THE EFFECT OF A FUNGAL PREPARATION ON TISSUE CULTURES OF *HOLARRHENA ANTIDYSENTERICA* (ROXB.) WALL.

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ABSTRACT

The addition of an aqueous extract from fruitbodies of *Tylopilus felleus* to tissue cultures of *Holarrhena antidysenterica* (Apocynaceae) caused the accumulation of an unknown compound in the culture medium. The compound was isolated and identified as 5-hydroxymethyl-2-furancarboxaldehyde (1). Moreover, biosynthesis of phenolic compounds was stimulated in response to the stress agents of the fungal preparation. Methyl ferulate (2) was the major phenolic constituent.

KEY WORDS: *Holarrhena antidysenterica* (Roxb.) Wall., tissue culture, fungal preparation, stress metabolites

INTRODUCTION

Stress agents (elicitors) may stimulate biosynthesis of some secondary metabolites in plant cells (Eilert 1987). Elicitor activity has been shown for compounds of fungal origin e.g. cell wall-derived β -1, 3-glucans (Ebel et al. 1976, Nichols et al. 1980). Polysaccharides of this type were separated from an aqueous extract of fruitbodies of *Tylopilus felleus* (Bull. ex. Fr.) P. Karst. (Boletaceae), one of them, called tylopilan, was characterized as a branched β -(1 $\rightarrow$ 3), (1 $\rightarrow$ 6)-D-glucan (Kohlmünzer et al. 1980, Defaye et al. 1988).

The present study describes the effect of an aqueous fungal extract on tissue cultures of *Holarrhena antidysenterica* (Roxb.) Wall. (Apocynaceae). The presence of alkaloids, i.e. conessine and conimine, characteristic natural antibacterial products of this species, was reported previously in its tissue culture (Dohnal et al. 1990).

MATERIAL AND METHODS

FUNGAL PREPARATION

Dried and powdered fruitbodies of *Tylopilus felleus* were exhaustively extracted with hot water. Combined extracts were lyophilized, 1 g of the lyophilizate corresponded to 3 g of dried material.

TISSUE CULTURE

A tissue culture of the tropical species *Holarrhena antidysenterica*, derived from the hypocotyl of sterile seedlings, was grown on Murashige and Skoog's basic medium (MS), supplemented with growth regulators (2,4-D - mg/kg; kinetin - 0.3 mg/kg), under constant illumination, ca. 1000 lux, at 28 °C. The stationary growth phase was achieved after ca. 3-4 weeks following inoculation (growth index (GI) = 3).

INCUBATION OF CALLUS TISSUES WITH THE FUNGAL PREPARATION

1. A dose-response test was conducted on MS agar (0.8%) medium containing 9 consecutive concentrations of the fungal preparation within the range of 0.05 - 4.0 %, calculated for dried fruitbodies. The tissue (250 mg) was transferred onto 20 ml of the medium in 5 replications per dose. After a four-week incubation period under the same conditions, GI was calculated as a percentage of the control.

2. A large scale experiment was carried out under stationary condition on MS liquid medium containing 0.6 % of the fungal preparation which was added to the callus culture three weeks after inoculation. Incubation was performed for 4 days under the same conditions as above.

EXTRACTION AND A PRELIMINARY CHROMATOGRAPHIC ANALYSIS

Lyophilized tissues and culture media were extracted separately by a previously described method (Dohnal et al. 1990) yielding crude acidic and alkaline extracts. The extracts were analysed by TLC on silica gel plates (Merck, Art. 5554). Spots were visualised by spraying with 20 % H₂SO₄ and with specific reagents for alkaloids and phenolics. The R_f values for conessine, conimine, 1 and 2 were 0.55, 0.15, 0.32 and 0.15, respectively, in the benzene-EtOAc-Et₂NH (6:3:1) solvent system.

ISOLATION AND IDENTIFICATION OF 5-HYDROXYMETHYL-2-FURANCARBOXALDEHYDE (1) AND METHYL FERULATE (2)

The experiment performed on a medium containing 0.6 % of the fungal preparation, yielded 50 g of lyophilized tissue (850 g, fresh wt) and 3.2 l of culture medium.

The combined crude acidic and alkaline extracts of the medium (144 g) were applied to a silica gel column (Merck, 70-230 mesh) packed in benzene. The column was eluted with

an increasing CHCl_3 gradient in benzene. The fractions eluted with a 1:1 benzene- CHCl_3 mixture contained 1 which was purified by preparative TLC on silica gel (Merck, Art.5745) in the same solvent system as above to give 53 mg of a yellow-brown solid. $^1\text{H-NMR}$ (300 MHz, DMSO-d_6) δ : 9.64 (1H, s, -CHO), 7.59 (1H, d, $J=3.5$ Hz, H-3), 6.70 (1H, d, $J=3.5$ Hz, H-4), 5.67 (1H, t br, -OH), 4.60 (2H, s, $-\text{CH}_2\text{-O}-$); IR (CHCl_3) cm^{-1} : 3408 (OH), 1668 (CO), 1616 ($\text{C}=\text{C}$), 1472, 1400, 1024; UV (MeOH) nm: 231, 249, 297; EI-MS m/z (rel. int. %): 126 (M^+) (82.4), 109 ($\text{M}-17$) $^+$ (23.2), 97 ($\text{M}-29$) $^+$ (100), 94 ($\text{M}-32$) $^+$ (18.5), 81 ($109-28$) $^+$ (5.2), 69 (24.8), 58 (22.6), 53 (21.2).

The crude alkaline tissue (60 mg) extract was chromatographed on a Chromatotron apparatus using a CH_2Cl_2 - MeOH (8:2) mixture as the mobile phase on silica gel (Merck, Art. 7741). The eluate containing the major component was subjected to prep. TLC on silica gel (Merck, Art. 5715), in CH_2Cl_2 , affording 13 mg of 2. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ : 7.62 (1H, d, $J=16$ Hz, H-8), 7.07 (1H, dd, $J=8$ and 2 Hz, H-6), 7.03 (1H, d, $J=2$ Hz, H-2), 6.92 (1H, d, $J=8$ Hz, H-5), 6.29 (1H, d, $J=16$ Hz, H= α), 3.93 s, 3.73 s (2 x OCH_3); IR (CHCl_3) cm^{-1} : 3440 (OH), 1705 (CO), 1645, 1610, 1520 ($\text{C}=\text{C}$), 1275, 1180; UV (EtOH) nm: 219, 235, 324; EI-MS m/z (rel. int. %): 208 M^+ (100), 177 ($\text{M}-31$) $^+$ (45.0), 149 ($\text{M}-31-28$) $^+$ (6.6), 145 ($177-32$) $^+$ (15.0), 117 ($145-28$) $^+$ (5.2), 85 (14.8), 55 (30.8).

RESULTS AND DISCUSSION

The effect of different doses of the fungal preparation containing β -1, 3-glucans, known as phytoalexin elicitors, on tissue cultures of *Holarrhena antidysenterica* was studied. It was found that doses lower than 0.1 % had a stimulating effect on callus growth (up to 20 %), while higher doses exerted an inhibitory effect; a 0.6 % dose reduced the growth to 50 % of the control culture. Doses higher than 2 % appeared to be toxic for plant tissues, causing tissue necrosis.

Extracts from tissues and culture media were analysed by TLC. The results of this analysis showed that the chromatographic profile of extracts containing less than 0.6 % of the

On the basis of these studies, a large scale experiment was carried out using a medium containing 0.6 % of the fungal preparation in order to obtain a sufficient amount of the material for isolation. The compound extracted from the culture medium was isolated by silica gel column chromatography and accounted for 0.1 % by weight of lyophilized tissue after 4 days of experiment. It was identified as 5-hydroxymethyl - 2-furancarboxaldehyde (1) (Fig.1.) (Shen and Mu 1990) on the basis of spectral analysis. The enclosed spectral data have reference value. The extract of the tissue was subjected to radial chromatography followed by prep. TLC affording the major component in ca. 0.03 % yield. Its identity with methyl ferulate (2) was confirmed by spectral methods.

The elicitors present in the fungal preparation did not stimulate the biosynthesis of alkaloids in the tissue culture. As a consequence of their action the furan derivative 1 was accumulated and the metabolism of phenolic compounds was accelerated. Phenylpropanoid-derived methyl ferulate (2) was found to be the major constituent of the phenolics. Previous reports have shown that polysaccharide fractions stimulate phenylpropanoid metabolism. The activity of the fractions resides in the glucan component and is believed not to be specific but, rather, a part of a general defensive response of plants (Ebel. et al. 1976, Eilert 1987).

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LITERATURE CITED

- DEFAYE J., KOHLMÜNZER S., SODZAWICZNY K., WONG E., 1988. Structure of an antitumor, water-soluble D-glucan from the carpophores of *Tylopilus felleus*. Carbohydrate Res. 173: 316-323.
- DOHNAL B., MIĘDZYBRODZKI J., WŁODARCZYK B., 1990. Alkaloids in the tissue culture of *Holarrhena antidysenterica*. Acta Polon. Pharmac. - Drug Res. 47: 71-74.
- EBEL J., AYERS A.R., ALBERSHEIM P., 1976. Host-pathogen interactions. Plant Physiol. 57: 775-779.
- EILERT U., 1987. Elicitation: methodology and aspects of application. In: Cell culture and somatic cell genetics of plants. Constabel F., Vasil I. K., (eds) Academic Press, San Diego-New York-Berkeley-Boston-London-Sydney-Tokyo-Toronto, vol. 4, pp. 153-196.
- KOHLMÜNZER S., GRZYBEK J., TANAKA M., 1980. Antitumor and cytotoxic activity of polysaccharides from *Tylopilus felleus*. Planta Med. 39: 231-232.
- NICHOLS E.J., BECKMAN J.M., HADWIGER L.A., 1980. Glycosidic enzyme activity in pea tissue and pea-*Fusarium solani* interactions. Plant. Physiol. 66: 199-204.
- SHEN Y., MU Q., 1990. New furans from *Cirsium chlorolepis*. Planta Med. 56: 472-474.

Fig.1. The chemical structures of isolated compounds 1 and 2.

preparation did not differ from the control. Trace amounts of previously described alkaloids (Dohnal et al. 1990) were detected in the tissues as well as blue-absorbing compounds in UV light. Doses of 0.6 % and over caused no changes in alkaloid production but stimulated biosynthesis of fluorescent compounds which gave coloured spots on chromatograms when sprayed with specific reagents for phenolics. Moreover, the formation of an unknown compound was observed for the first time in the tissue culture; it was found to be the main constituent of the culture medium.

WPLYW PREPARATU GRZYBOWEGO NA HODOWLĘ TKANKOWĄ
HOLARRHENA ANTIDYSENTERICA (ROXB.) WALL.

STRESZCZENIE

Przeprowadzono próby pobudzenia biosyntezy metabolitów wtórnych w tkance kalusowej *Holarrhena antidysenterica* (Apocynaceae) przez zastosowanie preparatu grzybowego - wodnego wyciągu z owocników *Tylopilus felleus*, zawierającego β -1, 3-glukany. Czynniki stresogenne występujące w preparacie spowodowały pobudzenie metabolizmu związków fenolowych, których główny składnik zidentyfikowano jako ester metylowy kwasu ferulowego. Ponadto w pożywce pohodowlanej stwierdzono obecność aldehydu 5-hydroksymetylo-2-furylowego, który nie występował ani w pożywce, ani w tkance kontrolnej.

SŁOWA KLUCZOWE : *Holarrhena antidysenterica* (Roxb.) Wall., hodowla tkankowa, preparat grzybowy, czynniki stresogenne