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Effect of *Botrytis cinerea* infection and elicitation on β-1,3-glucanase and chitinase activity in bean leaves and cell cultures

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Abstract

The activity of β-1,3-glucanase and chitinase in bean plants treated with *B. cinerea* products or/and infected and in cell cultures after application of fungal products has been studied. *Botrytis cinerea* infection and culture filtrates, ethanol precipitates, glucan and conidial extract treatment markedly enhanced the activity of both hydrolases. Cell cultures treated with *B. cinerea* products reacted similarly to intact plants. In plants pretreated with 2-day culture filtrate and conidial extract and then infected, β-1,3-glucanase and chitinase were induced stronger than after infection without pretreatment.

INTRODUCTION

The induction of β-1,3-glucanase and chitinase in plants infected with fungi and bacteria has been observed in many studies (M e i n s and A h l, 1989; M a u c h et. al., 1984). It has been suggested that activity increase of β-1,3-glucanase and chitinase is part of the biochemical defence of plants against pathogenic microorganisms, since chitin and β-1,3-glucan are major components of cell walls of many fungi and bacteria (B e n h a m o u et al., 1990). In support of this hypothesis, it has been shown that chitinase in combination with β-1,3-glucanase can partially decompose isolated fungal cell walls (M a u c h et al., 1988). These enzymes could not only degrade fungal wall constituents causing growth inhibition but also produce glycosidic fragments acting as elicitors of other defence reactions (K e e n and Y o s h i k a w a, 1983).

Some of biochemical defence responses observed in infected plants can be also induced by elicitors of pathogen origin. Results from several studies have demonstrated the increase in the activity of lytic enzymes β-1,3-glucanase and chitinase in response to treatment with elicitors (H u g h e s and D i c k e r s o n, 1991). Elicitors include multicomponent preparations as well as defined compounds and can vary considerably in chemical nature. They are thought to play an important role as signal molecules for the induction of plant defence mechanism.

Suspension cultures treated with elicitors provide a useful model for studying defence reactions of plants because the use of cultured cells instead of the whole plant facilities elucidation of highly complicated plant-pathogen interactions. Furthermore, it is worth examining whether defence reactions observed in infected plants occur in cell cultures with regard to the fact that *in vitro* cultured cells can be used as a tool in biotechnological methods developed to obtain plants resistant to pathogens.

In this work we compared changes in the activity of β-1,3-glucanase and chitinase in bean plants infected and treated with *B. cinerea* products and in suspension cultures after application of fungal products.

MATERIAL AND METHODS

Bean plants (*Phaseolus vulgaris* L., cv. Gold Saxa) were grown in a growth chamber at 20°C. At the age of two weeks plants were sprayed with *Botrytis cinerea* Pers. products: conidial extract, 2-day and 5-day culture filtrates, 2-day and 5-day ethanol precipitates and glucan. Three days later the secondary leaves of one part of elicited plants were inoculated with *B. cinerea* conidial suspension containing 5 x 10^5 conidia in 1 ml. After inoculation all plants were maintained at 100 % relative humidity. Two days later leaves were harvested and used for estimation of β-1,3-glucanase and chitinase activities. Plants nontreated with fungal products but infected were grown simultaneously. Nontreated and noninfected plants were used as control.

Bean cell suspension cultures were established from three-week-old callus tissues. Cell cultures were grown in Gamborg's B5 medium (D i x o n, 1985) supplemented with 0.2 mg/l 6-benzylaminopurine and 1 mg/l 2,4-dichlorophenoxyacetic acid in the dark at 23°C on a rotary shaker (120 rpm) and were transferred every 12 days. *B. cinerea* products (2 ml per 30 ml of culture containing 1 x 10^5 cells in 1 ml) were applied to cell cultures on the 6th day after subculture. Controls received an equivalent volume of sterile water. The enzymatic activity was assayed 24 and 48 h after treatment with *B. cinerea* products. Bean cells were counted in a hemocytometer.

Preparation of *B. cinerea* products: *B. cinerea*, a bean pathogen, was grown in surface culture at 24°C for 2 and 5 days in a medium composed of (in g/l): KH_2PO_4 – 1.75; $MgSO_4$ – 0.75; $CaCl_2$ – 0.5; glucose – 10.0; pectin – 10.0; pepton – 4.0, pH – 5.0. For 2-day cultures the medium was diluted 10 times. The cultures were grown in 1 l Erlenmayer flasks containing 100 ml of medium and 10 ml of inoculum.

The inoculum was a conidial suspension with a density of 1 x 10^6 conidia/ml for 2-day cultures and of 1 x 10^5 conidia/ml for 5-day cultures. The cultures were centrifuged after 2 and 5 days of culturing, respectively. The supernatants called 2-day and 5-day culture filtrates were used for elicitation. Ethanol precipitates from 2-day and 5-day cultures were obtained by adding to the equal volumes of the respective filtrates 96 % ethanol to a final concentration of 80 %. Glucan was prepared from 5-day culture filtrate by adding ethanol to 40 % saturation. The glucan precipitate was washed 2 times with distilled water and centrifuged. Before use, ethanol precipitates and glucan were resuspended in distilating water to the volume of the initial filtrates. *B. cinerea* potato-dextrose agar cultures were rinsed with 0.2 M NaCl in 0.01 M acetate buffer pH 5.0 to obtain conidial suspension containing 1 x 10^6 conidia/ml. The suspension was mixed for 30 min. at 10°C and centrifuged. The resulting conidial extract was used for elicitation.

Preparation of enzyme extracts: The leaves were homogenized in 1 M NaCl in 0.1 M acetate buffer pH 5.6, 1 : 1 w/v, mixed for 20 min. at room temperature and centrifuged. The sediment was washed with the same solution (1 : 4 w/v) and centrifuged again. Both supernatants were examined together for enzyme activities. The suspension cultures were centrifuged, sediments washed with 0.1 M acetate buffer pH 5.6 and centrifuged again. Both supernatants were examined for extracellular enzyme activities. To obtain the intracellular enzymes the sediments were homogenized in 1.0 M NaCl in 0.1 M acetate buffer pH 5.6, centrifuged and washed with the same solution.

Enzyme assays: The activities of β-1,3-glucanase and chitinase were determined by following the release of reducing groups according to Nelson's method (N e l s o n, 1944) using 0.5 % laminarin (Sigma) as a substrate for β-1,3-glucanase and 1 % chitin (Sigma) as a substrate for chitinase. The enzymatic activity was expressed in nkatal/g fr. wt. and in the case of suspension cultures in pkatal/10^5 cells. All results are the means of three separate experiments.

RESULTS

In bean plants infected with *B. cinerea* a considerable increase in β-1,3-glucanase and chitinase activity was observed (Fig. 1, 2). The activity of β-1,3-glucanase and chitinase increased 75 % and 200 %, respectively. In a subsequent experiment a variety of *B. cinerea* products were tested for their ability to induce these hydrolases. Treatment of bean plants with conidial extract, culture filtrates, ethanol precipitates and glucan resulted in a significant increase in both β-1,3-glucanase and chitinase activities (Fig. 1,2). The activity of β-1,3-glucanase and chitinase increased from 40 % to 65 % and from 100 % to 144 % above the control level, respectively. In plants treated with culture filtrate or ethanol precipitate from 2-day *B. cinerea* culture β-1,3-glucanase and chitinase activity increase was higher in comparison with that induced by 5-day culture products e.g. chitinase activity after 2-day culture ethanol precipitate treatment was about 50 % higher than that noted in 5-day culture ethanol

precipitate treated plants. However, when ethanol precipitates from either 2-day or 5-day cultures were applied the activity of the two hydrolases increased similarly to that after treatment with the corresponding filtrates.

Further we studied the activity of β-1,3-glucanase and chitinase after *B. cinerea* infection of bean plants pretreated with fungal products. In plants previously treated with conidial extract and 2-day culture filtrate and infected the activity of β-1,3-glucanase and chitinase increased about 25 % above the level in the infected, nontreated ones (Fig. 3, 4). The activity of both hydrolases after infection in plants pretreated with the other *B. cirenea* products was similar to that caused by infection without pretreatment.

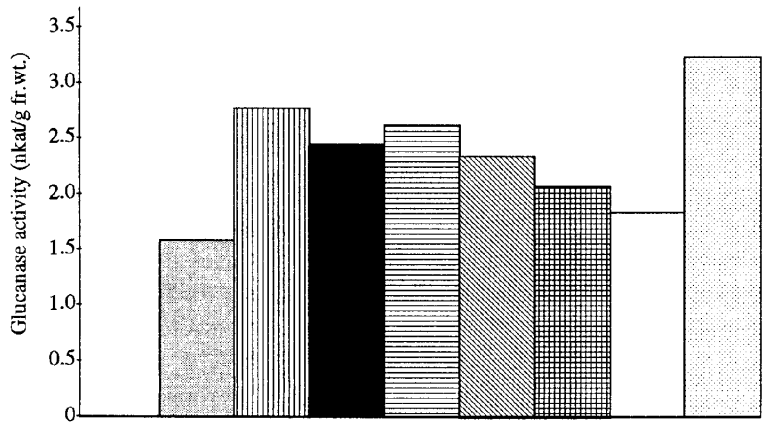
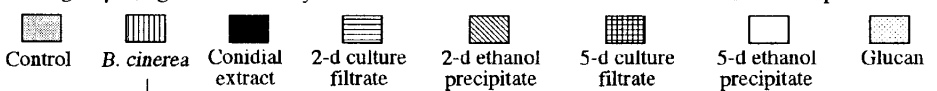


Fig. 1. β-1,3-glucanase activity in bean leaves after infection or treatment with *B. cinerea* products



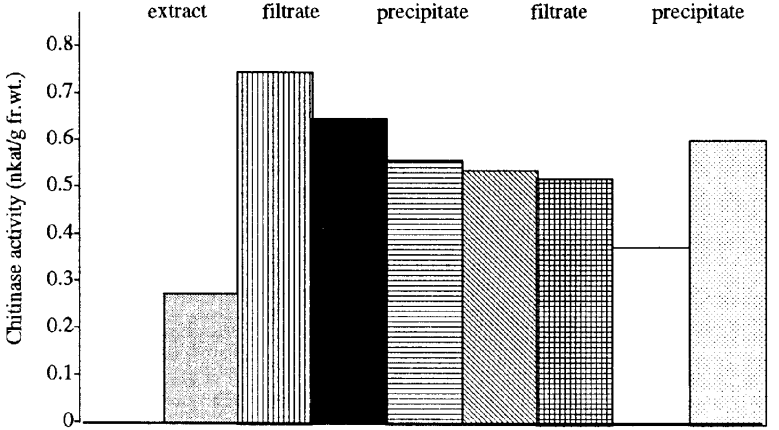
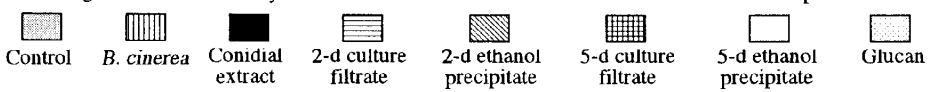


Fig. 2. Chitinase activity in bean leaves after infection or treatment with *B. cinerea* products



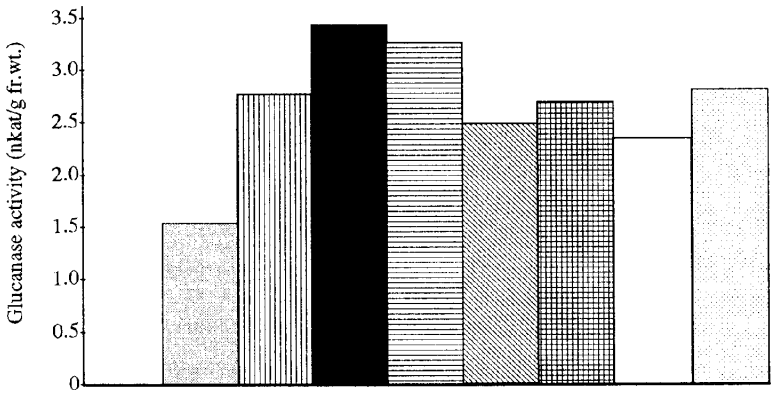
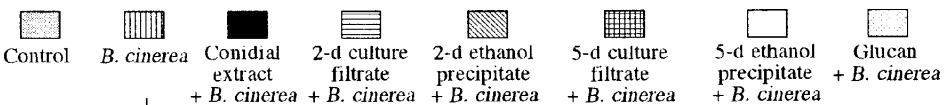


Fig. 3. β-1,3-glucanase activity in bean leaves pretreated with *B. cinerea* products and infected



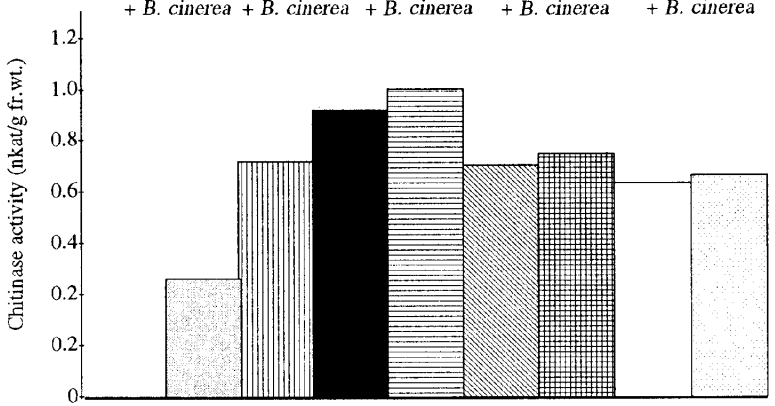
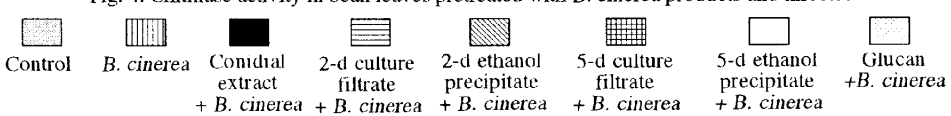


Fig. 4. Chitinase activity in bean leaves pretreated with *B. cinerea* products and infected



In bean cell suspension cultures treated with *B. cinerea* products the activities of β-1,3-glucanase and chitinase, both extra- and intracellular have been studied. Treatment with all tested fungal products resulted in a significant increase in chitinase and extracellular β-1,3-glucanase (Tab. 1, 2). The intracellular β-1,3-glucanase was not induced, except cultures treated with either conidial extract or 5-day culture filtrate (Tab. 1). The activity increase was already visible 24 h after elicitor application and persisted for 48 h. The highest activity of the studied enzymes, about 200--300 % of control, was detected in cultures treated with conidial extract and ethanol precipitates. In bean cultures the increase of extracellular chitinase and β-1,3-glucanase activity after treatment with culture filtrates was lower than that caused by ethanol precipitates, on an average about 55 % and 30 %, respectively. Addition of glucan to bean cultures enhanced the activity only of extracellular β-1,3-glucanase.

Table 1

β-1,3-glucanase activity (pkat/10^5 cells) in cell cultures treated with *B. cinerea* products

Treatment	Extracellular β-1,3-glucanase		Intracellular β-1,3-glucanase	
	Time after treatment (h)		Time after treatment (h)	
	24	48	24	48
Control	70.2	89.6	167.8	226.5
Conidial extract	258.3	198.0	236.6	376.0
2-d Culture filtrate	117.9	204.3	159.4	178.9
2-d Ethanol precipitate	160.0	263.4	181.2	190.3
5-d Culture filtrate	67.4	138.0	187.9	353.3
5-d Ethanol precipitate	101.1	162.2	206.4	212.9
Glucan	119.3	160.4	172.9	176.7

Table 2

Chitinase activity (pkat/10^5 cells) in cell cultures treated with *B. cinerea* products

Treatment	Extracellular chitinase		Intracellular chitinase	
	Time after treatment (h)		Time after treatment (h)	
	24	48	24	48
Control	41.2	46.5	6.1	5.5
Conidial extract	95.6	75.3	17.8	20.9
2-d Culture filtrate	51.9	56.7	12.6	14.6
2-d Ethanol precipitate	68.0	160.0	17.6	11.7
5-d Culture filtrate	88.6	55.8	11.3	6.1
5-d Ethanol precipitate	117.4	116.2	16.8	8.2
Glucan	48.6	48.8	5.7	6.0

DISCUSSION

We have demonstrated that β-1,3-glucanase and chitinase could be induced in bean cell cultures treated with *B. cinerea* products. Moreover, the response of cultured cells, except intracellular β-1,3-glucanase induction, was as effective as that in elicited intact plants. K o m b r i n k and H a h l b r o c k (1986) demonstrated β-1,3-glucanase and chitinase activity increase in parsley suspension cultures treated with cell wall elicitors from phytopathogenic fungi. K u r o s a k i and co-workers (1990) reported that *Chaetomium globosum* culture filtrate enhanced the activity of chitinase in carrot cultures. On the other hand in alfalfa cell suspensions *Colletotrichum lindemuthianum* cell wall extract did not induce an appreciable increase in β-1,3-glucanase and chitinase activities (D a l k i n et al., 1990).

Although much interest has been focused on the localization of elicitor induced lytic enzymes the existing data seem to be confusing. K o m b r i n k and H a h l b r o c k (1986) reported the induction of intracellular chitinase and β-1,3-glucanase in *Phytophthora megasperma* elicitor treated parsley cell cultures. According to K i r s c h et al. (1993) elicitor induced chitinase accumulated predominantly in the culture medium whereas β-1,3-glucanase was detected intracellularly. Our results demonstrated that in bean cell cultures *B. cinerea* products elicited extra- and intracellular chitinase and extracellular glucanase activity. The observed strong induction of the extracellular enzymes may favour the hypothesis of the antifungal function of these hydrolases because extracellular β-1,3-glucanase and chitinase are in position to attack the invading fungus before it penetrates into plant cells.

It was shown in a number of plant tissues that β-1,3-glucanase and chitinase are coordinately induced after infection or elicitor treatment (M e i n s and A h l, 1989; H u g h e s and D i c k e r s o n, 1991). Those reports correspond with the postulated common antifungal function of these two hydrolases. Similarly, we reported the simultaneous increase in β-1,3-glucanase and chitinase activities in response to infection or *B. cinerea* elicitor treatment.

Induction of defence reactions was achieved with a variety of compounds that were isolated from the cell walls, culture filtrates and cytoplasm of pathogenic microorganisms. B h a n d a l and co-workers (1987) reported that *Phytophthora megasperma* culture filtrate and cell wall preparation stimulated defence reaction in suspension cultures of soybean. According to K a u f f m a n n et al. (1993) a 32 kDa polipeptide secreted by *Phytophthora megasperma* elicited β-1,3-glucanase and chitinase in tobacco leaves. In bean both hydrolases were induced by cell wall elicitor from *Colletotrichum lindemuthianum* (H u g h e s and D i c k e r s o n, 1991) and in tobacco by salicylic acid (V a n d e n B u l c k e et al., 1990). B o w e n and H e a l e (1987) have shown that some components of germinating fluid of *B. cinerea* obtained from 2-day culture induced active resistance in carrot tissue. In this work we have reported that *B. cinerea* conidial extract, filtrates and ethanol precipitates from both 2-day and 5-day cultures and glucan enhanced β-1,3-glucanase and chitinase activity.

In a previous work we demonstrated that polygalacturonase from 2-day *B. cinerea* culture filtrate exhibited eliciting activity (U r b a n e k et al., 1991). Moreover it was shown that polygalacturonase accumulated during culture (U r b a n e k and Z a l e w s k a - S o b c z a k, 1984). We have observed that flitrate and ethanol precipitate from 5-day *B. cinerea* culture were not more effective in inducing enzyme activities that products obtained from the younger one. It could be due to qualitatively different enzyme composition of 2-day and 5-day culture products, assuming that enzymes were the active component in these elicitor preparations. Besides, some components of 5-day culture products accumulated during the culture could weaken their elicitor activity.

In contrast to the effect in plants, in bean cell cultures the increase in β-1,3-glucanase and chitinase activities after application of culture filtrates was lower

than that caused by ethanol precipitates. One of the explanations could be that some filtrate components negatively affected the cultured cells.

Montant and Thomas (1978) reported that *B. cinerea* secreted β-glucan consisting of 1 —> 3, 1 —> 6 linked glucosyl residues. Until now its function has not been clearly explained. We have observed that in bean plants glucan markedly inducted both β-1,3-glucanase and chitinase activities. However, in glucan elicited plants after infection with *B. cinerea* the activity of the two hydrolases was not higher than that in infected, nontreated ones. Glucan applied to bean cell cultures enhanced the activity only of β-1,3-glucanase, suggesting that it may act rather as β-1,3-glucanase substrate than as an elicitor.

Regarding the antifungal function of β-1,3-glucanase and chitinase it seems to be of particular interest that elicitor pretreatment may enhance plant response after subsequent infection. We found that in bean plants pretreated with *B. cinerea* 2-day culture filtrate and conidial extract and then infected the activity of β-1,3-glucanase and chitinase was induced stronger than only after infection. Plants with higher activity of enzymes capable of degrading fungal cell walls could be more resistant to diseases. Some positive correlation between the activity of β-1,3-glucanase and chitinase and inducted resistance has been observed (Tuzun et al., 1989).

In conclusion our results may be an indication as to the possible use of cell suspension cultures in studies on biochemical defence reactions because the response of elicitor treated cultures is similar to that of infected intact plants.

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Wpływ zakażenia *Botrytis cinerea* i elicytacji na aktywność β-1,3-glukanazy i chitynazy w liściach i kulturach zawiesinowych fasoli

Streszczenie

W pracy badano aktywność β-1,3-glukanazy i chitynazy w liściach fasoli (*Phaseolus vulgaris* L.) odm. „Złota Saxa" hodowanej w szklarni po potraktowaniu produktami otrzymanymi z hodowli *Botrytis cinerea* Pers. lub/i po zakażeniu oraz w kulturach zawiesinowych traktowanych produktami grzyba. Stwierdzono wyraźny wzrost aktywności obu enzymów w liściach zarówno po zakażeniu, jak i po potraktowaniu ekstraktem z zarodników, filtratami, osadami alkoholowymi i glukanem z hodowli *B. cinerea* Pers. Reakcja kultur zawiesinowych fasoli pod wpływem produktów *B. cirenea* Pers. była podobna do stwierdzonej w liściach. Indukcja β-1,3-glukanazy i chitynazy w liściach uprzednio traktowanych ekstraktem z zarodników i filtratem z 2-dniowej hodowli grzyba, a następnie zakażonych była silniejsza niż w liściach nietraktowanych po zakażeniu.