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New aspects on the infection mechanism of Botrytis cinerea Pers.

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Abstract

With the help of active types of oxygen, extracellular enzymes and mechanical processes, *Botrytis cinerea* is capable of infecting plant tissue. Whereas no clear correlation was found between the activities of protease, pectolytic or other cell wall degrading enzymes and virulence, a positive correlation between pathogenicity and the intensity of active types of oxygen released was apparent in some isolates. It is assumed that these toxins result from the activity of glucose or xylose oxidases. Antioxidants inhibited the infection. It is concluded from these results, that active types of oxygen play a decisive role in the infection process.

Additional keywords: *Vicia faba*, *Botrytis cinerea*.

Introduction

A number of detailed studies focussing on the mechanism by which *Botrytis cinerea* infects plant tissues have been published (Deverall and Wood, 1961a, b; Kovacs and Tüske, 1986; Verhoeff, 1986). The infection process of *B. cinerea* differs from that of the majority of other fungi. In particular the presence of certain sugars in the inoculum is essential for a successful artificial infection, a requirement that generally is not observed with other fungi. The importance of mechanical and enzymatic potential of *B. cinerea* in the infection process is controversial. Whereas Wasfy et al. (1978) state that cell wall-degrading enzymes, especially pectinases, play a very important role in pathogenicity, other authors (Papavizas and Ayers, 1965; Keen and Erwin, 1971; Zalewska-Sobczak, 1985) were not able to confirm the importance of these enzymes in the infection process.

The production of cuticle- and cell wall-degrading enzymes, such as cutinases, cellulases, hemi-cellulases and pectinases, by *B. cinerea* and other pathogenic fungi has been frequently demonstrated (Byrde et al., 1973; Urbanek and Zalewska-Sobczak, 1984; Salinas et al., 1986). However, a number of saprophytic fungi of different species and also timber decaying fungi produce and secrete these enzymes as well (Lyr, 1960), indicating that this feature alone does not imply pathogenicity. Also there have been no reports that *B. cinerea* produces toxins which are needed for pathogenicity, except for one report which demonstrated the secretion of a phospholipase (Shepard and Pitt, 1976).

Many factors can influence the infection process of *B. cinerea*. The epiphytic microflora and the waxy substances in the leaf surface of *Vicia faba* possess a certain inhibitory activity (Rosall and Mansfield, 1980), whereas artificial wounding of the cuticle favours the infection process (Doneche, 1986). Young leaves of *Vicia faba* are less susceptible to infection than old leaves (Deverall and Wood, 1961a, b) possibly a consequence of their higher anti-oxidant content.

Polyphenols inactivate the polygalacturonases of fungi relatively rapidly, a phenomenom observed for enzymes from other sources as well (Lyr, 1960). Phytoalexins certainly play an additional role in the protection of plants against an infection. However, despite their synthesis coupled with the accumulation of oxidized polyphenols, which is observed in *V. faba* following an infection by *B. cinerea*, these substances are not capable of restricting lesion development by the fungus under suitable conditions. Culture filtrates of *B. cinerea* and *B. fabae* cause stem sections of *V. faba* to macerate and turn brown (Deverall and Wood, 1961), which point to a still unexplained destruction of vacuoles or cell compartments. This process cannot be solely explained by the presence of cell wall degrading enzymes.

Typical for *Botrytis* is its relatively wide host range, the preferred infection of old, injured or by previous infections weakened, plant parts and its requirement for certain sugars in the infection medium for high pathogenicity. The requirement of these factors is difficult to explain if the activity of cell wall degrading enzymes would be solely responsible for infection. On the other hand, Deverall and Wood (1961a, b) were able to demonstrate that leaves with a high calcium content, which is characteristic for old tissues, were less sensitive to infection. A possible reason for this could be that Ca-pectate is a less suitable substrate for pectinases. Nevertheless old leaves are generally more easily infected than young leaves, an observation for which there is currently no explanation.

The sugars glucose, fructose, raffinose and maltose were found to favour the infection process (Harper et al., 1981), but not lactose, ribose, galactose, inositol, mannitol and sorbitol. The authors assumed that the fungus was able to produce more toxin in the presence of the stimulatory sugars, although evidence for such a toxin has not yet been presented.

Our investigations were aimed to establish whether a correlation exists between the ability to produce cell wall degrading enzymes and pathogenicity by studying a number of fungal isolates differing in pathogenicity. A second aim of our study was to find evidence for the production of the postulated toxin, which could explain the contradictory reports found in the literature.

Materials and methods

Origin and culture of the fungi. The isolates of *B. cinerea* used in this study included isolates from grapes and vine leaves collected in the field as well as reference isolates from culture collections. The isolates 1, 2 and 3 are wild types, isolate 4 is a laboratory strain with a high resistance towards dicarboximides (ED_{50} 500 mg l^{-1}). Isolates 5 and 6 are from grapes and, like isolate 7 from grape vine, are resistant to dicarboximide fungicides (ED_{50}: 3-8 mg l^{-1}). The strains 8, 10 and 12 are sensitive field isolates from which the dicarboximide resistant laboratory strains 9, 11 and 13 were obtained.

All isolates were cultured on 2% malt extract agar. Growth inhibition by chemicals

was determined in radial growth assays. Compounds were dissolved in acetone, and added to the cooled (50 °C) sterilized medium at the required final concentration. Plates were poured and inoculated with a mycelial plug from the edge of a growing colony. Inhibition was determined by comparing the colony diameters on fungicide amended and on control-plates.

Pathogenicity tests. Detached leaflets of *V. faba* cv. Fribo grown until the 4-leaf growth stage in soil under glasshouse conditions were used in pathogenicity tests. Half of each leaflet was spot-inoculated with a 10 μl-droplet of a conidial suspension (6 10^5 conidia ml^{-1}) containing various nutrients and sugars as indicated in Tables 2 and 3. The other half was inoculated with a conidial suspension in 3% glucose, and served as control. The inoculated leaflets were incubated in moist chambers at 22 °C under 12 h light periods. After 2 and 4 days, the number of lesions was counted and lesion types were classified according to the following scale: 1, no infection; 2, weak, light browning; 3, light browning; 4, strong browning local lesions and 5, black spreading lesions.

The number of lesions was multiplied with the classification number giving the infection ability. The relative infection ability is defined as the ratio of the infection ability in the experimental treatment and the infection ability in the control treatment multiplied by 100. In the pathogenicity tests, a set of isolates of *B. cinerea* with varying pathogenicity was used.

Biochemical studies.

Biochemical studies were carried out with crude mycelial extracts. To this end mycelium grown in shake cultures for 24 or 48 h at 20 °C in a liquid medium (Courson and Sisler, 1960) was homogenized in a mortar at 4 °C in 0.05 M phosphate buffer, pH 7.5. The homogenate was centrifuged at 3000× *g*. The supernatant served as the enzyme source.

A. Enzyme assays. The glucose oxidase activity was determined by the method described by Bentley (1963). Other sugar oxidases were determined according to this method using galactose or xylose as substrate. The activity of ascorbate oxidase was determined photometrically at 340 nm in a mixture of 0.9 ml 0.1 M phosphate buffer, pH 6.5, 0.15 ml 0.1 mM ascorbate, 0.15 ml 0.1 mM NAD and 0.2 ml enzyme suspension.

In addition to the assays on crude mycelial extract the formation and release of hydrogen peroxide in the mycelium of *B. cinerea* was directly detected half-quantitatively in agar plates. To this end, 5 ml of an alcoholic 0.1% benzidin solution containing 1 mg horse radish peroxidase were poured onto the colonized plates. After 30 min, the violet colouration was recorded according to the following scale: 0, no colour; +, slight violet colouration of the mycelium; + +, violet mycelium; + + +, dark violet mycelium; + + + +, dark violet colouration of the mycelium and also of the surrounding agar.

B. Determination of active oxygen in crude mycelial extracts of B. cinerea. Hydrogen peroxide was assayed in *B. cinerea* mycelial extracts with iodine-starch-complex as reagent and measuring absorbance in a colourimeter at 484 nm.

Superoxide anion radicals were determined by a method according to that of

Matkovics and Szabo (1984) based on the reduction of cytochrome C in the presence and absence of superoxide dismutase. The absorbance of reduced cytochrome C was registered at 550 nm in a spectrophotometer. Proteins were determined by the method of Lowry et al. (1951).

Results

B. cinerea is one of the few plant pathogens that needs sugars in artificial inoculations for infection. In agreement with other references (Deverall and Wood, 1961a, b; Harper et al., 1981) we found a significant effect of different sugars on the ability of *B. cinerea* to infect *V. faba*. Glucose or a complete medium strongly stimulates infection (Table 1). The infection ability decreased significantly with decreasing sugar concentration of the inoculum.

In further experiments, glucose was replaced by other sugars or sugar alcohols, i.e. arabinose, fructose, galactose, inositol, maltose, raffinose, rhamnose, sorbose, trehalose and xylose. The results are presented in Table 2. Mannose, maltose, fructose or xylose had stimulating effects similar to glucose. Galactose and raffinose were not as

Table 1. Effect of varying glucose concentrations in the inoculation medium on the infection ability of *Botrytis cinerea* isolate no. 1 on *Vicia faba*.

Inoculation medium	Concentration of glucose (%)	Relative infection ability
Aqua dest.	0	2
Complete nutrition cocktail according to Courson and Sisler (1960)	1.0	68
	2.0	74
	3.0	89
Glucose solution	0.5	13
	2.0	70
	3.0	100

Table 2. Effect of various monosaccharides in the inoculation medium at 3% w/v on the infection ability by *Botrytis cinerea* on *Vicia faba*.

Sugar	Relative infection ability by isolate No.:						
	1	2	3	4	5	6	7
Arabinose	0	0	0	0	0	0	0
Fructose	85	38	46	78	62	27	78
Galactose	50	27	164	68	87	76	107
Glucose	100	100	100	100	100	100	100
Inositol	0	0	0	0	0	3	0
Mannose	106	72	123	114	125	68	106
Raffinose	53	81	60	47	0	94	69
Xylose	33	89	90	83	40	76	84

effective. The addition of rhamnose, sorbose or trehalose stimulated infection to only a small degree. Inositol and arabinose, however, were completely ineffective. No significant differences were observed in the behaviour of the individual isolates.

In order to rule out any possible nutritional effects of the sugars in the stimulation of infection, the same sugars were used in an agar medium as sole carbon source. The results indicated that all *B. cinerea* isolates studied reacted similarly to the different sugars. Whereas mannose and maltose could replace glucose in the growth test, xylose proved to be unsuitable as a nutrient source, although this sugar strongly stimulated infection. This indicates that the stimulatory effect of certain sugars was not a result of an increased energy source. Further evidence for this was obtained in experiments with inositol and arabinose. Both sugars could effectively be utilized as energy source in agar media but did not stimulate infection.

The stimulatory effect of glucose and xylose on the infection would suggest that *B. cinerea* produces sugar oxidases like glucose and xylose oxidases, as has been described for other fungi by Lyr (1962). In studies using isolates with different pathogenicity, no strong correlation could be found between the potential of peroxide generating sugar oxidases and the ability of isolates to infect bean leaves (Table 3). In contrast, the xylose oxidase activity correlated well with the stimulatory effect of xylose with respect to the infection of bean leaves by different *B. cinerea* isolates (Table 4).

Hydrogen peroxide is excreted into the medium. Its presence in cultures of *B. cinerea* on malt extract agar was demonstrated with benzidin and horse radish peroxidase

Table 3. Activities of hydrogen peroxide producing flavin enzymes from different isolates of *Botrytis cinerea* and their pathogenicity on levaes of *Vicia faba* cv. *Fribo*.

Isolate no.	Relative pathogenicity[1]	Enzyme activity[2] (Units)		
		glucose oxidase	galactose oxidase	ascorbate oxidase
1	100	2.01	2.74	2.84
2	40	1.39	1.51	0.40
4	86	1.68	2.61	2.78
5	93	0.40	0.52	1.08
8	59	1.16	1.45	1.64
9	65	3.76	4.66	1.60
10	8	2.34	2.63	1.06
11	70	2.68	2.96	1.50
12	77	2.65	3.22	0.43
13	75	2.35	2.81	0.43

[1] Determined in the presence of 3% glucose in the inoculation medium and defined as the ratio of the infection ability of the isolate indicated and that of isolate 1 multiplied by 100.
[2] Enzyme activities were determined in the crude mycelial extracts. The reaction mixture contained 1.0 ml 0.5 M phosphate buffer, pH 6.5, 0.5 ml 5% substrate, 0.5 ml enzyme suspension, 0.1 ml peroxidase solution from horse radish (1 mg ml^{-1}) and 0.05 ml 0.1% p-phenylendiamine. One enzyme unit is defined as the activity giving an increase of 0.01 absorbance unit at 485 nm per mg protein per min.

Table 4. Activity of xylose oxidases from different isolates of *Botrytis cinerea* and their pathogenicity in a xylose containing inoculum (1% xylose) on leaves of *Vicia faba* cv. *Fribo*.

Isolate no.	Relative infection ability	Enzyme activity[1] (units)
1	33	0.12
2	89	1.41
4	83	0.97
5	40	0.45
6	76	1.00
7	84	1.21
8	24	0.94
9	94	1.93
10	10	1.21
11	63	2.13
12	88	2.89
13	82	1.61

[1] Enzyme activities were determined in the crude mycelial extracts. The reaction mixture contained 1.0 ml 0.05 M phosphate buffer, pH 6.5, 0.5 ml 5% xylose solution, 0.5 ml enzyme suspension, 0.1 ml peroxidase solution from horse radish (1 mg ml^{-1}) and 0.05 ml 0.1% p-phenylendiamine. One enzyme unit is defined as the activity giving an increase of 0.01 absorbance unit at 485 nm per mg protein per min.

Table 5. Influence of different sugars on growth of *Botrytis cinerea* isolate no. 1 *in vitro* and on release of peroxide. (Hydrogen peroxide was measured directly on agar dishes).

Sugar	Concentration (%)	Relative growth of mycelium (%)	Release of hydrogen peroxide	Relative infection ability
Arabinose	1.0	35	+	0
Galactose	1.0	94	+ + +	50
Glucose	1.0	100	+ + + +	100
Inositol	1.0	59	+	0
Mannose	1.0	92	+ + + +	106
Trehalose	1.0	62	+	5
Glucose + Gluconic acid lactone	0.5 + 0.5	121	+ + +	75
Xylose	1.0	0	n.d.[1]	89
Glucose + Xylose	0.5 + 0.5	83	+ + + +	n.d.[1]

[1] n.d. = not determined.

(Table 5); similarly the presence of active oxygen in liquid cultures could also be established.

In all cases there was a significant correlation between the intensity of peroxide production *in vivo* and the type and concentration of sugars used in the nutrient medium

Table 6. Action of various anti-oxidative agents on infection ability of *Botrytis cinerea* on *Vicia faba*. (The inoculation medium contained 2% malt extract for the stimulation of infection).

Agent	Concentration (%)	Relative infection ability	
		isolate no. 1	isolate no. 4
Control	0	100	100
Benzoic acid	0.025	10	46
Gluconic acid lactone	0.100	100	11
Catalase[1]	1.000	80	33
1-Phenyl-2-thiourea	0.050	0	53
Propane-1-diol	0.050	75	22
Thiourea	0.050	23	25

[1] Activity of catalase = 100 000 units ml^{-1}.

(Table 5). A reduction in the glucose concentration in agar from 1 to 0.2% resulted in a rapid reduction in the peroxide production. At a concentration of 0.05% glucose, it was not possible to measure peroxide although mycelial growth by *B. cinerea* was good. These results correlate well with those made with artificial inoculations; infection was reduced when the glucose concentration in the inoculum was below 1%. From this it can be concluded that active types of oxygen play an important role both in the infection process and in softening of the host tissue.

Furthermore, gluconic acid lactone, a competitive inhibitor of glucose oxidase, inhibited infection by *B. cinerea*, as did a series of antioxidants and radical scavengers (Table 6). All compounds tested for an effect on infection following inoculation exhibited no inhibition of conidial germination or mycelial growth of *B. cinerea* on solid media. This indicates that the processes which were influenced by the antioxidants and the inhibitor of glucose oxidase are those which are very important for infection.

Discussion

In the current literature a clear correlation between the pathogenicity of *B. cinerea* isolated in different host-parasite combinations and the potential to produce cell wall degrading hydrolases does not exist (Lorenz and Pommer, 1987). The production of cell wall degrading enzymes by fungi is so widespread that it is unlikely that a specific relationship between the production of these enzymes and pathogenicity exists.

To date the questions as to whether *B. cinerea* produces a toxin and why aged tissue is more susceptible are still unanswered. The requirement of certain chemical substances (e.g. sugars) as additives to artificial inoculation is also typical. Our data indicating that glucose, fructose, mannose, maltose, galactose and xylose stimulated the infection of *V. faba* confirm literature data. It is of interest to note that xylose itself had no nutritional value to the fungus. In contrast, inositol and arabinose could be utilized as a C-source but were not stimulatory for infection.

Further observations indicated that *B. cinerea*, and possibly other fungal species as

well, produce sugar oxidases. *Trametes versicolor* is known as a producer of glucose and xylose oxidase (Lyr, 1962). When glucose is oxidized into gluconic acid, hydrogen peroxide is produced, which on its turn can be converted to other forms of superoxide and hydroxyl radicals, which are highly toxic and capable of destroying relatively inert material, such as cutin. The cytotoxicity most probably results from the oxidative destruction of the unsaturated lipids in the membrane, amongst other reactions, which results in the collapse of the tissue, a typical symptom of an infection by *B. cinerea*. Young tissue generally has a high degree of membrane stability. It has been shown that cytokinine application to cabbage delayed senescence and reduced at the same time the degree of *Botrytis* infection (Bochow and Schicknick, 1984).

The results presented here show that *B. cinerea* can produce a range of flavin oxidases. The presence of glucose-, xylose-, galactose- and ascorbate-oxidase could be documented. As the substrates for these enzymes can be released by cell wall degrading hydrolases, conditions are met which favour the production of hydrogen peroxide. The substrates, however, are not present on the intact cuticula and must be supplied with the inoculum. This could explain why *B. cinerea* operates mostly as a wound parasite under field conditions.

In our studies, a correlation between oxidase activity and pathogenicity was found in particular with the xylose oxidase. However, the values found for enzyme activity *in vitro* should not be overestimated, as these values are highly dependent on the age of mycelium, culture conditions, inducers and effectors.

Unfortunately the actual enzyme activities *in situ* during the infection process are very difficult to determine. The role of the hydrolytic enzymes in overcoming penetration barriers such as the cuticle has not yet been determined. In addition to cutinolytic enzymes, cutin and suberin are very susceptible to oxidative breakdown.

Apparently superoxide and free oxygen radical play an important role in the infection process. It could be shown that both catalase and α-tocopherol acetate could significantly inhibit infection of *V. faba* leaflets by *B. cinerea*. Gluconic acid lactone, a specific inhibitor of glucose-1-oxidase, could reduce both the production of peroxide by *B. cinerea* as well as the infection of *V. faba*. These results are supported by work reported by Rist and Lorbeer (1984a, b) who observed a higher infection of onion leaves by *B. cinerea* after they had been treated with ozone, indicating a weakening of the host tissue through the active oxygen.

From the results it can be concluded that the stimulation of infection by *B. cinerea* after addition of certain sugars to the artificial inoculum is not a result of nutritional effect but probably due to the active forms of oxygen formed. The sugars also favour the destruction of the cell membranes of the host, a typical symptom caused by infection of this fungus. As a result, the contents of the vacuoles could be released and react with cytoplasmic peroxidases which in turn result in the development of characteristic *B. cinerea* lesions on *V. faba*.

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Samenvatting

Nieuwe aspecten aangaande het infectiemechanisme van Botrytis cinerea Pers.

Met behulp van reactieve zuurstofverbindingen, extracellulaire enzymen en mechanische processen is *Botrytis cinerea* in staat planten te infecteren. Terwijl er geen duidelijke positieve correlatie gevonden werd tussen activiteiten van proteases, pectolytische enzymen en celwandafbrekende enzymen enerzijds en pathogeniteit anderzijds, werd wel een positieve correlatie waargenomen tussen pathogeniteit van sommige isolaten en het vrijmaken van reactieve zuurstofverbindingen door deze isolaten. Er wordt aangenomen dat deze toxische zuurstofverbindingen vrijkomen door activiteit van glucose- en xylose-oxidasen. Antioxidantia remden het infectieproces. Uit deze resultaten werd geconcludeerd dat de reactieve zuurstofverbindingen een beslissende rol spelen in het infectieproces van *B. cinerea*.

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