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Elicitor of the β-Thujaplicin Accumulation in Callus Cultures of *Cupressus lusitanica*.*1

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The addition of yeast extract to callus cultures of Cupressus *lusitanica* leads to a large increase in the production of β- thujaplicin that acts as a phytoalexin of the treated cultures. γ- Thujaplicin was not formed in any appreciable amounts. The content usually reached 8-16 mg/g of dry weight of callus in 6 days of incubation after the addition of yeast extract. This accumulation of β- thujaplicin suggested the presence of elicitor components in the yeast extract. Therefore, the extract was fractionated by means of ethanol precipitation, ribonuclease treatment and complexation with the Fehling reagent. Some fractions of the extract actively induced the β- thujaplicin accumulation in the Cupressus ***Zusitanica*** callus. The most effective fraction was purified about 50 - fold over unfractionated extract, and was a polysaccharide which was composed of mainly mannose, glucose, and to a lesser degree ribose residues.

INTRODUCTION

Plants frequently accumulate phytoalexins, low molecular weight toxins which inhibit the growth of microorganisms, in their tissues to express resistance to invading pathogens (Darvill and Albersheim, 1984). This response is regarded as one of the defence systems characteristic of plant cells that lack immune systems present in animal cells (Kurosaki, 1990). Some of elicitors which induce the phytoalexin formation originate from the microbial cell wall. Thus glucan fragments (Albersheim et al., 1981) and chitosan (Hadwiger and Beckman, 1980) were identified as elicitors of the phytoalexin production in soybeans and pea-pod tissues, respectively.

Some heart wood constituents are likely to be phytoalexins because they are of the fungicidal and insecticidal natures and are sometimes formed in the uninjured sapwood tissue adjacent to the damaged cells when trees are damaged (Hart, 1989). Therefore, if phytoalexin production in cultured cells of softwood species can be controlled by elicitation, that would be a good model system for the biochemical study of heart wood constituents.

Recently Sakai et ***al.*** (1993) reported that β- thujaplicin, a typical heart wood constituent of the Cupressaceae species, was accumulated in the callus culture of Cupressus *lusitanica* when treated with yeast extract and culture filtrates of some fungi. In this paper we deal with the partial purification and characterization of the elicitor present in yeast extract for the β-thujaplicin production in C. *lusitanica* callus cultures,

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MATERIALS AND METHODS

Identification of phytoalexin

Three grams of *Thuja plicata* wood meal were extracted with 100 ml of methanol. The methanol extract was evaporated and the residue successively subjected to silica gel column chromatography and a preparative thin layer chromatography using benzene-methanol -acetic acid (20:1:1) as an eluent. Thujaplicins were visualized by spraying with iron (III) acetate upon thin layer chromatography plates. The thujaplicin-rich fraction was subjected to ^1H NMR. The spectrum was recorded in $CDCl_3$ on a spectrometer JNM-GSX 400 using tetramethylsilane as an internal standard and the composition of α-, β- and γ- thujaplicins was estimated. Identification of the phytoalexin in C. *lusitanica* callus was performed by means of co - chromatography with the wood thujaplicins on gas chromatography (GC). GC column and temp. : Neutrabond-1 (25 m $\times$ 0.53 mm I.D.) and at 130-180°C with program rate 2°C/min.

Purification of the elicitor

1) Ethanol precipitation

Commercial yeast extract (DIFCO) was used throughout this work. For the purification of the elicitor from yeast extract, we followed the ethanol precipitation procedure of Hahn *et al.* (1978). An aqueous solution of yeast extract (150 g/l) was fractionated into the 80%-ethanol-soluble (YE2) and the precipitate (YE3). The latter was dissolved in 1000 ml of water, and fractionated into the 60%-ethanol soluble (YE4) and the precipitate (YE5). Unfractionated yeast extract was designated as YE1.

Sugar and protein contents in each fraction were determined by the phenol -sulfuric acid method (Dubois et *al., 1956)* and with a protein assay kit with standard II (BIO-RAD Lab.), respectively.

2) Enzymatic treatment and acid hydrolysis of YE5

YE5 (180 mg / 10 ml) was incubated with 2.5 μ g (905 units) of ribonuclease (Worthington Biochemical Co.) in 5 ml of 0.2 M Tris-HCl buffer (pH 7.5), 2.5 ml of 0.02 M EDTA and 2.5 ml of reagent grade water for 24 h at 37°C. The enzymatic treatment was terminated by autoclaving for 2 min at 120°C. After centrifuging the reaction mixture, the supernatant was evaporated to 10 ml, applied to a Sephadex G-75 column (35 cm $\times$ 3 cm I.D.) and eluted with reagent grade water at 1.7 ml /min. Absorbance was monitored at 280 nm, and at 490 nm by the phenol -sulfuric acid method. The fractions eluted from 51 to 136 ml of elution volume and from 136 to 225 ml were separately collected and designated as YE5A and YE5B, respectively (cf. Fig. 3).

For acid hydrolysis, 180 mg of YE5 was heated for 1 h at 120°C in 10 ml of 3% trifluoroacetic acid which was vacuum-evaporated after the hydrolysis.

3) *Fractionation with the Fehling reagent*

The elicitor -active fraction (YE5A) was further fractionated into soluble and precipitate fractions with the Fehling reagent according to the method of Peat *et al.* (1961). The supernatant (YE5Aa) and precipitate (YE5Ab) were separately acidified to pH 3.0 with 1 M HCl, and then chromatographed on the above-mentioned Sephadex

G-75 column to remove inorganic salts. The fractions, which eluted between 51 and 136 ml, were collected.

Analysis of the sugar composition

The elicitor fractions (2-15 mg) were hydrolyzed in 72% H_2SO_4 (2 ml) at 30°C for 1 hour and then the reaction mixture was diluted and hydrolyzed in 3% H_2SO_4 at 100°C for 8 hours according to the method of Selvendran et al. (1979).

The sugar moieties were converted to alditol acetates according to Sutherland et *al.* (1992) except where the reduction was performed with 7 mg $NaBH_4$ for 90 min and that 0.05 ml of pyridine and 0.1 ml acetic anhydride were employed for acetylation. A GC analysis was carried out with 3% ECNSS-M on Gas Chrom Q (100-120 mesh) in a glass column (2 m *x* **3.0** mm) at 170-200°C with a program rate of 2°C/min.

Culture condition

The cell culture of C. *lusitanica* (Sakai et *al.,* 1993) grown on the Gamborg B5 medium (Gamborg *et al.,* 1968) containing 2% sucrose, 10^{-5} M NAA, 10^{-8} M BAP and 0.27% gellan gum in the dark at 25°C was transferred to a fresh medium every 3 - 4 weeks. Prior to the start of the present experiment the cultures had been maintained *in vitro* for more than three years.

Assay for elicitor activity

A 1 ml aliquot of solution of each yeast extract fraction was added dropwise onto about 1 g of C. *Zusitanica* callus on the growth medium described above. Water (1 ml) was added for the control experiments. After a six -day incubation, the callus was pulverized with a pestle and mortar and extracted twice with 6 ml of ethyl acetate. β-Thujaplicin content in the ethyl acetate extract was determined by means of GC after decomposing thujaplicin-iron complexes by treating the extract with a mixture of 6 M HCl and 10% potassium ferrocyanide. A GC analysis was performed as described above for the phytoalexin identification.

RESULTS AND DISCUSSION

Identification of phytoalexin

^{1}H NMR spectra indicated that a thujaplicins mixture isolated from *T. plicata* contained P-and y-thujaplicins (about 1:2) but no a-thujaplicin. Gas chromatograms of the thujaplicin mixture and methanol extracts from C. *hsitanica* callus are shown in Fig. 1. It is noteworthy that *C. Zusitanica* callus culture specifically accumulated β-thujaplicin but almost no y-thujaplicin, even though Zavarin *et al.* (1967) reported the presence of both β- and y- thujaplicins in heartwood samples of *C. lusitanica*. β-Thujaplicin, a characteristic heartwood constituent of Cupressaceae species, possesses an antimicrobial activity which gives strong durability to wood (Trust et *al.*, 1973) and this compound was produced in a considerable amount when *C. Zusitanica* callus was treated with some fungal culture filtrates or yeast extract (Sakai *et al.,* 1993). Therefore, β-thujaplicin may be regarded as one of phytoalexins in C. *lusitanica* callus and yeast extract is expected to contain an elicitor component.

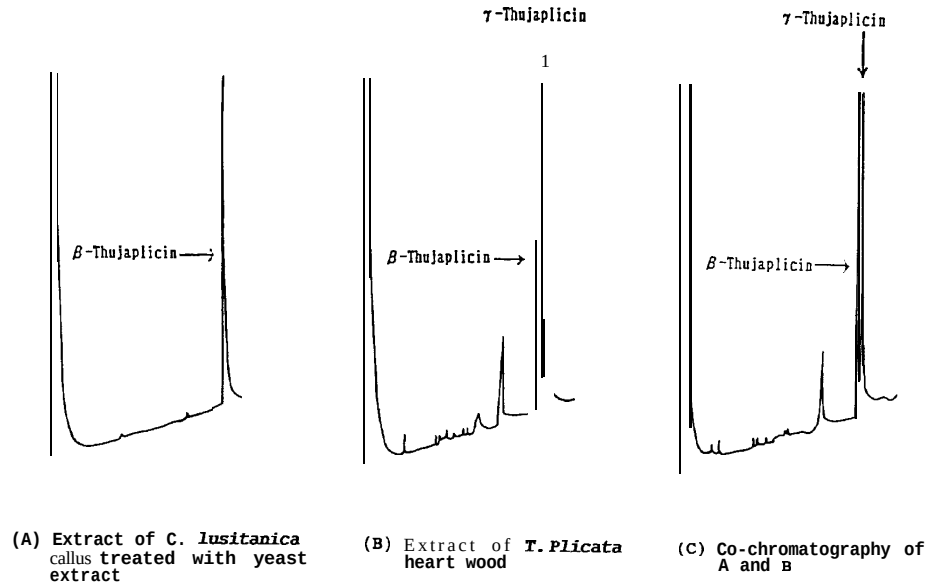

Fig. 1. Identification o phytoalexin.

Purification of the elicitor from yeast extract

1) Ethanol precipitation

The yeast extract solution was fractionally precipitated by changing the ethanol concentration of the solution. Thus, YE1 was designated as unfractionated yeast extract; YE2 and YE3 as 80%ethanol soluble and insoluble, and; YE4 and YE5 as 60% ethanol soluble and insoluble, respectively.

Contents of β-thujaplicin in the callus treated with YE1 through YE5 are shown in Fig. 2. The elicitor activities of YE1, YE3 and YE5 were at almost the same level (8 mg of β-thujaplicin/d.w.g. of callus) whereas YE4 had rather weak activity (5 mg/ d.w.g). YE2 possessed very weak elicitor activity (1 mg/d.w.g). The weight of YE5 accounted for only 12% of unfractionated yeast extract YE1 (Table 1) in spite of its strong elicitor activity. This indicates that YE5 is the most purified fraction. Therefore, further purification of the elicitor was attempted with YE5.

2) Enzymatic treatment and acid hydrolysis of YE5

Yeast extract contains large amounts of nucleic acids and their fragments. YE5 contained as little as 0.6% protein in spite of its large absorptivity at 280 nm (Table 1). Furthermore, the protein content was not proportional to the absorptivity at 280 nm of each fraction. These results suggest that the substances responsible for absorption at 280 nm were not proteins but mainly nucleic acid derivatives. Accordingly YE5 was digested with a ribonuclease prior to the gel filtration chromatography to show if nucleic acids have any distinct effects on the elicitor activity of the yeast extract. Substances that absorbed at 280 nm were markedly depolymerized, indicating that these substances were ribonucleic acids, while molecular the weight distribution of

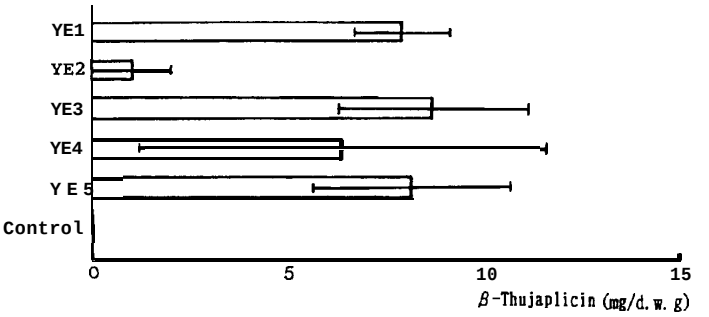

Fig. 2. Elicitor activity of YE1-YE5.
Note : Error bar shows standard deviation.

Table 1. Properties of fractions obtained by the ethanol-precipitaion method.

	Yield (%)	Sugar content (%)	Protein content (%)	Absorptivity at 280 nm (l/g·cm)
YE1	100.0	19.0	0.27	1.607
YE2	61.3	11.2	0.26	1.838
YE3	38.6	31.6	0.29	1.601
YE4	27.5	18.2	0.16	1.375
YE5	12.0	58.9	0.60	2.550

polysaccharide was kept unchanged (Fig. 3). Consequently, we successfully fractionated YE5 into a polysaccharide-rich fraction (YE5A), and another fraction which was rich in nucleic acid fragments (YE5B). Ribonuclease-treated YE5 held its elicitor activity the same as untreated YE5. The polysaccharide-rich fraction, YE5A, induced the β-thujaplicin accumulation in callus at the same level as YE5 did. The elicitor activity of YE5B was rather small (Fig. 4).

Both polysaccharide and nucleic acid in YE5 were markedly degraded by acid hydrolysis (data not shown). Content of β-thujaplicin in C. *lusitanica* callus treated by acid hydrolyzed YE5 was as small as that in control (Fig. 4). This suggests that elicitor in YE5 was completely degraded by acid hydrolysis. These results suggest that the elicitor in yeast extract was polysaccharide and support the observations of Brodelius et *al.* (1989), Hahn *et al.* (1978) and Sumaryono *et al.* (1991).

3) *Fehling precipitation*

It was confirmed by Phaff et al. (1977) that a large amount of mannan was present in yeast extract. We, therefore, employed the Fehling precipitation in order to fractionate YE5A into precipitate (YE5Ab) and supernatant (YE5Aa) fractions. The non-precipitated fraction, YE5Aa, elicited the accumulation of 6 mg/d.w.g of β thujaplicin which was about half the elicitor activity of YE5A (Fig. 4). The decrease of elicitor activity of YE5Aa as compared with YE5A is possibly due to the loss of a portion of YE5Aa during gel filtration on a Sephadex G 75 column for removing

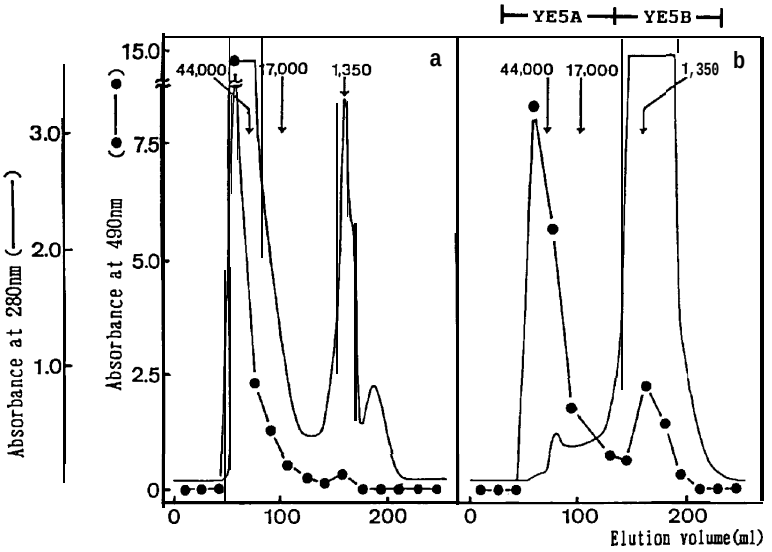

Fig. 3. Molecular weight distribution of YE5 treated with ribonuclease.
Notes: Arrows (↓) designate elution volume of molecular weight markers.
a: YE5, b: YE5 treated with ribonuclease.

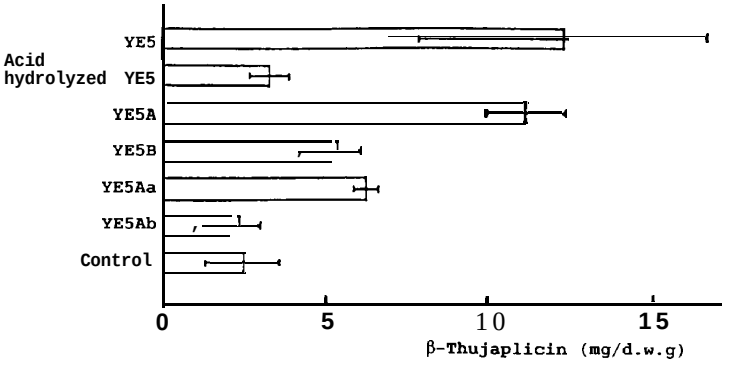

Fig. 4. Elicitor activity at different stages of purification.
Note: Error bar shows standard deviation.

inorganic salts. YE5Ab which is considered to be mannan did not possess elicitor activity.

Figure 5 shows the dose-response of β thujaplicin accumulation in C. *lusitanica* callus for the elicitor fractions at the different stages of purification. YE5Aa with the highest specific activity stimulated the accumulation of about 11 mg of β-thujaplicin/ d.w.g at the concentration of 0.2 mg/ml. On the other hand, 10 mg/ ml of YE1 stimulated about 12 mg of β- thujaplicin /d.w.g accumulation. Thus, YE5Aa was purified about 50-fold over unfractionated yeast extract.

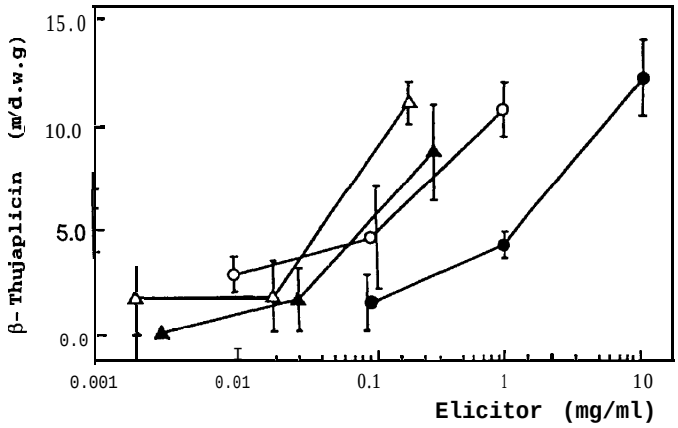

Fig. 5. Dose-response curves of elicitor fractions at different stages of purification.
Note: Error bar shows standard deviation.
Legend: ●: YE1, ○: YE5, ▲: YE5A, △: YE5Aa.

Table 2. Sugar composition of elicitor fractions.

	Yield" (%)	Sugar content (%)	Sugar composition (%) Mannose	Glucose	Ribose
YE5A	3.3	76.1	85.3	12.4	2.4
YE5Aa	0.8	99.6	50.9	45.4	3.7
YE5Ab	2.0	91.2	94.4	5.6	Trace

1) Yeild is expressed as the percent on yeast extract.

Analysis of the sugar composition

The biotic components with elicitor activity are heterogeneous in chemical structure and size as elicitors of different structures have been isolated not only from phytopathogenic fungi but also from the cell walls of plants themselves (Darvill and Albersheim, 1984).

Sugar compositions of elicitor fractions were, therefore, analyzed and listed in Table 2. The most purified fraction, YE5Aa, contained mannose, glucose, and ribose residues in the ratio of 51:45:4. This result leads to the conclusion that the elicitor of the β-thujaplicin accumulation in C. *lusitanica* callus isolated from yeast extract may be a glucomannan. It was reported that yeast extract elicited accumulation of rosmarinic acid in *Orthosiphon aristatus* cell suspension cultures (Sumaryono *et al.*, 1991). Two elicitors in yeast extract were shown to be carbohydrate polymers having molecular weights of *ca.* 22,000 and 7,000 and containing mainly mannose, glucose, and to a lesser degree galactose. These elicitors of the rosmarinic acid production are quite similar to the elicitor of the β-thujaplicin accumulation in C. *lusitanica* callus, although there is a small difference in their sugar compositions.

The elicitor -inactive fraction, YE5Ab, was composed of almost solely mannose. This suggests that mannan is not elicitor of β-thujaplicin accumulation. This was

further confirmed by the fact that commercial yeast mannan did not show any detectable elicitor activity.

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