* species a low external Ca^{2+} concentration decreases the hyphal extension rate. Calcium blockade with calcium channel blockers also decreased the hyphal growth unit and extension rate of hyphae of the genera *Fusarium* and *Phytophtora* (Robson *et al.* 1991*a*,*b*; Temperli *et al.* 1991).

The importance of extracellular calcium for normal growth of *Neurospora crassa* and reversal effects of a calcium channel blocker, verapamil, have been reported recently (Dicker and Turian 1990). In this paper, we describe effects of extracellular Ca^{2+} ions, three calcium channel blockers (verapamil, nifedipin and nitrendipin) and a known morphogen cyclosporin A on growth and morphology of *B. cinerea*.

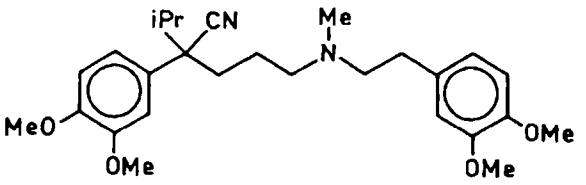
MATERIALS AND METHODS

Microorganism. Botrytis cinerea strain 4-22 was obtained from the *Culture Collection of Microorganisms of the Department of Microbiology, Biochemistry and Biology, Slovak Technical University, Bratislava.*

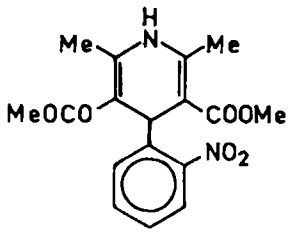
Culture medium. Malt extract containing 2.0 % (*W*/*V*) agar (*Oxoid*), pH 6.2, was used. The original concentration of Ca^{2+} in the medium was 0.6 mmol/L as determined by Dr. Z. Hladký (*Department of Analytical Chemistry, Slovak Technical University, Bratislava*) by atomic absorption spectrometry.

Compounds tested. Calcium channel blockers, nifedipin and nitrendipin were kindly provided by Dr. Z. Mahrla (*Institute of Drug Research, Modra,* Slovakia), verapamil was purchased from *Sigma.* Cyclosporin A (as ramihyphin A, Baráth *et al.* 1974) was prepared in our laboratories. $CaCl_2 \cdot 6H_2O$ was purchased from *Lachema* (Brno, Czech Republic).

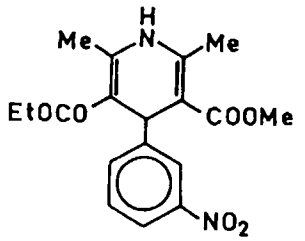
Colony growth. $CaCl_2$ was dissolved in distilled water, other substances in dimethyl sulfoxide (Me_2SO) and their solutions were added to the culture medium after its sterilization at 50 °C. Me_2SO



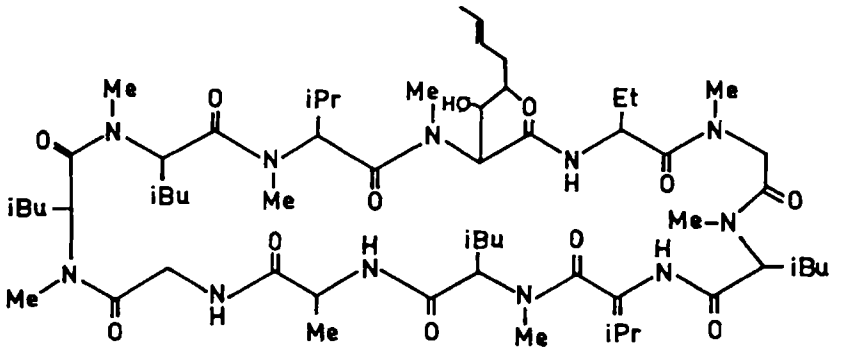
verapamil



nifedipin



nitrendipin



cyclosporin A

alone was added to controls. The final concentration of Me_2SO was the same in all cases (1 %, *V/V*). Ten-mL volumes of the culture medium with and without the above compounds were then poured into Petri dishes (d = 90 mm). Vegetative inoculum on filter paper discs was prepared according to Betina *et al.* (1972) and individual discs were placed in the center of the agar plates. Diameters of colonies growing at 25 °C were measured at intervals.

Hyphal extension and morphology. In addition to measurements of diameters of colonies on agar plates in Petri dishes with and without the compounds tested, extension and morphology of hyphae were checked microscopically at intervals. The Internode Length Unit (ILU) described by Armetrout *et al.* (1986) was used to quantify branching frequency in *B. cinerea* induced by verapamil and cyclosporin A. ILU is the mean distance (μm) between branch junctions (internodes). Each value shown in Table I was obtained as an average of 30 measurements of the length of the first internode from the apical end of the hypha. The ILU was determined by measuring the lengths of the internodes from the photomicrographs of 3-day-old colonies. Two further techniques were used to study morphogenetic effects. Discs of filter paper (6 mm diameter) containing known amounts of the compounds tested were placed either at the edges of growing colonies on agar plates in Petri dishes or to edges of microcolonies on microscopic slides as described elsewhere (Betina and Mičeková 1973). Microphotography was performed in situ after staining with 0.5 % (*W/V*) methyl blue in lactophenol.

RESULTS AND DISCUSSION

The Ca^{2+} ions present in the malt extract agar used (0.6 mmol/L) were insufficient for optimal growth. Exogenous calcium added at concentrations of 10, 50 and 100 mmol/L stimulated growth of colonies by 13, 23, and 40 %, respectively. The highest concentration added, 500 mmol/L, inhibited growth by 42 %.

Calcium channel blockers, when added alone at concentrations of 1.0 and 0.5 mmol/L, inhibited the growth of colonies as follows. Nifedipin (Fig. 1 *left*) at concentrations of 1.0 and 0.5 mmol/L caused a 71 and 66 % inhibition, respectively; it was the most effective of the three blockers tested, At the same concentrations, nitrendipin caused a 60 and 54 % inhibition, respectively. Verapamil was the least effective, causing a 48 and 13 % inhibition, respectively.

When calcium was added simultaneously with its blockers but at concentrations exceeding those of the blockers 10 to 100 times, it caused a decrease of their inhibitory effect (Fig. 1 *right*). A higher excess (like 500-fold) decreased the stimulatory effect of lower concentrations of Ca^{2+} ions.

Cyclosporin A (ramihyphin A) was more effective than the channel blockers. Complete inhibition was observed at 0.5 μmol/L. Addition of Ca^{2+} ions together with cyclosporin A did not significantly reverse its inhibitory effect. Again, 500 mmol/L Ca^{2+} inhibited growth concomitantly with cyclosporin A.

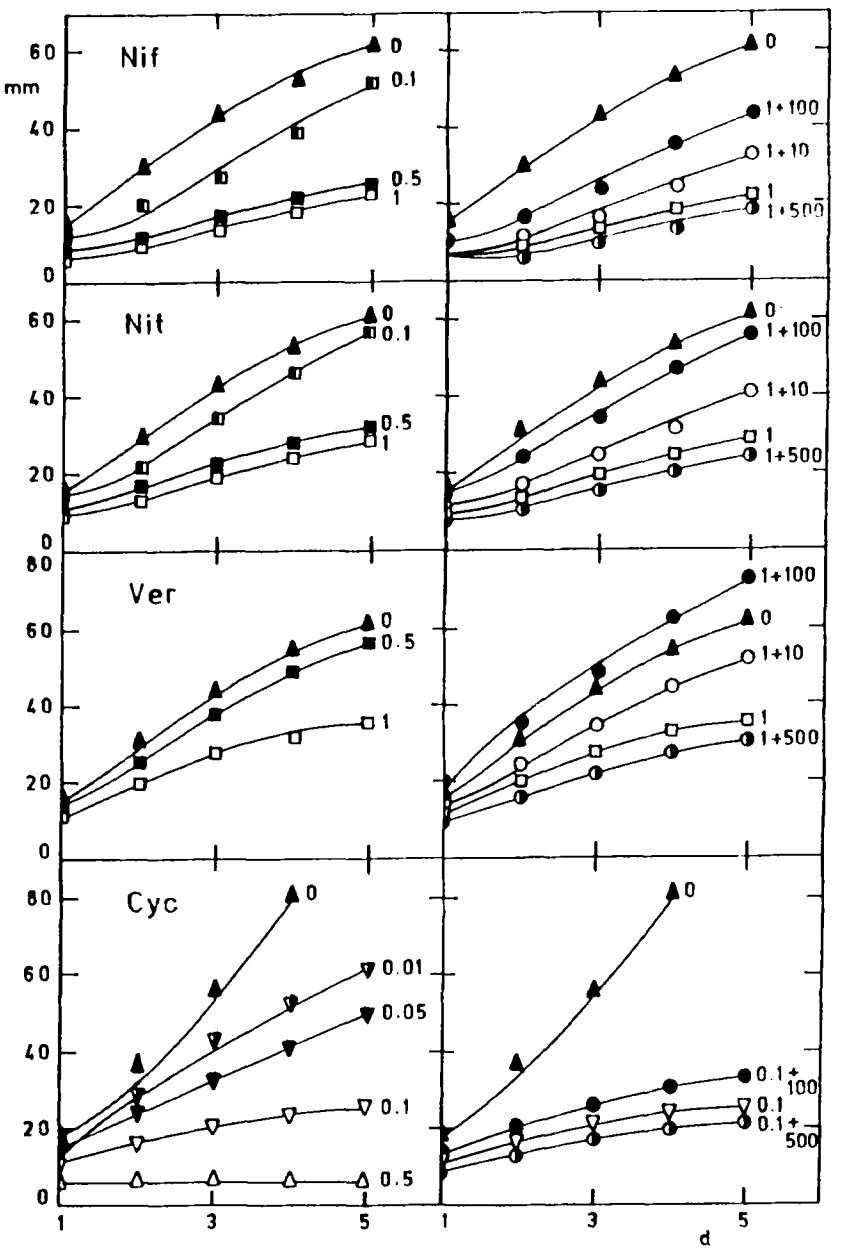


Fig. 1. *Left*: inhibition of growth (colony diameter, mm) of *B. cinerea* by nifedipin (**Nif**), nitrendipin (**Nit**), verapamil (**Ver**) and cyclosporin A (**Cyc**); *right*: the reversal of inhibition by external Ca^{2+}. *Numbers at curves*: concentration: mmol/L for Nif, Nit, Ver, Ca^{2+}; μmol/L for Cyc.

The calcium channel blockers and cyclosporin A elicited profound changes in the morphology of hyphal tips, mainly their branching and curling. At the same time, swelling and bulging of hyphae was also observed in some cases and was accompanied by the release of the cytoplasmic content. Both calcium channel blockers and cyclosporin A were effective in inducing these changes. The typical morphogenic effect of the tested compounds are presented in the Fig. 2–4. The onset of the morphological changes induced by calcium channel blockers was dependent on their concentration. At concentrations of 0.5 mmol/L there were no morphological changes observed, despite the inhibition of growth. At 1 mmol/L concentrations, the changes started after about 3 d of cultivation (Fig. 3). The onset of changes could be accelerated when a sterile disc soaked in the solution of the calcium channel blocker

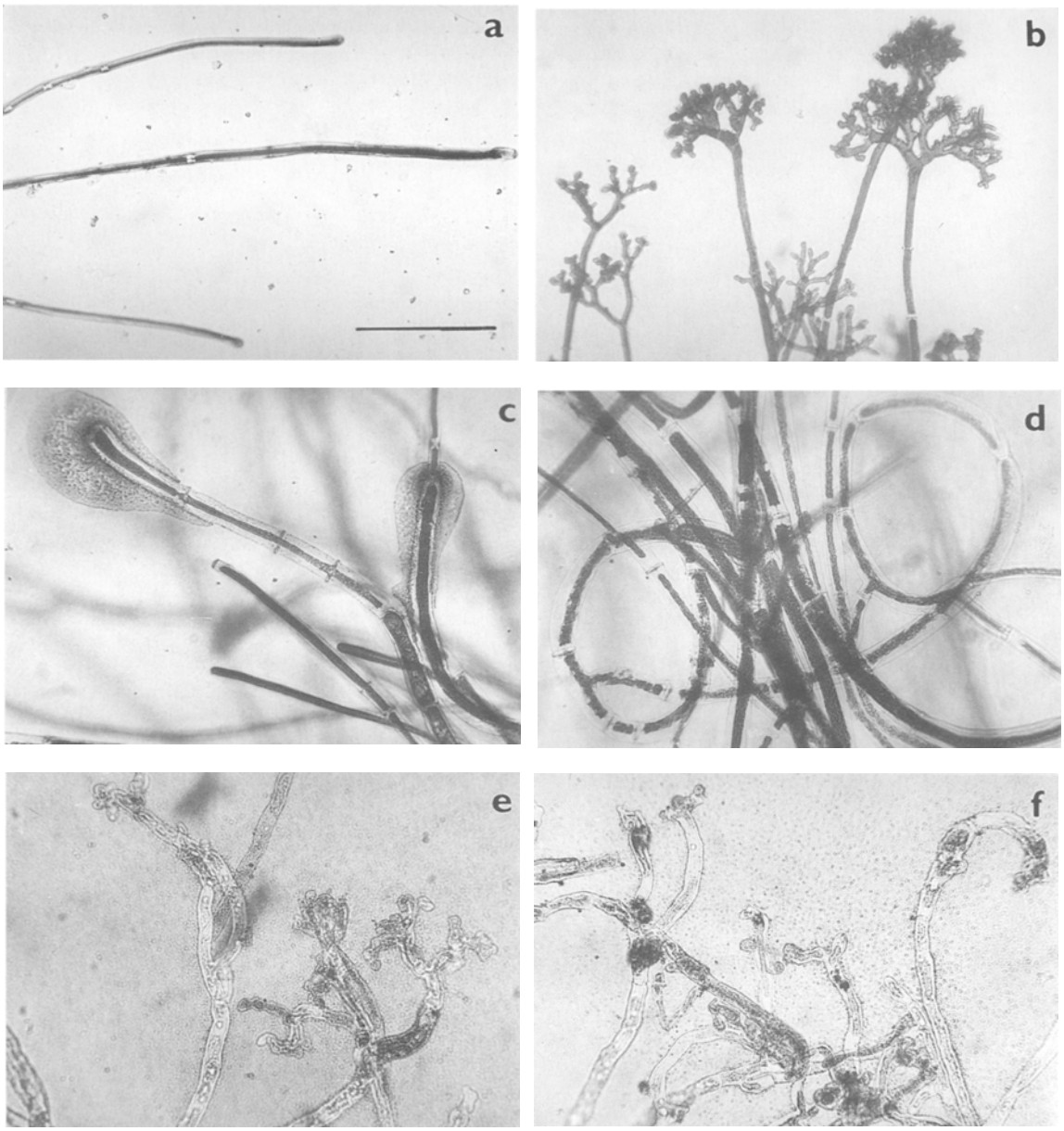


Fig. 2. Changes in morphology of *B. cinerea* hyphae induced by "locally" applied calcium channel blockers. Paper discs containing each 200 μg of the drug tested were put close to the growing edge of the colonies cultivated on the microscopic slides covered with malt agar. Photomicrographs were taken 7 h later; **a** – control, **b** – nitrendipin, **c, d** – nifedipin, **e, f** – verapamil. Bar represents 50 μm.

(200 µg) was placed close to the growing edge of the colony. In this case morphological changes were observed after 7 h (Fig. 2). The addition of Ca^{2+} exceeding the concentration of the calcium blocker up to 500 times reversed in part the morphological changes elicited by calcium blockers but complete abolition of these changes was never observed (Fig. 3e,f; Table I). Lower concentrations of Ca^{2+} were progressively less effective in this respect. Similarly, approaching the growing edge of the colony with a paper disc soaked with 5 µg of cyclosporin A tested decreased the time necessary for eliciting morphological changes by cyclosporin A up to 4 h instead of the about 20 h necessary for the effect of the

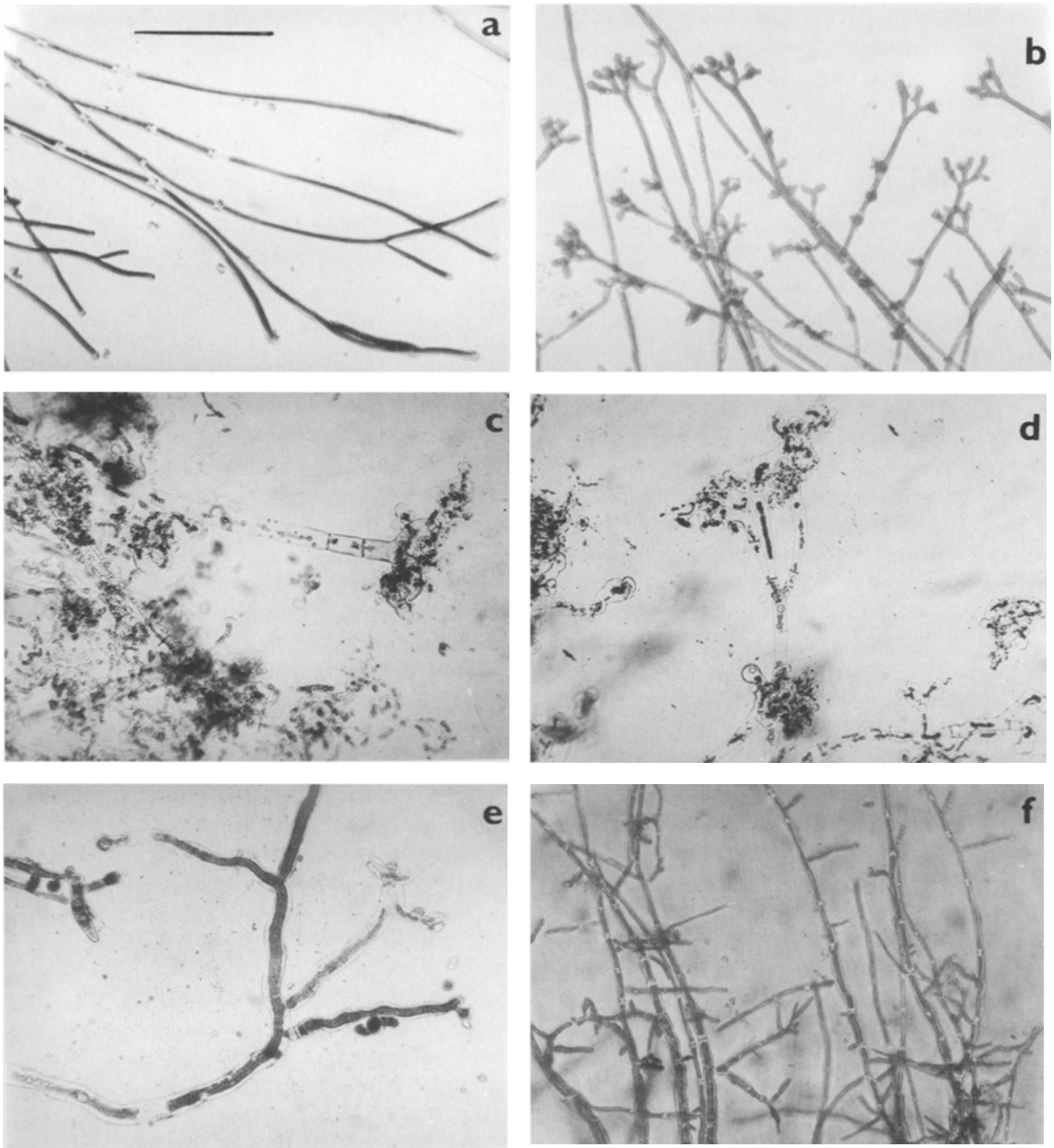


Fig. 3. Changes in morphology of *B. cinerea* hyphae induced by calcium channel blockers dissolved in the medium; *B. cinerea* was cultivated for 3 d in malt agar containing: **a** – Me_2SO (1 %), or 1 mmol/L nitrendipin (**b**), nifedipin (**c**, **d**), verapamil alone (**e**), verapamil + 500 mmol/L Ca^{2+} (**f**). Bar represents 50 µm.

cyclosporin A dissolved in the medium. The effect of cyclosporin A on hyphal morphology could not be reverted by the addition of excess of Ca^{2+} (Fig. 4a,b; Table I). The morphological changes induced

either by the calcium channel blockers or cyclosporin A tested were not uniform but depended on the drug tested, its concentration, and also on the method of drug application (*i.e.*, "general" application – when the drug was dissolved in the cultivation medium, or "local" application – when the drug was applied from the paper disc placed close to the edge of the colony). No unequivocal relationship between these parameters and a particular morphological change could be generalized from our experiments.

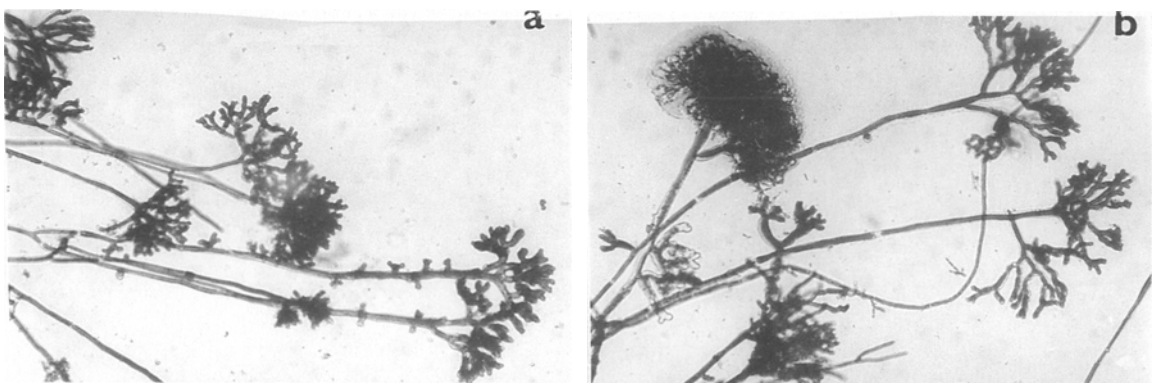


Fig. 4. Effect of cyclosporin A on the hyphal morphology of *B. cinerea* cultivated for 20 h in malt agar containing: 0.1 μmol/L cyclosporin A (**a**), 0.1 μmol/L cyclosporin A + 500 mmol/L Ca^{2+} (**b**). Bar represents 50 μm.

Table I. The effect of verapamil, cyclosporin A and their combination with Ca2+ on the branching frequency in *B. cinerea*

Compound	Concentration μmol/L	Ca^{2+} mmol/L	ILU[a] μm
Verapamil	0	0	156
		100	160
		500	150
	500	0	144
	1000	0	60
		100	65
		500	110
Cyclosporin A	0	0	156
		100	158
		500	160
	0.01	0	55
	0.05	0	25
	0.1	0	22
		100	23
		500	21

[a]Internode length unit.

Recently, several observations were made which revealed the relationship among the fungal growth, morphology, differentiation and homeostasis of Ca^{2+} ions. The species included *Neurospora crassa* (Reissing and Kinney 1983; Dicker and Turian 1990), *Fusarium graminearum* (Robson *et al.* 1991*a,b*), and *Ceratocystis ulmi* (Muthukumar *et al.* 1984). The tools which made this possible were drugs used as coronary vasodilatants in human medicine – the phenylalkylamine verapamil or the dihydropyridine derivatives nifedipin, nitrendipin, *etc.*, regarded generally as calcium channel blockers (*see* Triggle 1982, *for review*), and EGTA. *B. cinerea* could be also added to the microorganisms listed above. These data suggest that the Ca^{2+} homeostasis perturbation, namely the restriction of the Ca^{2+} influx is the primary cause of the subsequent inhibition of growth and of the triggering of morphological changes.

Comparison of the morphological changes exerted by calcium channel blockers and by cyclosporin A showed that a similar change in morphology could be elicited probably without an apparent involvement of calcium ions because the effect of cyclosporin A used could not be reversed by the excess of external Ca^{2+} ions. It should be pointed out that these observations are not necessarily in contradiction if we take into account the fact that the final target of the action of calcium channel blockers is not the calcium homeostasis but calcium-dependent processes which could be directly affected by the actions of individual cyclosporin A. The identification of these targets is not possible on

the basis of the results we obtained so far and requires a direct biochemical or molecular genetic approaches.

This work was supported in part, by the *Slovak Grant Agency* (Grant no. 1/990629/93).

REFERENCES

BARÁTH Z., BARÁTHOVÁ H., BETINA V., NEMEC P.: Ramihyphins – antigungal and morphogenic antibiotics from *Fusarium* sp. S-435. *Folia Microbiol.* **19**, 507–511 (1974).

BARÁTHOVÁ H., BETINA V., BARÁTH Z., NEMEC P.: Morphogenic effects of ramihyphin A in filamentous fungi. *Folia Microbiol.* **20**, 97–100 (1975).

BETINA V., MIČEKOVÁ D., NEMEC P.: Antimicrobial properties of cytochalasins and their alteration of fungal morphology. *J.Gen.Microbiol.* 71, 343–349 (1972).

BETINA V., MIČEKOVÁ D.: Morphogenic activity of cytochalasins, cyanein and monorden in *Botrytis cinerea*. *Z.Allg.Mikrobiol.* **13**, 287–298 (1973).

BETINA V., NEMEC P., DOBIAS J., BARÁTH Z.: Cyanein, a new antibiotic from *Penicillium cyaneum*. *Folia Microbiol.* **7**, 353–357 (1962).

BRIAN P.W., CURTIS P.J., HEMMING H.G.: A substance causing abnormal development of fungal hyphae produced by *Penicillium jaczrewskii*. III. Identity of "curling" factor with griseofulvin. *Trans.Brit.Mycol.Soc.* **29**, 173–179 (1946).

DICKER W.J., TURIAN G.: Calcium deficiencies and apical hyperbranching in wild-type and the "frost" and "spray" morphological mutants of *Neurospora crassa*. *J.Gen.Microbiol.* **136**, 1413–1420 (1990).

DREYFUSS M., HARRI E., HOFFMAN H., KOBEL H., PACHE E., TSCHERTER H.: Cyclosporin A and C. New metabolites from *Trichoderma polysporum* (LINK ex PERS) RIFAI. *Eur.J.Appl.Microbiol.* **3**, 125–133 (1976).

MURHUKUMAR G., NICKERSON K.W.: Ca(II)-calmodulin regulation of fungal dimorphism in *Ceratocystis ulmi*. *J.Bacteriol.* **159**, 390–392 (1984).

PROKSA B., UHRÍN D., KOVÁČIK V., VOTICKÝ Z., BETINA V.: Identity of the antibiotic ramihyphin A and cyclosporin A. *Folia Microbiol.* **36**, 141–143 (1991).

PITT D., POOLE P.C.: Calcium induces conidiation in *Penicillium notatum* in submerged cultures. *Trans.Brit.Mycol.Soc.* **76**, 219–230 (1984).

REISSIG J.L., KINNEY S.G.: Calcium as branching signal in *Neurospora crassa*. *J.Bacteriol.* **154**, 219–230 (1983).

RICHMOND D.V., PRING R.J.: The effect of benomyl on the fine structure of *Botrytis fabae*. *J.Gen.Microbiol.* **66**, 79–94 (1971).

ROBSON G.D., WIEBE M.G., TRINCI A.P.J.: Involvement of Ca^{2+} in the regulation of hyphal extension and branching in *Fusarium graminearum* A 3/5. *Exp.Mycol.* **15**, 263–272 (1991a).

ROBSON G.D., WIEBE M.G., TRINCI A.P.J.: Low calcium concentrations induce increased branching in *Fusarium graminearum*. *Mycol.Res.* **95**, 561–565 (1991*b*).

ROSEN B.: Calcium transport in microorganisms, pp. 187–216 in *Membrane Transport of Calcium* (E. Carafoli, Ed.). Academic Press, London 1982.

SEKIGUCHI J., GAUCHER G.M.: Conidiogenesis and secondary metabolism in *Penicillium urticae*. *Appl.Environ.Microbiol.* **33**, 147–158 (1977).

TEMPERLI E., ROOS U.P., HOHL H.R.: Germ tube growth and the microtubule cytoskeleton in *Phytophtora infestans*: Effects of antagonists of hyphal growth, microtubule inhibitors, and ionophores. *Mycol.Res.* **95**, 611–617 (1991).

TRIGGLE D.J.: Biochemical pharmacology of calcium blockers, pp. 121–134 in *Calcium Blockers. Mechanisms of Action and Clinical Application* (S.F. Flaim, R. Zelis, Eds). Urban and Schwarzenberg, Baltimore 1982.