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The effect of poplar bark components on the activity of polygalacturonase and cellulase produced by *Dothichiza populea* Sacc. et Bri.*

INTRODUCTION

Pectolytic and cellulolytic enzymes produced by parasitic fungi and bacteria play an important role in plant diseases. The first group of enzymes like polygalacturonase cause maceration of tissues and death of host cells (Albersheim et al., 1969; Bateman and Millar, 1966; Hall and Wood, 1973; Mullen and Bateman, 1971), while cellulase in the case of woody plants, is responsible for degradation of xylem cells (Nilsson, 1974).

Under natural conditions *Dothichiza* spores penetrate the host plant through bud scale scars and leaf scars (Gremmen, 1958). Mycelium growth and further development of the fungus may be inhibited by substances produced by the host such as polyphenols (Glattes, 1971; Pukacka, 1975); chlorogenic acid and other phenolic compounds have been identified from poplar bark, to a total of 2 - 3 percent of the fresh weight (Pukacka, 1975).

It has been shown that extracellular polygalacturonases of some pathogens are strongly inhibited by polyphenols and the products of their oxidation such as catechin (Byrde et al., 1973; Hunter, 1974), chlorogenic and caffeic acid (Lyr, 1965; Patil and Dimond, 1967), gallo/ellagitannins and m-digallic acid (Bhatia et al., 1972).

The aim of the present study was to investigate the influence of substances present in the bark upon the activity of polygalacturonase and cellulase of the pathogen *D. populea*. It was shown that poplar bark extracts can inhibit both cell wall degrading enzymes, especially after their partial oxidation by phenolases present in the bark.

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MATERIALS AND METHODS

THE PLANT MATERIAL AND PATHOGEN

Research was carried out with *Populus* PK-127 No. 15 (=*P. maximowiczii*×*P. laurifolia*) a resistant clone, and *P. nigra* and P. PK-137 No 9 (=*P. nigra*×*P. nigra* '*Italica*') both susceptible to *D. populea*.

A pure culture of *D. populea* grown on Horak's solid medium (P u k a c k a, 1975), containing maltose and glucose as source of energy was used for inoculation of poplar bark. This culture was isolated from *P.*×*euroamericana* '*Robusta*' by K o z ł o w s k a (1971).

PREPARATION OF CRUDE POLYGALACTURONASE (PG) AND CELLULASE (C_x) FROM *D. POPULEA*

The enzymes of *D. populea* were obtained from a culture growing in a liquid medium containing 1 percent sodium polypectate (Sunkist Growers, Inc.) or 0.75 percent carboxymethylcellulose (Koch-Light) as the sole source of carbon. One-or two-week old cultures of mycelium were filtered and the filtrates saturated with ammonium sulphate at 0°C. Precipitates were dissolved in small quantities of 0.05 M citrate buffer pH 5.0 and dialysed for 72 hours in distilled water at 3°C. Every 24 hours the water was changed, the dialysates centrifuged (6000 g for 5 min.) and supernatant used as crude PG or C_x.

THE EFFECT OF POPLAR BARK EXTRACTS ON THE ACTIVITY OF PG AND C_x FROM *D. POPULEA*

From two-year old fresh shoots of poplars PK-127 and PK-137 the bark was peeled off and cut up. Five gram samples were extracted three times with hot 80 percent ethanol. The combined extracts were evaporated to dryness and the residue dissolved in 10 ml of hot water.

To 2 ml of the crude enzyme preparation containing the PG or C_x enzymes from *D. populea*, poplar bark extracts were added in aliquots of 1.0, 0.5 or 0.25 ml (corresponding to 500, 250 and 125 mg fresh weight of poplar bark; see Table 1) and the sample made up to 3 ml with citrate buffer of pH 5.0. The mixtures were kept at 30°C for either 3 or 24 hours. After that time to each sample 5 ml of 1 percent sodium polypectate or 0.75 percent carboxymethylcellulose in 0.05 M citrate buffer of pH 5.0 was added. The viscosity of the samples was measured at $t=0$ and following further incubation at 30°C for $t=3$ hours. The measurements were made with an Ostwald viscosimeter. The activity of PG and C_x enzymes was expressed as the difference (t_0-t_3), where t_0= efflux time

at zero time and t_3=efflux time after 3 hrs of incubation. For comparative purposes the reaction mixture with water (the blanc sample) was used instead of the extracts from poplar bark.

THE EFFECT OF OXIDIZED EXTRACTS FROM POPLAR BARK ON THE ACTIVITY OF PG AND C_x ENZYMES FROM *D. POPULEA*

Extracts from poplar bark were oxidized with an enzymatic preparation from the bark of PK-127 and carried out in the following manner.

Bark from two-year-old freshly harvested poplar shoots was fragmented, frozen in liquid nitrogen and ground to a fine powder. The powdered bark samples were transferred to a Jena glass filter G_4, washed several times with cooled acetone and then dried under vacuum. A 500 mg sample of the acetone powder was mixed with 1.5 g polyvinylpyrrolidone Polyclar AT prepared according to Knypl and Chylińska (1974) and 5 ml of 0.1 M phosphate buffer of pH 6.0. The sample was ground thoroughly in a mortar cooled on ice. After an hour the homogenized material was centrifuged (10 000 g for 5 min.) and the supernatant used for the oxidation of extracts from poplar bark. 1.0, 0.5 or 0.25 ml of the extract from the bark of PK-127 or PK-137, 0.5 ml of the oxidizing solution and 1 ml of PG or C_x enzymes from *D. populea* were incubated at 30°C for 3 and 24 hours. After incubation, 5 ml of sodium polypectate or carboxymethylcellulose was added to the samples as in the previous experiments. Viscosity was measured at the time the substrate was added (t_0) and after 3 hrs incubation at 30°C (t_3). The control sample had all the components except the extracts from poplar bark.

THE ACTIVITY OF PG AND C_x ENZYMES FROM POPLAR BARK TISSUE INOCULATED WITH *D. POPULEA*

One-year-old shoots of the poplar PK-127 and *P. nigra* were cut up into 15 cm pieces, washed thoroughly in a detergent solution, disinfected by immersing in 0.5 percent $HgCl_2$ for one minute and washed again with water, then placed in test tubes with enough distilled water to cover the lower cut surfaces. Representative batches of the cuttings were incoulated at two places by putting agar discs with mycelium of *D. populea* on the wounds. The control shoots were wounded only by using a cork borer. After 10 and 20 days the cuttings were sampled for analysis. From a 1 cm zone around the inoculation point the bark was removed, frozen in liquid nitrogen and ground to powder. The ground bark was washed several times with cooled acetone on Jena glass filter G_4 and then dried under vacuum. 30 mg samples of acetone powder were ground with 1 g of PVP, Polyclar AT in 5 ml citrate buffer 0.05 M of pH 5.0. After

30 min. the material was centrifuged (10 000 g for 5 min.) and the supernatant made up to 9 ml with the buffer. It acted as the crude enzymatic preparation.

The reaction mixture contained 4 ml of substrate (0.75 percent of sodium polypectate or 0.3 percent of carboxymethylcellulose in citrate buffer of pH 5.0) and 2 ml of crude enzymatic preparation. The samples were incubated at 30°C for 8 hours. Viscisity was measured before and after incubation. The activity of PG and C_x was expressed as $t_0 - t_8$/mg protein, where t_0=efflux time at zero time and t_8=efflux time after 8 hrs of incubation with substrate. The protein was estimated using the method of Potty (1969).

RESULTS

The effect of poplar bark extracts on the activity of PG and C_x enzymes of *D. populea* is presented in Table 1 and 2. Even after 3 hours of incubation with various extracts the activity of the enzymes studied declines. It is evident that the extracts from the resistant poplar PK-127 generally inhibit the activity of PG and C_x more effectively than the analogous extracts from the susceptible poplar PK-137.

Table 1

Effect of extracts from poplar bark on the activity of polygalacturonase (PG) and cellulase $(C)_x$ from *Dothichiza populea* culture filtrates

Poplar clone	Poplar bark extract mg	PG[a]		C^a_x	
		3 hrs	24 hrs	3 hrs	24 hrs[b]
PK-127 (resistant)	500	19	16	13	3
	250	23	19	27	18
	125	24	23	33	25
PK-137 (susceptible)	500	23	11	23	12
	250	29	21	29	29
	125	27	22	37	40
Water	0	27	25	44	45

[a] Activity of PG and C_x is expressed as the decrease in viscosity (Δt in seconds) in the reaction mixture containing the fungal enzyme along with the substrate and the poplar bark extract during 3 hrs of incubation at 30°C, pH 5.0.

[b] Crude enzyme PG and/or C_x was preincubated with bark extracts at 30°C for 3 or 24 hours.

The oxidized extracts of poplar bark inhibit the activity of PG and C_x from *D. populea* much more strongly than the nonoxidized ones and the degree of inhibition depends on the origin of extract and the duration of preincubation with the fungal enzyme.

The activity PG and C_x from tissues of poplar bark increases in the inoculated shoots. This is almost three-fold in the case of poplars susceptible to *D. populea* and very slight in the case of the resistant poplar PK-127 (Tab. 3).

Table 2

Effect of oxidized extracts from poplar bark on the activity of PG and C_x from *D. populea* culture filtrate

Poplar clone	Poplar bark extract mg	PG[a]		C^a_x	
		3 hrs	24 hrs	3 hrs	24 hrs[b]
PK-127 (resistant)	500	0	0	21	9
	250	3	0	26	15
	125	4	0	28	25
PK-137 (susceptible)	500	8	1	25	26
	250	11	2	31	27
	125	11	2	32	33
Water	0	15	13	38	41

[a] As in Table 1 above.

[b] Crude enzyme PG and/or C_x was preincubated with bark extracts and poplar phenoloxidase at 30°C for either 3 or 24 hours.

Table 3

Influence of *D. populea* infection on PG and C_x activities in poplar cuttings

Poplar clone	10 days[a]				20 days[a]			
	PG		C_x		PG		C_x[b]	
	H	I	H	I	H	I	H	I
P. nigra	11	31	20	34	13	30	22	36
PK-127	11	14	10	15	10	10	9	12

[a] Time after inoculation.

[b] Activity of PG and C_x is expressed as decrease in viscositi (seconds) of sodium polypectate or carboxymethylcellulose per mg protein in sample at 30°C, pH 5.0.

H-healthy, I-infected poplar cuttings.

DISCUSSION

The fungus *Dothichiza populea* Sacc. et Bri. produces extracellular hydrolytic enzymes such as pectinases (polygalacturonase and pectin methylesterase) and cellulase (C_1 and C_x) in a medium with pectin or cellulose as the sole source of carbon (Pukacka, 1972). The object of this paper was to establish the effect of poplar bark substances on the activity of polygalacturonase (PG) and cellulase (C_x) from *D. populea*. For this purpose, ethanol extracts were prepared from the bark of poplars resistant and susceptible to *Dothichiza* bark necrosis. Both the ethanol extracts were found to contain a considerable amount of phenolic substances such as chlorogenic acid and catechol (Pukacka, 1975). The phenolic substances can inactivate several enzymes including pecto and cellulolytic enzymes of many pathogens (Byrde et al., 1960; Bhatia et al., 1972; Hunter, 1974; Lyr, 1965; Patil and Dimond, 1967). In the experiments presented here a phenol — phenoloxidase system was isolated from the host and combined in vitro with the

pathogen's exoenzymes. We observed a distinctly inhibitory effect on the activity of fungal PG and C_x exerted by the extracts from poplar bark and products of their oxidation. The bark extract from the resistant poplar PK-127 gave a stronger inhibition of the fungal enzyme than the same from a susceptible poplar (Tab. 1 and 2). However it should be mentioned that the degree of inhibition does not depend on the total amount of phenolic compounds in the bark, because the poplars studied did not differ from each other in this respect (Pukacka, 1975).

In the other experiment the activity of PG and C_x enzymes was studied in tissues of poplar bark inoculated with *D. populea* (Tab. 3). An almost three-fold increase in the activity of those enzymes was observed in the bark of susceptible poplars after inoculation. It is also likely that this increase is caused by the presence of fungal PG and C_x in the bark tissue, since resistant bark did not show a significant increase in the cell wall degrading enzymes. Other authors (Byrde et al., 1973; Mullen and Bateman, 1971) using the isoelectric focussing technique were able to distinguish between the fungal pectolytic exoenzymes and the host enzyme following infection. The results obtained indicate that a factor exists inhibiting the activity of PG and C_x in the bark of a resistant poplar. Since the activity of this factor was enhanced by polyphenolase oxidation it is assumed that it is a phenolic substance. It was previously shown (Pukacka, 1975) that balsam poplars are characterised by higher phenoloxidase activity of their bark tissue than black poplars. The present experiments have shown that the phenol-phenoloxidase system may be an important element in the mechanism of resistance of poplar bark against *D. populea*.

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SUMMARY

The fungus *Dothichiza populea* Sacc. et Bri., causing bark necrosis of poplar, produces extracellular pectolytic and cellulolytic enzymes. The ethanol extract from a resistant balsam poplar hybrid PK-127 inhibits the activity of polygalacturonase and cellulase from *D. populea* to a greater extent than an analogous extract from the bark of the susceptible black poplar PK-137. A stronger action is demonstrated by bark extracts treated with a preparation phenoloxidase from bark tissues. The polygalacturonase and cellulase activities in inoculated bark of susceptible poplars are twice as high as those of the resistant hybrid.

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STANISŁAWA PUKACKA

Wpływ składników kory topoli na aktywność poligalakturonazy i celulazy Dothichiza populea Sacc. et Bri.

Streszczenie

Grzyb *Dothichiza populea* Sacc. et Bri., który wywołuje chorobę kory topoli produkuje zewnątrzkomórkowe enzymy pektolityczne i celulolityczne. Ekstrakty etanolowe z kory topoli odpornej PK-127 (*P. maximowiczii*×*P. laurifolia*) hamują aktywność poligalakturonazy i celulazy *D. populea* w większym stopniu niż analogiczne ekstrakty z kory topoli wrażliwej PK-137 (*P. nigra*× *P. nigra 'Italica'*). Bardziej silne działanie wykazują ekstrakty traktowane preparatem fenoloksydazy z tkanki kory. Aktywność poligalakturonazy i celulazy w zakażonej korze topoli wrażliwej jest dwukrotnie wyższa niż w odpornej.

СТАНИСЛАВА ПУКАЦКА

Влияние компонентов коры тополей на активность полигалактуроназы и целлюлазы Dothichiza populea Sacc. et Bri.

Резюме

Гриб *Dothichiza populea* Sacc. et Bri., вызывающий заболевания коры тополей, выделяет внеклеточные пектолитические и целлюлолитические ферменты. Этаноловые экстракты из коры устойчивого тополя PK-127 (*P. maximowiczii*×*P. laurifolia*) тормозят активность полигалактоуреназы и целлюлазы *D. populea* в большей степени, чем аналогичные экстракты коры тополя чувствительного PK-137 (*P. nigra*×*P. nigra 'Italica'*). Более сильное действие было у экстрактов, обработанных фенолоксидазой из тканей коры. Активность полигалактоуреназы и целлюлазы в зараженной коре устойчивых тополей в два раза выше, чем у чувствительных.