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Role of phenolic compounds in the resistance of poplars to the fungus *Dothichiza populea* Sacc. et Bri.

INTRODUCTION

Phenolic compounds are very common in the plant kingdom. In view of their toxicity to microorganisms even at very low concentration they have been studied by many authors as factors taking part in the resistance of plants to fungal and bacterial infections. A review of these studies has been given by P r i d h a m (1960) and K o s u g e (1969).

In the present investigation phenolic compounds existing in poplar bark were investigated and attempts were made to explain their participation in the resistance of poplars to infection by the fungus *Dothichiza populea*. This pathogen is the causative agent of a poplar bark disease, and it is known that black poplars are more susceptible than the balsam poplars (D o n a u b a u e r, 1966; S i w e c k i, 1977). Many authors have reported the existence in poplar bark of substances that are toxic to *D. populea* (B u t i n and L o e s c h c k e, 1960; K o z ł o w s k a, 1971; P u k a c k a, 1975).

In the present study most toxic substances to *D. populea* were sought in ethanolic extracts of bark from various balsam and black poplar cultivars.

MATERIALS AND METHODS

1. PATHOGEN AND THE HOST

For the studies use was made of a pure culture of *D. populea* mycelium isolated by K o ł o w s k a (1971) from *Populus 'Robusta'* and propagated on Horak's medium at a temperature of 25°C (P u k a c k a, 1975).

The studies were conducted on healthy one-year-old shoots of poplars from a collection belonging to the Institute of Dendrology of the Polish Academy of Sciences. The cultivars used were from sections *Aigeiros* and *Tacamahaca* and one *P. alba* from section Leuce. They were:

Aigeiros	*Tacamahaca*
P. nigra 'Italica'	*P. trichocarpa*
PK-137 cl. 9 (*P. nigra 'Italica'* × *P. nigra*)	*PK-127* cl. 15 (*P. maximowiczii* × *P. laurifolia*)
P. deltoides	*P. tacamahaca*
P. 'Robusta'	*P. laurifolia*
P. angulata 'Cordata'	*P. balsamifera*

Material for study was taken in the spring of 1978. In the collection no instances were observed of natural infection of poplars by *D. populea.*

2. GROWTH OF *D. POPULEA* MYCELIUM ON A MEDIUM CONTAINING EXTRACTS FROM POPLAR BARK

The method of preparing extracts and studying their effects on the growth of *D. populea* has been described in the work of Pukacka (1975) with minor modifications. Onto the medium a disk of the mycelium 3 mm in diameter was placed. At appropriate times during incubation at 25°C the diameter of the mycelium was measured and substracting the initial 3 mm the percentage growth relative to the control was calculated. The control consisted of a mycelium grown in a similar manner on the same medium but without the extracts form poplar bark.

3. ISOLATION OF COMPOUNDS INHIBITING THE GROWTH OF *D. POPULEA* FROM ETHANOLIC EXTRACTS OF POPLAR BARK

For the studies use was made of extracts from the bark of poplars *PK-127* (15) and *PK-137* (9) representing sections *Tacamahaca* and *Aigeiros* respectively. Ethanolic extracts from 2.5 g samples of bark were spotted onto plates with a silica gel GF 254. These were developed ascending in a solvent composed of chloroform: ethyl acetate: formic acid (50 : : 40 : 10). After developing the chromatograms were dried at room temperature for 24 hours and then five characteristic zones were identified on them under UV 360 nm light. From the zones the gel was removed and eluted in 96% ethanol. The ethanol was evaported to dryness and the residue was introduced into 50 ml of the medium for *D. populea* growth and the effect of these eluates on the mycelial growth was estimated.

4. IDENTIFICATION OF PHENOLIC COMPOUNDS CONTAINED IN THE ELUATES

Ethanolic extracts after elution of the gel from various zones of the chromatogram were evaporated to dryness. The residue was dissolved in 20 ml of distilled water and subjected to acid hydrolysis in 1 N HCl,

at 90°C for 30 min. The hydrolyzates were extracted 3 times with ethyl ether. After evaporating the ether the residues were analysed qualitatively. For the identification of phenolic compounds use was made of two dimentional paper chromatography and thin layer chromatography. Use was made of Whatman no. 1 paper and silica gel GF 254. The chromatograms were developed in the following solvents : benzene : acetic acid: water (1 : 1 : 1), 3% acetic acid, chloroform : acetic acid (95 : 5). Pure phenolic reagents were used as standards for identification.

5. ESTIMATION OF THE CONTENT OF PHENOLIC SUBSTANCES IN THE BARK

A general quantitative estimation of the phenolic and o-diphenolic compounds was made by the method of Swain and Hillis (1959) and the level of chlorogenic acid by the method of Zucker and Ahrens (1958).

6. DETERMINATION OF THE CONTENT OF SALICYLIC AND GENTISIC ACIDS

Ethanolic extracts from 1 g samples of bark of the studied poplars were subjected to acid hydrolysis and then extracted trice with ethyl ether. The ether fractios were evaporated to dryness and the residue was picked up in a small quantity of ethanol. The whole amount was spotted onto plates with the silica gel GF 254 and developed in the solvent chloroform : acetic acid (95 : 5). Under UV 360 nm light spots of salicylic and gentisic acids were identified. The gel from these spots was taken and eluted with 96% ethanol. Then at a sufficient dilution the spectra of these compounds were drawn in UV light in a SECORD spectrophotometer. The content of salicylic and gentisic acids in the studied eluates was identified with the help of calibration curves. These curves were prepared drawing spectra of salicylic and gentisic acids from ethanolic solutions at various concentrations. The extinction values at $\lambda = 300$ nm for salicylic acid and $\lambda = 325$ nm for gentisic acid are directly proportional to the concentration of these compounds in the sample.

RESULTS

Effect of ethanolic extracts from the bark of the studied poplars on the growth of *D. populea* mycelium is presented in Fig. 1. A.B.C. Ethanolic extracts from the bark of balsam poplars inhibit the growth of *D. populea* mycelium distinctly more than the extracts from the bark of black poplars. A particularily strong inhibiting effect was observed by extracts from *P. trichocarpa, P. tacamahaca* and *PK-127* cl. 15. This

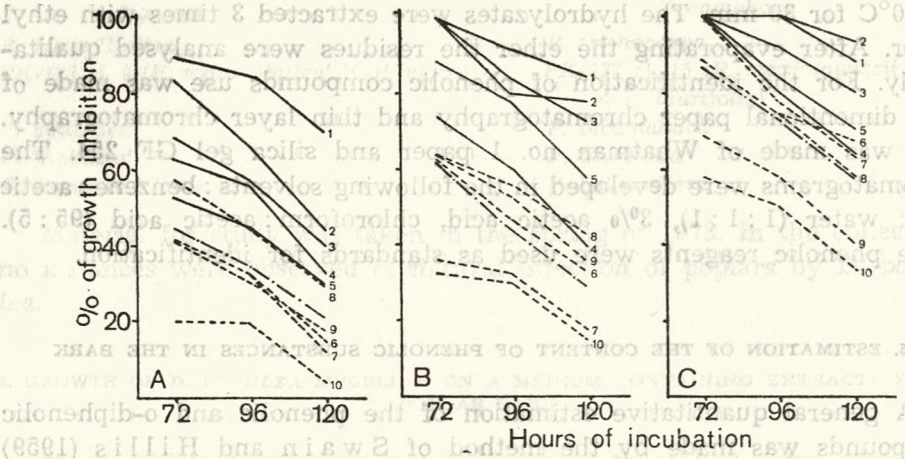

Fig. 1. Inhibition of *D. populea* mycelial growth by ethanolic extracts from poplar bark: A — 2%, B — 3% and C — 4% extracts

——— poplars from section *Tacamahaca:* 1. *P. trichocarpa*, 2. *P. tacamahaca*, 3. *PK-127* (15) (*P. maximowiczii* × *P. laurifolia*), 4. *P. balsamifera*, 5. *P. laurifolia*; — — — — poplars from section *Aigeiros*, 6. *PK-137* (9) (*P. nigra 'Italica'* × *P. nigra*), 7. *P. nigra 'Italica'*, 8. *P. 'Robusta'* 9. *P. deltoides*, 10. *P. angulata 'Cordata'*; —.—. *P. alba*

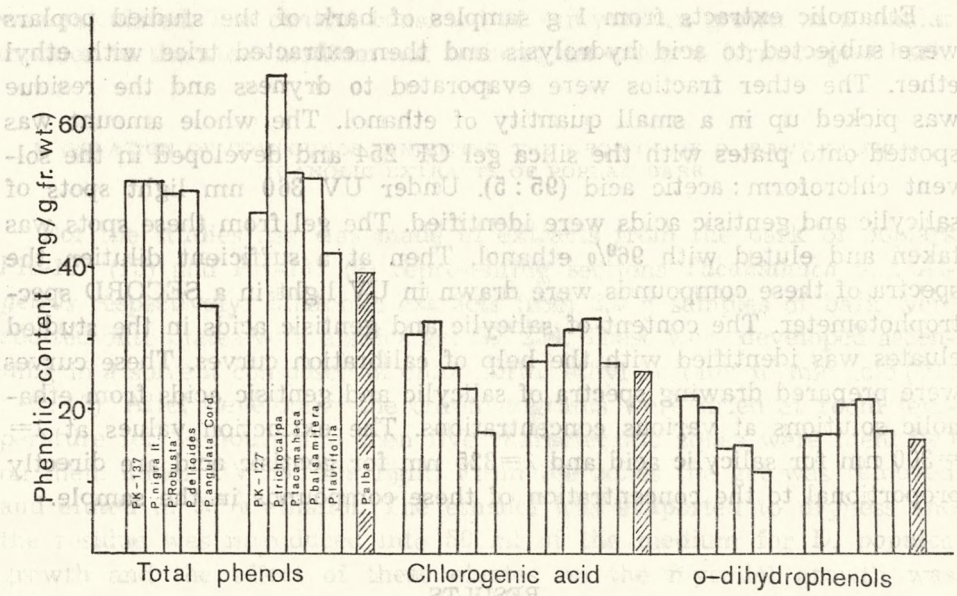

Fig. 2. Content of phenolic compounds (mg/g fresh wt.) in the bark of poplars from sections *Aigeiros*, *Tacamahaca* and from *P. alba*

is visible already at a concentration of 2% and as the concentration increases the inhibiting effect increases also. The effect of extracts from the bark of *P. alba* on the growth of the mycelia is similar to that of the black poplars.

From further experiments it appears that the degree of inhibition of the growth of *D. populea* mycelia by extracts from bark of the studied poplars is not correlated with the total content of phenolic compounds in the bark, nor with the content o-dihydrophenols or chlorogenic acid (Fig. 2).

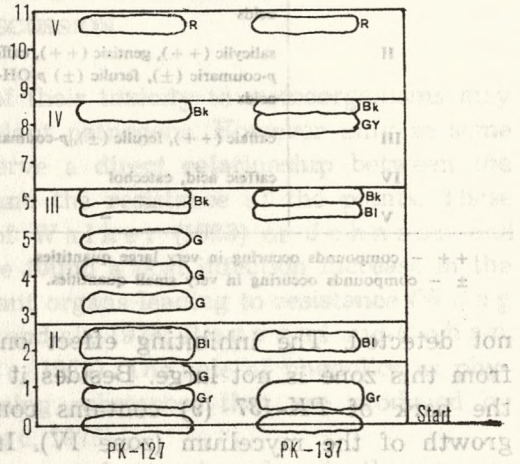

Fig. 3. Chromatogram of ethanolic extracts from the bark of poplars *PK-127* (15) and *PK-137* (9) divided into zones. On the chromatogram spots are marked as seen in uV light in ammonium fumes, and the colour of these spots is indicated (Gr — gray, G — green, Bl — blue, Bk — black, Y —yellow, R — red)

The split up of the chromatograms into characteristic zones is presented in Fig. 3 and the effect of eluted substances from individual zones on the growth of *D. populea* is illustrated in Table 1.

On the basis of the data presented in Table 1 it is possible to show the most important differences between the properties of the extracts from the bark of *PK-127* (15) and *PK-137* (9). Most inhibitive to the growth of *D. populea* mycelium were substances from zone II of the extract from *PK-127* (15). In that zone large quantities of salicylic and gentisic acids were found and other phenolics in lower concentrations (Tab. 2). The analogous zone from extract of *PK-137* (9) bark contained also salicylic acid, but in much lower quantities and gentisic acid was

Table 1

Effect of phenolic compounds eluted from chromatograms on the growth of *D. populea* mycelium

Chromatogram zone	% inhibition of mycelium growth			
	PK – 127 cl. 15 (*P. max.* × *P. laurifolia*)		*PK – 137* cl. 9 (*P. nigra* 'Italica' × *P. nigra*)	
	72h	96h	72h	96h*
I	36	25	30	23
II	80	70	14	8
III	20	4	20	17
IV	14	8	+15	+30
V	0	0	0	0

\+ – percentage of mycelial growth stimulation.
* Time of incubation at 25°C.

Table 2

Phenolic compounds after hydrolysis of eluates from individual zones on the chromatograms from the bark of poplars *PK-127* (15) and *PK-137* (9)

Zone	*PK – 127* (15)	*PK – 137* (9)
I	caffeic, *p*-coumaric, salicylic (±), gentisic (±) acids	caffeic (±), ferulic, *p*-coumaric, salicylic (±) acids
II	salicylic (+ +), gentisic (+ +), caffeic (±), *p*-coumaric (±), ferulic (±) *p*-OH-benzoic acids	salicylic, *p*-coumaric, caffeic (±) acids
III	caffeic (+ +), ferulic (±), *p*-coumaric acids	caffeic acid, quercetin
IV	caffeic acid, catechol	quercetin (+ +), catechol
V	–	–

+ + – compounds occuring in very large quantities.
± – compounds occuring in very small quantities.

not detected. The inhibiting effect on mycelial growth by the eluate from this zone is not large. Besides it was found that the extract from the bark of *PK-137* (9) contains compounds clearly stimulating the growth of the mycelium (zone IV). In the eluates that stimulate the growth of the mycelium primarily quercetin was found. In the case of eluates from the bark of *PK-127* (15) in none of the zones was any stimulation of mycelial growth observed.

Table 3

Content of salicylic and gentisic acids in the bark of balsam, black and white poplars in ng/g of fresh weight. Sequence of cultivars corresponds to decreasing ability of bark extracts to inhibit *D. populea* mycelial growth as seen in Fig. 1

Poplar cultivar	Salicylic acid	Gentisic acid
Tacamahaca		
P. trichocarpa	6 700	1200
P. tacamahaca	6 700	270
PK – 127 (15)	10 200	176
P. balsamifera	4 600	176
P. laurifolia	5 000	90
Aigeiros		
PK – 137 (9)	3 500	50
P. nigra 'Italica'	3 900	48
P. 'Robusta'	3 600	76
P. deltoides	3 400	110
P. angulata 'Cordata'	2 800	76
Leuce		
P. alba	3 600	270

A detailed quantitative analysis has shown that in the bark of balsam poplars the content of salicylic and gentisic acids is distinctly higher than in the bark of black poplars (Tab 2). A particularly high concentration of salicylic acid was observed in the bark of *PK-127* (15), while

in the bark of *P. trichocarpa* a high content of gentisic acid was observed. The level of salicylic acid in the bark of *P. alba* is similar as in the black poplars and it is possibly for this reason a bark extract from this poplar has a low ability to inhibit the mycelial growth of *D. populea.*

DISCUSSION

Phenolic compounds in view of their toxicity to microorganisms may participate in the combating of plant pathogens. However only in some instances was it possible to observe a direct relationship between the presence of specific compounds and the resistance of the plants. These were the now classical studies of Walker (1923) or Johnson and Shaal (1957). Some authors have found a post infection increase in the level of phenolic compounds in plant organs leading to resistance (Wong and Preece, 1978; Carrasco and al., 1978; Reuveni and Cohen, 1978; Brown and Swinburne, 1971). The role of phenolics as phytoalexins is also known these being substances that are produced by plants as a result of infection (Kuć, 1976).

The fungus *D. populea* attacs young shoots of poplars in the nursery or on plantations. Observations conducted in the field and experiments with artificial infection have shown that balsam poplars are much more resistant to this pathogen than black poplars (Donaubauer, 1964; Siwecki, 1977). Hepting (1971) mentions *P. alba* among the most susceptible poplars to *D. populea* in USA. So far reports are lacking on the occurence of disease caused by *D. populea* on this poplar in Poland. In the present study *P. alba* was also included to compare its properties with those of the balsam and black poplars.

Poplar bark contains a great variability of phenolic compounds. They occur in the free state or bound in the form of glucosides. Butin and Loeschcke (1960) and Kozłowska (1971) have shown that water or ethanolic extracts from the bark of some poplar cultivars may inhibit at some concentration the germination of spores and mycelial growth of *D. populea.* Extracts from balsam poplars contained clearly fewer fungistatic substances than from black poplars. Butin and Loeschcke (1960) have separated with the help of paper chromatography from the bark of *P. trichocarpa* several fractions the most active of which contained primarily catechol while another fraction with somewhat lower activity contained a phenolic glucoside identified later as trichocarpin (Loeschcke and Francksen, 1964). Both studies of Pukacka (1975) and Butin and Loeschcke (1960) have shown that almost all phenolic compounds occuring in the bark of poplars are toxic to *D. populea* at appropriate concentrations. They do not however occur always at those concentrations in the fresh weight of bark.

In the present study the effect of ethanolic extracts from the bark of 10 varieties of black and balsam poplars and of *P. alba* on the growth of *D. populea* mycelium has been investigated. It was found that the extracts from the balsam poplars clearly inhibit more the growth of *D. populea* mycelia than those from the black poplars (Fig. 1 A - C). This however is not dependent on the total content of phenolic compounds in the bark, nor on the levels of chlorogenic acid or o-diphenolic compounds (Fig. 2). Further studies have shown that the most toxic fraction of the studied extracts contained mainly salicylic and gentisic acids (Tab. 1 and 2). The content of these compounds in the ethanolic extracts from bark of the studied poplars is correlated with their toxicity (Fig. 1, Tab. 3). Since further studies by the author did not discover any increase in the total content of phenolic compounds in the bark, nor any increase in the level of any individual phenolic compound following inoculation of poplars with the pathogen *D. populea*, it has to be assumed that phenolic compounds play a role in the pre-infection resistance of poplars to this disease. Salicylic acid is of particular importance since its concentration equivalent to the content in the bark of some poplars is capable of inhibiting growth of the fungus in vitro conditions (Pukacka, 1975).

A positive correlation between the content of salicylic and gentisic acids and the resistance of poplar cuttings to artificial infection with *D. populea* has been found by Tomaszewski et al. (1972 - 1975). Salicylic and gentisic acids occur in the bark of poplars exclusively in a bound form, however they can be released by the enzymes of the pathogen (Pukacka, 1975) as well as by the enzymes of the host (Tomaszewski et al., 1972 - 1975).

The inhibiting effect on *D. populea* mycelium by the ethanolic extracts of *P. alba* is similar as in the case of black poplars. Similar also is the content of salicylic acid in the bark of this poplar. Absence of incidence of *Dothichiza* bark canker on *P. alba* in Poland would have to be explained by lack of ecological conditions for the development of this disease or other resistance mechanisms operating in the bark of this poplar.

SUMMARY

The present study summarizes the results of studies on the role of phenolic compounds in the resistance of poplars to the fungus *D. populea*. The studies were conducted on 10 cultivars of balsam and black poplars and on *P. alba*. It is generally known that balsam poplars are more resistant to infection by *D. populea* than black poplars and so far on *P. alba* incidence of this disease was not reported in Poland. The conducted studies show that ethanolic extracts from the bark of balsam

poplars inhibit more growth of *D. populea* mycelium than do the extracts from black poplars. The inhibiting effect is not correlated with the total content of phenolic compounds in the bark nor with the content of o-diphenolics or of chlorogenic acid. On the other hand it is correlated with the content of salicylic and gentisic acids. In the case of infection of poplars by *D. populea* the phenolic compounds appear to have only a pre-infection role in resistance.

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Udział związków fenolowych w odporności topoli na grzyb Dothichiza populea Sacc. et Bri.

Streszczenie

Niniejsza praca podsumowuje wyniki badań nad rolą związków fenolowych w odporności topoli na grzyb *D. populea*. Badania prowadzono na 10 odmianach topoli balsamicznych, czarnych oraz *P. alba*. Ogólnie wiadomo, że topole balsamiczne są bardziej odporne na porażenie przez *D. populea* niż czarne, natomiast u *P. alba* jak dotąd, nie stwierdzono symptomów choroby wywołanej przez tego patogena. W wyniku przeprowadzonych badań stwierdzono, że wyciągi etanolowe z kory topoli balsamicznych bardziej hamują wzrost grzybni *D. populea* niż z czarnych. Efekt hamowania nie jest skorelowany z ogólną zawartością związków fenolowych ani też z zawartością o-dwufenoli i kwasu chlorogenowego w szczególności. Jest on natomiast zgodny z zawartością kwasu salicylowego i gentyzynowego. W przypadku infekcji topoli przez grzyb *D. populea* związki fenolowe wydają się mieć znaczenie jedynie w odporności przedinfekcyjnej.

СТАНИСЛАВА ПУКАЦКА

Роль фенольных соединений в устойчивости тополей к грибу Dothichiza populea Sacc. et Bri.

Резюме

Настоящая работа является обобщением результатов исследований, целью которых было выяснение роли фенольных соединений в устойчивости тополей к заражению грибом *D. populea*. Исследования велись на 10 разновидностях тополей: бальзамических, черных и *P. alba*. Общеизвестно, что бальзамические тополя более

устойчивы к заражению *D. populea*, чем тополя черные, а у *P. alba* до сих пор не найдено симптомов заболевания вызванного этим патогеном. В результате проведенных исследований найдено, что этаноловые вытяжки с коры бальзамических тополей в большей степени тормозят рост гифов гриба *D. populea*, чем из черных. Не найдено корреляции между эффектом торможения роста и общим содержанием фенольных соединений, о-дифенолов и хлорогеновой кислоты. Этот эффект хорошо согласуется с содержанием салициловой и гентизиновой кислоты. При инфекции тополей грибом *D. populea* фенольные соединения имеют, по всей вероятности, значение в прединфекционной устойчивости.