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Production of cytokinin-like substances and ethylene by the ectomycorrhizal fungus *Cantharellus cibarius*

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It was found that the hardwood form of *Cantharellus cibarius* (strain 5400) produced less cytokinin-like substances than the coniferous form (strain 5410). Among the active substances the following were detected: 2iP, 2iP riboside and zeatin. No significant differences in ethylene production between both strains in the presence or absence of methionine (considered to be the precursor of this gas) were noted.

Key words: *Cantharellus cibarius*, ectomycorrhizal fungus, cytokinin-like substances, ethylene.

INTRODUCTION

There exists firm evidence thast plant growth regulators, among them cytokinins and ethylene produced by plants, are essential for their growth and development. Plant growth regulators are also produced by various microorganisms living in association with plants (Gogala 1991). There is also evidence that plant growth hormones produced by bacteria can increase growth rates and improve yields of the host plant (Barea, Brown 1974). Different microorganisms both saprotrophic and pathogenic have been found to produce cytokinins (Phillips, Torrey 1972; Kampert, Strzelczyk 1980; Strzelczyk, Kampert 1983; Evidente et al. 1991).

Plant growth hormones affect not only the growth and development of the plant but they are also of importance in establishing and functioning of

mycorrhizae (S l a n k i s 1973; C r a f t s, M i l l e r 1973). Among the plant growth regulators cytokinins are of special interest. They stimulate cell division, modify cell enlargement, delay senescence, inhibit root elongation and protect the plant against pathogens by increasing the synthesis of phenolic compounds (D e k h u i j z e n 1976). Therefore, it is not surprising that among plant growth regulators auxins and cytokinins are being considered of utmost importance in plant mycorrhizal fungus interrelationships. Cytokinins may modify the growth of cortical cells in such a manner as to facilitate the invasion of the root by the fungus. They also cause mobilization of nutrients to the region of mycorrhizal association (C r a f t s, M i l l e r 1974).

Ethylene is an endogenous growth regulator that is produced by higher plants as well as by microorganisms. This gas affects plant growth and some of the biochemical processes in plants. Fungi are known to produce ethylene often in correlation with infection or pathogenesis (A r s c h e r, H i s l o p 1975; G r a h a m, L i n d e r m a n 1980).

Cantharellus cibarius is an ectomycorrhizal fungus of many forest trees (T r a p p e 1986). It is also an appreciated edible mushroom of significant economical importance. Yet because of many reasons this species was not intensively studied (D a n e l l 1994). To our knowledge papers published on this fungus are not numerous. Only one paper on *C. cibarius* has been published in Poland (P a c h l e w s k i, S t r z e l c z y k, K e r m e n 1996). Because no data on the production of plant growth regulators by this fungus are available, the synthesis of cytokinin-like substances and ethylene in two strains of *C. cibarius* was carried out.

MATERIALS AND METHODS

O r i g i n o f t h e i s o l a t e s. Two isolates of the ectomycorrhizal fungus *C. cibarius* hardwood (strain No 5400) and coniferous (strain No 5410) strains were studied. Pure cultures were obtained from fruit bodies of this fungus growing under oak (No 5400) and pine (No 5410) as described in detail by P a c h l e w s k i, S t r z e l c z y k and K e r m e n (1996).

C u l t u r e c o n d i t i o n s. Both strains were cultured and stored on potato dextrose agar (Difco) slants. In this study they were grown in Lamb's (1974) medium-glucose 10 g, NH_4Cl 500 g, $MgSO_4.7H_2O$ 50 mg, k_2HPO_4 50 mg, Na EDTA 1 ml/l of 5 ppm Fe solution, thiamine 1 mg, biotin 0,01 mg. distilled water 1000 ml, pH 5,8-6,0. Erlenmeyer flasks (500 ml) containing 200 ml of the above medium were inoculated with two discs (1 cm in diameter) cut from a 7 day-old agar (potato dextrose agar, Difco-PD) cultures of the fungi.

The inoculated flasks were incubated at 26°C for 14 days. Subsequently the mycelium was separated from the medium by filtration on filter paper and dried at 85°C to constant weight. The experiments were set in quintuplicate.

E x t r a c t i o n of cytokinin-like substances (CLS). The supernatants were adjusted to pH 2.5-3.0 with 1 N HCl and passed through a Dowex 50 W × 8 (Merck) cation exchange column H^+ form, 50-100 mesh. The column was washed with 500 ml of double distilled water and CLS were eluated with 340 ml of 2 N NH_4OH followed by 680 ml of 5 N NH_4OH. The combined eluates were evaporated to dryness in vacuo at 80-85°C to remove ammonia. The dry residue (from 1000 ml) of culture fluid was dissolved in 2 ml of 35% ethanol and applied to Sephadex LH-20 (Pharmacia, Uppsala) column 90 × 25 cm and eluated with 35% ethanol. Four fractions, 10 ml each, were collected and evaporated to dryness at 85°C.

B i o s s a y (in triplicate) was performed using soybean callus according to M i u r a and M i l l e r (1969). Three pieces of soybean — *Glycina max* (L.) Merill var. *acme* callus c 45-50 mg each were placed in each flask. The cultures were grown in an illuminated chamber at continuous illumination of 50 lux at 28°C for 25 days. The total amount of CLS produced was calculated from a standard response curve prepared for pure kinetin (Serva) and expressed as equivalents of kinetin (E.kin μg/g dry weight of mycelium).

G a s c h r o m a t o g r a p h y. This method was employed for more accurate identification of the cytokinin-like substances. For this purpose gas chromatograph Shimadzu GC-14 A was used. The samples (ethanol extracts) were dried at N_2 and silylated with BSA (N,O-bis(trimethylsilyl)acetamide) and kept for 1 hr at 80-85°C. Samples (1 μl) were injected onto a capillary column SE-54-DF-0,50 (25 m × 0,32 ID). The carrier gas was N_2 at a flow rate of 4 cm^3/min. The following cytokinin-like substances were used as standards: 2iP — (6(γ, γ-dimethylallylamino)purine, R2iP — (6(γ, γ-dimethylallylamino) purine riboside, zeatin riboside (ZR) and zeatin (Z). The conditions of the experimetn were as follows:

2iP — temperature column 200°C, temperature of detector and injector 260°C, 2iPR — temperature column 260°C, temperature of detector and injector 280°C, ZR — temperature column 280°C, temperature of detector and injector 320°C; Z — temperature column 230°C, temperture of detector and injector 280°C.

E t h y l e n e d e t e c t i o n. The fungi were cultured in sterile bottles with rubber stoppers. Some of them received 400 mg/l of methionine filter sterilized (millipore 0,45 μm) as the precursor of ethylene. Each 100 ml bottle containing 50 ml of liquid Lamb's medium was inoculated with one disc (1 cm) cut from a PD culture. The cultures were grown for 14 days at 26°C.

After 14 days of growth 1 ml ethylene-in-air were withdrawn from the flasks and analysed by gas chromatograph Shimadzu GC-14 A with Porapak P column 2 m × 1,8 (80-100 mesh). The conditions of the experiment were as follows: temperature of the column 100°C, temperature of the detector and injector 120°C. Subsequently the mycelium was separated from the medium by filtration on filter paper and dry mass was determined after drying to constant weight at 85°C. Data were evaluated statistically using the following methods: 2-factors ANOVA comparing the effects of methionine (2) and strain (1) on ethylene production and the Newman-Keuls multiple range test (comparison of averages $p \leqslant 0{,}05$).

RESULTS

It was found that *C. cibarius* strain 5400 produced less CLS than strain 5410 per gram of dry mycelium. However, the differences were not statistically significant. Thus it may be assumed that both strains produced in fact the same amount of these substances (Table 1). The largest increase of soybean callus caused by the medium of the strain 5410 was noted in fraction numbers 16-20 which correspond to 2iP and 2iP riboside and in fraction 36-40 which corresponds to zeatin when compared with the standards (Fig. 1). The 2iP and zeatin were synthesized only by the coniferous form 5410 as shown by gas chromatography (Fig. 2). No cytokinin-like substances were found in the strain 5400 when gas chrmatolography was used. Both strains of *C. cibarius* produced ethylene. However no effect of methionine, which is assummed to be the precursor of this gas, in both strains was noted (Table 2).

Table 1
Production of cytokinin-like substances by *Cantharellus cibarius*

Strain number	Quantity of CLS (in equivalents of kinetin)		Fraction No
	µg/ml medium	µg/g dry mass	
5400	0.0060	0.0132	16-32
5410	0.0048	0.0169	16-24; 40

Table 2
Production of ethylene by *Cantharellus cibarius*

Strain number	Quantity of ethylene in nM/g dry mass ± S.E.	
	without methionine	with methionine
5400	12186.72 ± 7571.72	7827.51 ± 5789.44
5410	3575.03 ± 395.98	6079.43 ± 2078.91

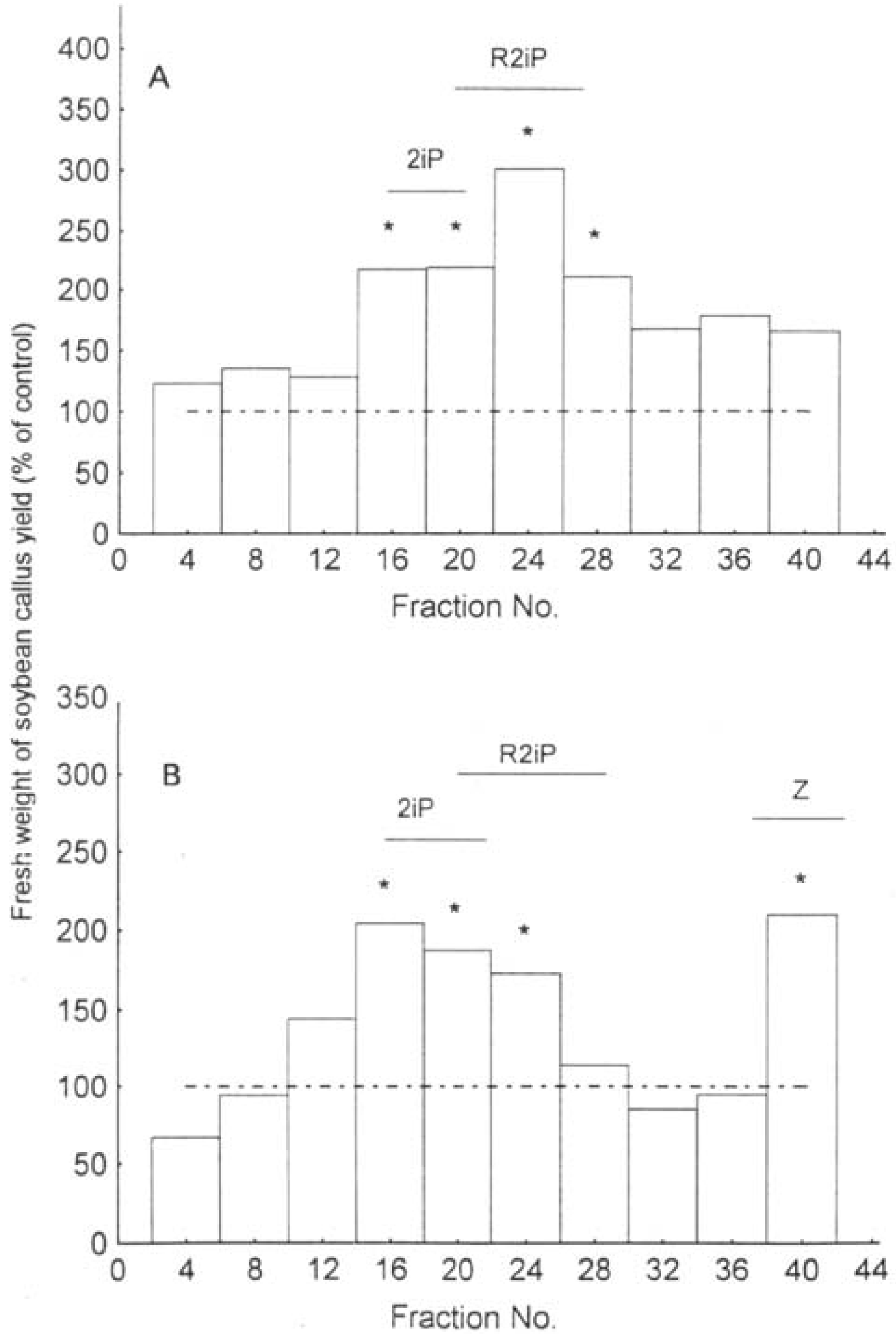

Fig 1. Column chromatographic analysis of CLS produced by *Cantharellus cibarius* 5400 (biotest soybean callus)
Horizontal dashed line — control 100%; * — significant stimulation as compared to control [t-test (n = 3) $p \leqslant 0.05$]; 2iP — 6(γ, γ-dimethylallylamino) purine; R2iP — 6(γ, γ-dimethylallylamino) purine riboside; Z — zeatin

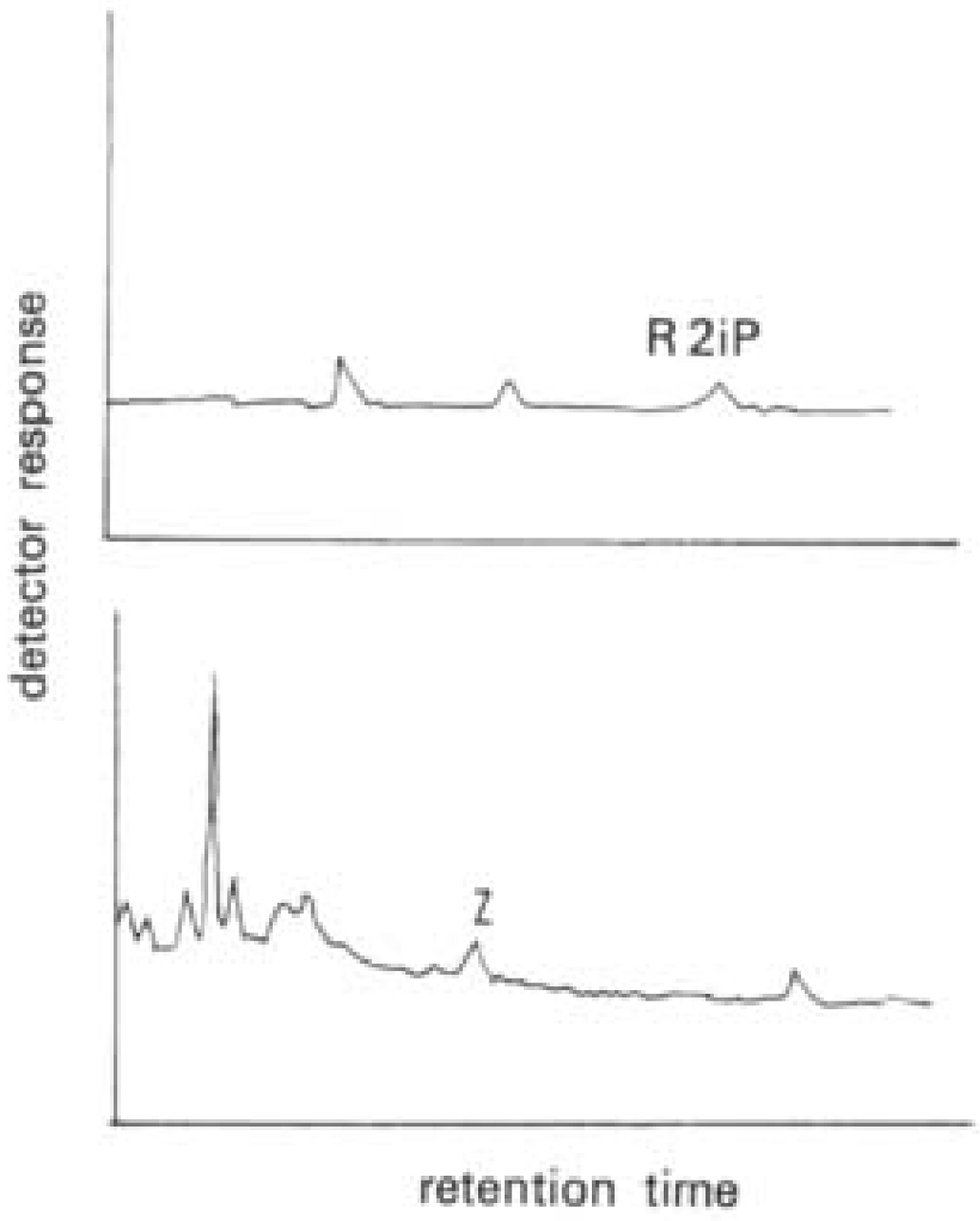

Fig. 2. Gas chromatographic analysis of CLS produced by *Cantharellus cibarius* 5410
R2iP — 6(γ, γ-dimethylallylamino) purine riboside; Z — zeatin

DISCUSSION AND CONCLUSIONS

Among the numerous possible effects that soil microorganisms may exert on plants is the production of plant growth regulators (K a t z n e l s o n 1965; G a r b a y e 1991; G o g a l a 1991). Because of intensive development of microorganisms in the root zone it might be expected that the greatest amount of these substances would be produced therein by them (K a t z - n e l s o n 1965). Among the metabolites affecting mycorrhiza formation and functioning plant growth regulators are being considered of importance (H o r a k 1965; S l a n k i s 1973; G o g a l a 1991). Auxins and cytokinins were most often studied (K a m p e r t, S t r z e l c z y k 1980; S t r z e l - c z y k, K a m p e r t 1983; S t r z e l c z y k at al. 1989) as they are thought to be of the utmost importance in plant mycorrhiza relationships. On the other hand data on the production of ethylene, especially by mycorrhizal fungi, are scarce and have been investigated only recently (G o g a l a 1991). However it is assumed that ethylene in addition to auxins and cytokinins plays an important role in mycorrhiza formation and functioning (S l a n k i s 1973). S m i t h and R e s t a l l (1971) indicated that ethylene was normally produced under anaerobic conditions. However, it was found that the importance of such conditions depends upon the provision of substrates. When glucose and methionine (which is supposed to be a precursor in ethylene

production) are provided, the production of ethylene by *Mucor hiemalis* and by soil is stimulated (Lynch, Harper 1974). No significant differences in ethylene production between both strains studied in our work (with and without methionine) were found. Also cytokinins were synthesized by both forms of *Cantharellus cibarius*.

Fungi form the largest and probably the most important portion of the microbial population of forest soils. Yet most research on these organisms concerned ecological and taxonomical rather than physiological studies (Mańka, Truszkowska 1958; Kowalski 1974). Therefore the objective of this work was to study the production of cytokinin-like substances and ethylene by the important and poorly studied fungal species *Cantharellus cibarius*. Such studies are certainly of biological and ecological importance. However we are aware of the danger of extrapolation from studies *in vitro*. Nevertheless it seem that it is essential to isolate organisms in order to determine their physiological, biochemical and other activities and to find out what they are capable of doing under controlled conditions. Earlier studies and the present data indicate that the production of plant growth regulators is quite common among symbiotic and non-symbiotic soil microorganisms (Strzelczyk et al. 1989). The plant growth regulators studied were produced by both strains of *C. cibarius*.

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Wytwarzanie substancji typu cytokininy oraz etylenu przez ektomikoryzowy grzyb *Cantharellus cibarius*

S t r e s z c z e n i e

Stwierdzono, że *Cantharellus cibarius* pochodzący z lasu liściastego wytwarzał mniej substancji typu cytokininy aniżeli zebrany w lesie iglastym. Wśród aktywnych substancji wykryto 2iP, 2iP hydrazyd i zeatynę. Stwierdzono nieznaczną różnicę w wytwarzaniu etylenu w obecności metioniny (uważanej ze prekursora tego gazu) przez obydwa szczepy.