CZECH MYCOL. 47 (4), 1994

Production of abscisic acid and cytokinins in static liquid culture by Schizophyllum commune

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Janitor A. and Vizárová G. (1994): Production of abscisic acid and cytokinins in static liquid culture by Schizophyllum commune – Czech Mycol. 47: 293–302

The superficial cultivation of fungus Schizophyllum commune Fr. in static liquid cultures showed production of abscisic acid – type inhibitor (ABA) and isopentyl – adenine type cytokinins (2iP) by this fungus. The analyses were done after 28 days of cultivation.

Key words: production abscisic acid, cytokinins, static liquid culture, Schizophyllum commune Fr.

Janitor A. and Vizárová G. (1994): Produkcia kyseliny abscisovej a cytokinínov hubou Schizophyllum commune v tekutých kultúrach. – Czech Mycol. 47: 293–302

Pri povrchovej kultivacii huby Schizophyllum commune Fr. v statických tekutých kultúrach bolo zistené, že huba produkuje do média inhibítor typu kyseliny abscisovej (ABA) a cytokiníny typu izopentyl-adenín (2iP). Analýzy boli robené po 28 dňoch kultivácie.

INTRODUCTION

During the last years the literature takes notice of the ability of some saprophytic and parasitic fungi to synthesize abscisic acid (ABA) (Crocoll et al. 1991). Abscisic acid a natural plant growth regulator has been first identified in *Cercospora rosicola* in 1977 (Assante et al. 1977) and *Fusarium culmorum* in 1984 (Michniewicz et al. 1984). On the other hand it is well known that some hemibiotrophic fungi (*Monilia* sp., *Cytospora* sp., *Helminthosporium* sp., *Cercospora* sp., *Taphrina* sp., *Botrytis* sp., etc) show the ability to produce some phytohormones – cytokinins (Kern and Neaf-Roth 1975, Vizárová 1975, Arrora and Mandahar 1979, Strzelczyk and Kempert 1983, Mills and Van Staden 1978, Mazin et al. 1980). The present work deals with the production of the above – mentioned substances by wood destroying parasitic – saprophytical fungus *Schizophyllum commune* Fr.

MATERIAL AND METHODS

An isolate of the parasitic – saprophytical fungus *Schizophyllum commune* Fr. (Sch/5), obtained from Slovakian apricot cultures was used for our experimental

works.In comparison with many isolates of Slovak and foreign provenance the isolate showed the greatest activity in connection with the pathological injury of apricot vessel system (Janitor 1989). The fungus was cultivated in a liquid medium (Lilly et Barnet 1953) containing the following compounds: 15 g glucose, 0.5 g asparagine, 0.25 g $MgSO_4.7H_2O$, 0.75 g KH_2PO_4, 25 mg thiamin, and 5 mg biotin in 500 ml of distilled H_2O (pH of the medium before inoculation was 5.0). The cultures were inoculated with a solid inoculum (five pieces per 1 cm^2 in 0,4 l Erlenmeyer flasks) and kept in an incubator at 25 °C. After 28 days of cultivation the analysis was made (Fig. 1).

Fig. 1 Mycelium growth in Erlenmayer flasks after 28 days of cultivation.

Extraction and purification of phytohormones

a) Cytokinins

method of column thickening of cytokinins by help of ion – converter Dowex in H^+ cycle (50-80 mesh.) was applied. This method was also used for thickening of isotopes. The pH of the filtrate before separation of cytokinins was 5.2. The filtrate was further purified on the Dowex column (20 cm long. 3 cm in diameter). The resulting filtrate showed pH of 3-3.2. The lowered pH indicated separation of low basic substances. The absorbed compounds of cytokinins with a similar character were removed from the column by 200 ml of 0.1 M NH_4OH in 70 % ethanol. The

eluate obtained was evaporated in vacuum to dryness and dissolved in 10 ml of 96 % ethanol and reevaporated. Then 10 ml of alkaline water (pH 7,8) was added to the evaporate and extracted with n-butanol (2:1 v/v) for 24 hours. Subsequently n-butanol was evaporated to dryness. The residue was dissolved in 96% ethanol. The solution was filtered on DEAE cellulose column in vacuum (3x2 cm). The cytokinins obtained were analyzed by thin-layer chromatography (TLC), gas liquid chromatography (GLC), mass spectrometry (MS) and by biotest (Vizárová et Vozár 1984, Palni et al. 1985, Lethan 1968). As the stamdards following commercial preparations were used: F. A. Sigma, zeatin, zeatin ribosid and isopentyl-adenine (2iP).

b) Abscisic acid – type inhibitor (ABA)

The filtrate after column thickening on Dowex 50 (in H^+ cycle pH=3,0) was extracted 3 times with ethylacetate. The mixed ethylacetate extracts were evaporated in vacuum to dryness at 35 °C. The residue was dissolved in methanol and laid on thin layers of silica gel. The developing mixture was benzene-ethylacetate-acetic acid (100/20/5). For rechromathography a mixture of benzene-acetone-acetic acid (70/30/1 v/v) was used (Rypák and Kamenická 1986). The R_F position responsible for ABA was determined with a UV lamp at 254 nm. This position was further used for ABA detection by a biotest according to Nikolajeva and Daleckaja (1963). The method is based on inhibition of seed germination of mustard seeds and on biotest based on the determination of growth principe of wheat segment. Gas liquid chromatography (GLC) was performed by method of methylation modified by Vozár and Vizárová (1992) (unpublished). Methanol extract was evaporated to dryness dissolved in 2 ml of benzene and in 2 ml of the methylic agent (BF methanol) and methylated at the temperature of 92 °C for 5 minutes in the dark. A glass column (3 % OV-17 on WAW chromosorb 80-100 mesh.) was used to carry out GLC separation. Conditions were as follows: t = + 200 °C, detector t = + 230 °C, injector = + 250 °C. Shimadzu CR3A computer was used for automatic registration.

RESULTS AND DISCUSSION

The results obtained from the analysis of the culture medium showed the ability of this fungus to produce ABA and cytokinins into the culture medium. The methods of purificaton and identification of inhibitors applied in our experiments demostrated a considerable production of abscisic acid (ABA). TLC, GLC and biotest (Fig. 2, 3a, b) were used for its determination. Our results correspond to the literature dates with the following fungi as ABA producers: *Agrocybe praecox, Alternaria alternata, Coprinus domesticus, Cunninghamella echinulata,' Mucor spinosus, Polyporus brumalis, Rhizopus arrhizus, Rhizopus nigricans, Trametes*

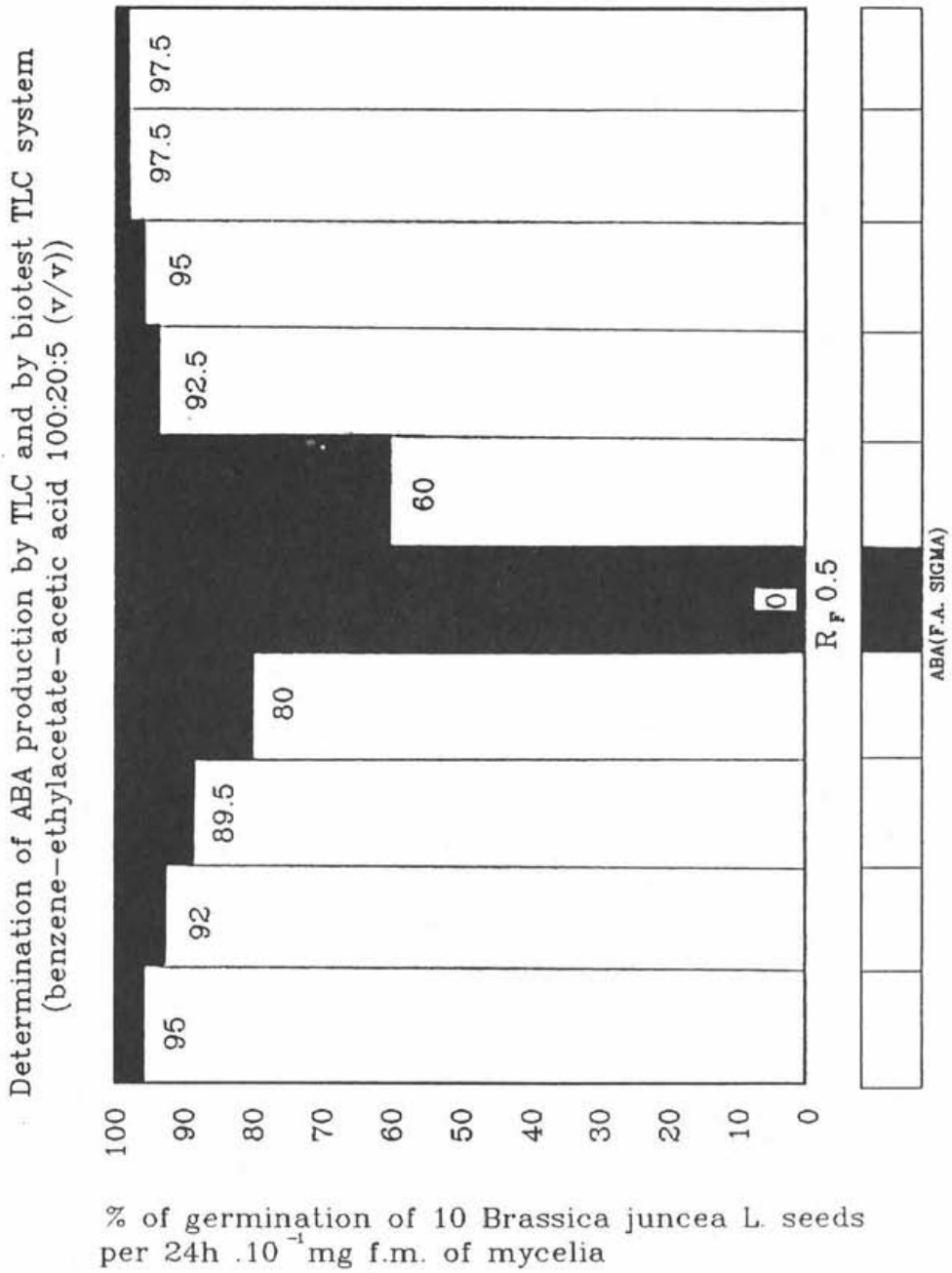

Determination of ABA production by TLC and by biotest TLC system
(benzene–ethylacetate–acetic acid 100:20:5 (v/v))
95
92
89.5
80
0
60
92.5
95
97.5
97.5
R_F 0.5
ABA(F.A. SIGMA)
100
90
80
70
60
50
40
30
20
10
0
% of germination of 10 Brassica juncea L. seeds
per 24h .10 $^{-1}$ mg f.m. of mycelia

Fig. 2

versicolor (Crocoll et al. 1991), as well as *Cercospora pini-densiflorae, Cercospora theate, Cercospora fici, Verticillium dahliae* (Okamoto et al., 1988), and fungus *Fusarium culmorum* (Michniewicz et al., 1984). All studies have been based on a work dealing with ABA production by *Cercospora rosicola* (Assante et al., 1977). Our studies suggest the ability of fungus *Schizophyllum commune* to produce an inhibitor of the ABA type in the culture medium together with the ability of their biosynthesis. These results are corresponding with the work of Dorffling and Peterson (1984), who identified ABA in fungi of the genus *Botrytis, Ceratocystis, Fusarium* and *Rhizoctonia.* Although only GLC and TLC methods and biotest could be used in our studies for identification of ABA, we suppose that our results contribute to the extension of the knowledge about the ability of the parasitic-saprophytical fungus *Schizophyllum commune* Fr. to biosynthetize and produce ABA also as well as other parasitic and saprophytic species mentioned above. Similar methods were used for identification of ABA in Ficus superba var. Japonice (Uede et al., 1991).

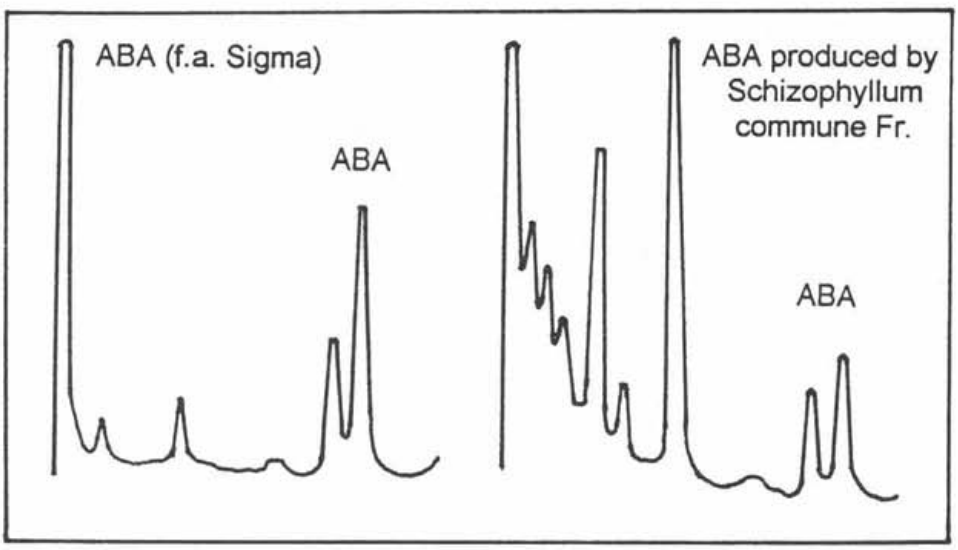


Fig. 3a **Fig. 3b**

To the contrary, we found the above – mentioned fungus being able to produce cytokinins in the culture medium. TLC (one – and two – dimensional), GLC, MS and biotest were used for determination of cytokinins. Isopentyl – adenine (2iP) was identified by the two chromatography methods (Fig. 4, 5a, 5b). Our results may complete the information in literature about the ability of hemibiotrophic fungi

to produce cytokinins (Mandahar et Angara 1987). The recent literature provides contradictory data on the individual cytokinin production by fungi. For example in Fusarium moniliforme var. subglutianus, a high level of isopentenyl-adenine and low levels of zeatin and zeatin riboside were identified in infected plant cells by using of HPLC (Van Staden et Nicholson 1989). To the contrary in healthy plant tissues high levels of zeatin and zeatin riboside were determined. In the fungus Cylindrocarpon destructans, zeatin and zeatin riboside were identified (Strzelczyk et Kemptner 1983). Our results showed 2iP production. Similar results concerning 2iP production in a culture medium were obtained in the bacterium Corynebacterium fasciens (Phillips et Torrey 1970). Our results might be in correlation with some literature data where in cells infected with parasitic fungi an amount of 2iP was identified by using HPLC and GLC methods (Nicholson et Van Staden 1988, Vizárová et al., 1988).

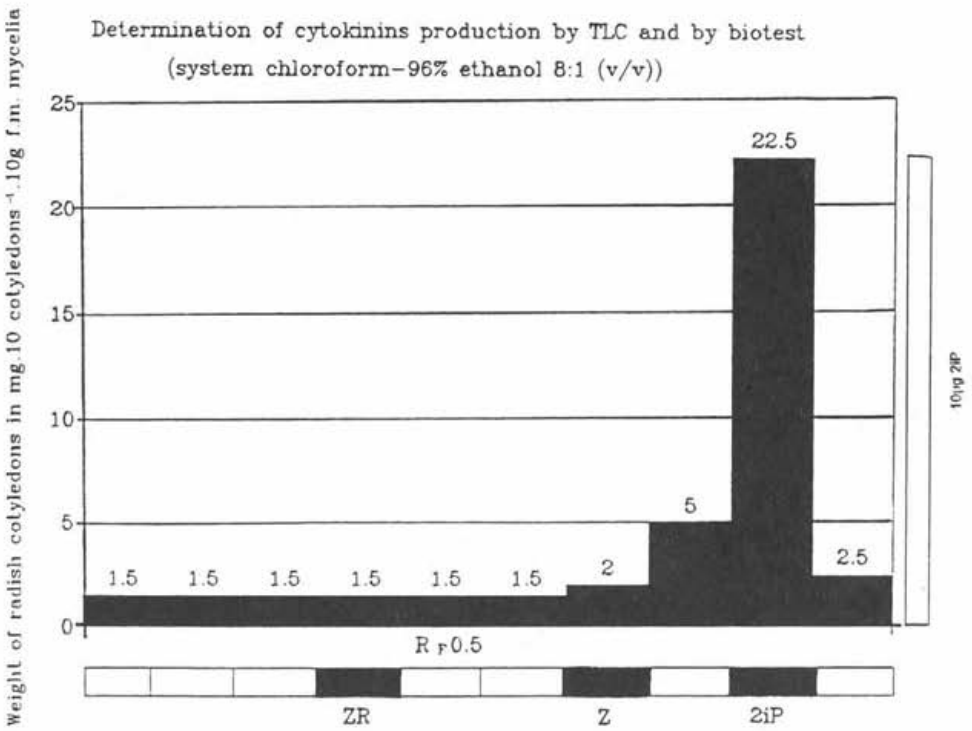
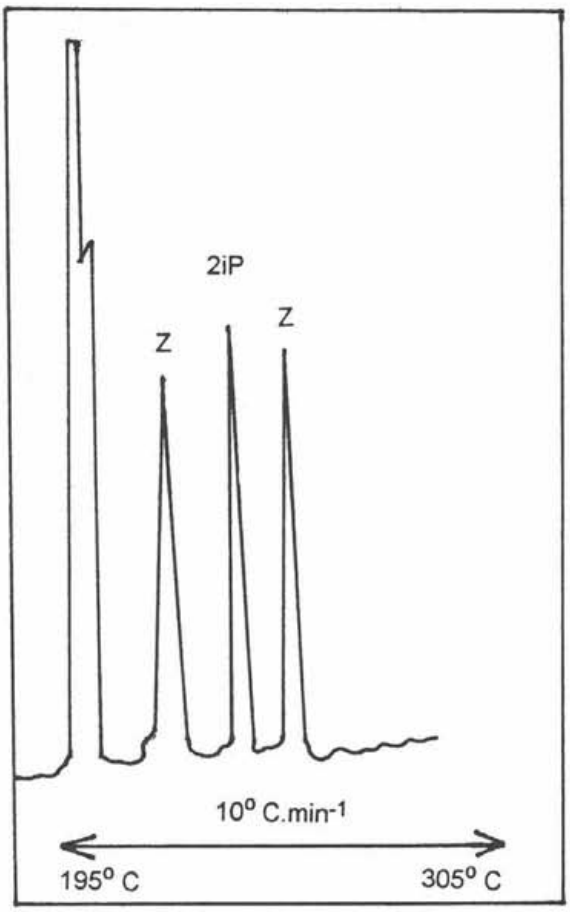


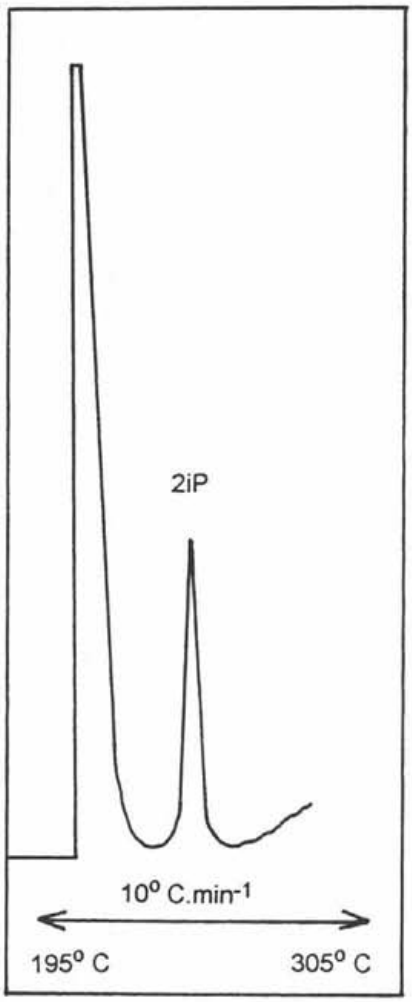
Fig. 4

According to the important function of ABA and cytokinins in plant metabolism and with the fact that their participation in plants depend on their concentration and a donor activation causing metabolic changes these compounds might be an



TMS derivates of cytokinins (f.a.SIGMA) determined by GLC as TMS derivatives.
Z = zeatin, 2iP = isopentyl adenine, ZR = zeatin ribaside
Column 2% OV 101 CHROMOSORBE WAW - MDCS 80 - 100 mesh.
Gas flow: N2 = 30 $cm^3.min^{-1}$, H2 = 30 $cm^3.min^{-1}$, air = 300 $cm^3.min^{-1}$
temperature from 195° C to 305° C.

Fig. 5a



Cytokinins produced by Schizophyllum commune Fr. determined by GLC as TMS derivatives. Column 2% OV 101 CHROMOSORBE WAW - MDCS 80 - 100 mesh. Gas flow: N2 = 30 $cm^3.min^{-1}$, H2 = 30 $cm^3.min^{-1}$, air = 300 $cm^3.min^{-1}$ temperature from 195° C to 305° C.

Fig. 5b

important product of fungal metabolism during their growth and development. We suppose that our primary data concerning the ability of the fungus Schizophyllum commune to produce these compounds into the culture medium may elucidate the mechanism of the pathogene-host plant interaction and also the processes connected with the changes of metabolism of the attacked woody-plants by the above mentioned fungus.

Although we had no possibility to apply mass spectroscopy we cannot eliminate the possibility to identificate also another inhibitor besides ABA e. g. jasmonic acid (Lopez et al., 1987).

A c k n o w l e d g e m e n t s

The autors thank Mrs. M. Verbová and Miss. L. Lanczová for their technical help and Dr. I. Vozár for GLC.

REFERENCES

ARRORA R. K. and MANDAHAR C. L. (1979): Secretion of cytokinins by Helminthosporium turcicum in liquid cultures. Acta Phytopath. Sci. Hung.14: 7-11.

ASSANTE G., MERLINI L. and NASINI G. (1977): +/– abscisic acid a metabolite of the fungus Cercospora rosicola. Experientia 33: 1556-1557.

CROCOLL C., KETTNER J. and DORFFLING K. (1991): Abscisic acid in saprophytic and parasitic species of fungi. Phytochemistry 30: 1059-1060.

DORFFLING K. and PETERSON W. (1984): Abscisic acid in phytopathogenic fungi of the genera Botrytis, Ceratocystis, Fusarium and Rhizoctonia. Z. Naturforsch. 39: 683-686.

JANITOR A. (1989): Parasitism of the Schizophyllum commune Fr. on apricot. IX. Int. symp. apric. cultur.Caserta, Italy, 123 pp. (Abstract)

KERN H. and NAEF-ROTH S. (1975): Zur Bildung von Auxin und Cytokininen durch Taphrina-Arten. Phytopath. Z. 83: 193-222.

LETHAN D. S. (1968): A new cytokinins bioassay and the naturally occuring cytokinin complex. In: F. Wightman and G. Setterfield (eds.) Biology and Physiology of plants growth substances: 11-31, Runge Press, Ottawa.

LILLY V. I. and BARNET G. (1953): Fiziologia Gribov II. (Physiology of fungi II): 220-279, Moscow.

LOPEZ R., DATHE W., BRUCKNER C., MIERSCH O. and SEMBDNER G. (1987): Jasmonic acid in different parts of the developing plants. – Biochem. Physiol. Pflanz. 182: 195-201.

MANDAHAR C. L., ANGRA R. (1987): Involvement of cytokinins in fungal pathogenesis. – Res. Bull. Sci., Punjab University, 38: 35-49.

MAZIN V. V., IVANOVA G. A., ČURIKOVA V. V. and TALIEVA M. N. (1980): Cytokinin activity of the culture medium of the fungus Botrytis anthophyla Bond a fungus provoking flower moulding process. Mikologija i fytopathologia 14 (5): 385-388. (In Russian)

MIILS L. J., VAN STADEN J. (1978): Extraction of cytokinins from maize smut tumors of maize and Ustilago maydis cultures. Physiol.– Plant Patholog.13: 73-80.

MICHNIEWICZ M., MICHALSKI L., CZERWINSKA E. and KRUZSKA G. (1984): Control of growth and development of isolates of Fusarium culmorum (W.G.Sm.) Sacc. of different pathogenicity to wheat seedlings by plant growth regulators IV. abscisic acid. Acta Physiol. Plant (Pol.) 6: 55-64.

MICHNIEWICZ M., CZERWINSKA E. and LAMPARSKA K. (1986): Stimulatory effect of abscisic acid on the growth and development of Cylindrocarpon destructans (Zins.) Scholl. – Bull. Polish. Acad. Sci. Biol. Sciences 34: 55-59.

NICHOLSON R. I. D. and VAN STADEN J. (1988): Cytokinins and mango flower malformation. I. Tentative identification of the complement in healthy and malformed inflorescences. – J. Plant Physiol., Vol. 132: 720-724 pp.

NIKOLAJEVA M. G. and DALECKAJA T. V. (1963): Growth of mustard seeds for estimation of biological activity of abscisic acid. Trudy Bot. Inst. AM ZSSR ser. 4: 16-19. (In Russian)

OKAMOTO M., HIRAI N., KOSHIMIZU K. (1988): Biosynthesis of abscisic acid in Cercospora pisidensiflorae. Phytochemistry 27: 2099-2103.

PALNI L. M., TAY S. A. B., MANDI S. K., PIANCA D. J., DE KLERK G. J. M., WONG O. C., LETHAN D. S. and MACLEOND J. K. (1985): Cytokinin biosynthesis in plant tumor tissues. – Biol. Plantarum (Praha) 27: 195-203.

PHILLIPS H. L. and TORREY J. G. (1970): Cytokinin production by Rhizobium japonicum. Physiol. Plant. 23: 1057-1063.

RYPÁK M. and KAMENICKÁ A. (1986): Growth regulators, vest and germination of tree seeds. Veda-Bratislava, 150 pp. (In Slovak)

STRZELCZYK E., KEMPERT M. (1983): Production of cytokinin like substances by Cylindrocarpon destructans (Zins.) Schott. isolates pathogenic and non pathogenic to fir (Abies alba Mia) seedlings. Phytopath. Z. 106: 90-96.

UEDE J., MIZUMOTO T. and KATO J. (1991): Quantative changes of abscisic acid and methyl jasmonate correlated with vernal leaf abscission of Ficus superba var. japonica. Bioch. Physiol. Pflanzen 187: 203-210.

VAN STADEN J., NICHOLSON R. I. D. (1989): Cytokinins and mango flower malformation II. The cytokinins complement produced by Fusarium moniliforme and the ability of the fungus to incorporate ($8^{1}4C$) adenine into cytokinins. – Physiol. Molec. Plant Pathology 35: 423-431.

VIZÁROVÁ G. (1975): Contribution to the study of cytokinin production by phytopathogenic fungi.– Biol. Plantarum (Praha) 17: 380-382.

VIZÁROVÁ G., SHASKOVA L. S. and ANDREEV L. N. (1988): On the question of the relationship between free zeatin content and resistance of wheat to biotrophic fungi. Acta Phytopath. Enth. Hung. 23: 385-392.

VIZÁROVÁ G. and VOZÁR I. (1984): Free endogenous cytokinin content in the seed of barley and wheat cultivars with different resistance to powdery mildew.– Bioch. Physiol. Pflanzen 179: 767:777.