Nachrichtenbl. Deut. Pflanzenschutzd., **45** (1), S. 7–10, 1993, ISSN 0027-7479.

Institut für Mikrobiologie, Technische Universität Braunschweig, Braunschweig

Importance of antibiotically active metabolites of Mycosphaerella brassicicola (Duby) Lindau for the estimation in evaluating host susceptibility to ring spot disease of cabbage

Zur Bedeutung antibiotisch aktiver Metaboliten von Mycosphaerella brassicicola (Duby) Lindau für die Abschätzung der Anfälligkeit des Kohls gegenüber der Ringfleckenkrankheit

Von Monika Götz und C. Boyle

Abstract

Production of antibiotically active metabolites by *Mycosphaerella brassicicola* was observed on three different solid media and in broth cultures. Comparisons between the algicidal and fungicidal activities of the extracts suggested the presence of at least four antibiotically active substances. Extracts of potato dextrose broth cultures of *M. brassicicola* inhibited the germination of seeds of host plants whereas non-host seeds were not affected significantly. The relative level of susceptibility of white cabbage to *M. brassicicola* correlated with the inhibition of seed germination by extracts of the filtrate and mycelium. Extracts of filtrate of broth cultures showed an inhibition of the main root growth and of the side root development of seedlings. Due to the high concentrations of toxins, seedlings of susceptible and resistant cultivars did not differ in their reaction to the antibiotically active metabolites.

Zusammenfassung

Auf drei unterschiedlichen Medien wurde in Fest- und Flüssigkultur die Produktion antibiotisch aktiver Metaboliten von *Mycosphaerella brassicicola* untersucht. Ein Vergleich der algiziden und fungiziden Aktivitäten der Extrakte läßt auf mindestens vier antibiotisch wirksame Substanzen schließen. Extrakte der Kartoffel-Dextrose-Flüssigkultur von *M. brassicicola* hemmten die Keimung der Wirtspflanzensamen, Samen von Nicht-Wirtspflanzen wurden nicht signifikant beeinträchtigt. Die relative Anfälligkeit von Weißkohl gegenüber *M. brassicicola* korrelierte mit der Hemmung der Wirtssamenkeimung durch die Filtrat- oder Mycelextrakte. Kulturfiltratextrakte bewirkten eine Hemmung des Hauptwurzelwachstums und der Seitenwurzelentwicklung von sieben Tage alten Weißkohlkeimlingen. Entsprechend der hohen Toxinkonzentrationen unterschieden sich die Befunde anfälliger und resistenter Sorten nicht in ihrer Reaktion nach der Einwirkung antibiotisch aktiver Metaboliten.

Introduction

Mycosphaerella brassicicola (Duby) Lindau causes the ring spot disease of brassicas (Weimer, 1926; Nelson & Pound, 1959; Dring, 1961). Since its discovery in France more than 190 years ago it has been found in other countries of western Europe. It has also caused yield losses in New Zealand, Australia, Peru and Ecuador. The fungus is most often reported on cauliflower and cabbage, however, it can attack many brassica species (Sherf & Mac Nab, 1986). Cabbage *(Brassica oleracea* L. *var. capitata)* is the most important vegetable culture in Germany where a serious epidemic of ring spot disease occurred in 1985. Especially in Dithmarschen and on Fehmarn losses up to 40 to 50 % of the average crop of white and red cabbage were recorded (Zornbach, 1988).

In order to avoid future crop damage and high costs for fungicides, it is necessary to test all cabbage cultivars with respect to their susceptibility. For this purpose it is advantageous to find a simple and reliable method to determine the susceptibility of a cultivar by using its seeds or young plants.

The presence of a chlorotic zone surrounding the lesions caused by *M. brassicicola* as well as histological studies of the first stages of infection showed that mesophyll cells adjacent to the hyphae had been destroyed (Götz et al., in prep.). These observations also suggested that antibiotically active substances are involved in the infection process.

This paper deals with the detection and biological activity of antibiotically active secondary metabolites produced by *M. brassicicola*.

Materials and Methods

Culture conditions: Fungal isolates were obtained by collecting ascospores discharged from infected white cabbage leaves. Single spore isolates were cultivated on potato dextrose agar (= PDA), vegetable juice agar (= V8) and mineral salt agar (= SNA) at 20 °C in the dark.

Screening: A screening for antibiotically active metabolites occurred on PDA, SNA and V8 solid media. After 4, 7, 9, 14, 21, 31 and 41 days of incubation at 20 °C in the dark, each solid culture was sprayed with an aqueous suspension of one of seven indicator organisms (Table 1). After 2–7 days zones of inhibition were measured.

For the screening in liquid cultures flasks containing PDA, V8 or SNA medium were inoculated with preculture of the corresponding medium. The cultures were incubated on a shaker at 20 °C and 120 rpm in the dark. In order to control the test uninoculated medium was incubated in the same manner. After 7, 14, 21, 31 and 41 days of incubation the mycelium was separated from the medium by filtration.

Extraction: In order to extract active metabolites from the liquid medium acetoacetate and the medium filtrate were combined at the ratio of 1:1, shaken for 5 min and after separation of the two phases the water phase was extracted for the second time. All organic phases were combined and

evaporated. The residue was resuspended in methanol, resulting in a concentration factor of 100.

The mycelium was washed twice with distilled water, lyophilisated, homogenized and extracted with methanol for 48 h. The methanol was separated from the mycelium by filtration and concentrated in the rotary evaporator.

Plate diffusion tests: A plate diffusion test according to ZÄHNER (1965) was conducted to determine the antibiotic activity of the mycelial and medium extracts against seven indicator organisms (Table 1).

Plant tests: For the germination test and the following experiments extracts of mycelium grown in PDA liquid culture and the corresponding filtrate were used.

To test the activity of the toxins against seeds, the extracts were diluted with distilled water (1:9). As a control, methanol was diluted to the same ratio. Filters were placed in petri dishes and soaked with the extracts or the control. The seeds of *Medicago sativa, Lepidium sativum, Brassica napus* and of the white cabbage cultivars 'Lennox', 'Erdeno' and 'Carlton' were placed in the prepared petri dishes and incubated at 20 °C with 12h:12h light:dark period. After 3, 5, and 8 days of incubation the percentage of germinated seeds was determined.

Effects of the antibiotically active metabolites on young plants were determined by incubating seeds from the cultivars 'Lennox' and 'Erdeno' in a humid chamber for 7 days. In order to avoid the phytotoxic effects of methanol it was evaporated from the mixture. The 7 day old seedlings were incubated in titer plates (∅ 10 mm, depth 25 mm) with 1,5 ml of the aqueous extract per well at 20 °C with a 12h:12h light:dark period. After 4 days, 1 ml of the aqueous extract was added to each seedling. Three days later, the lengths of the seedlings including the roots were measured.

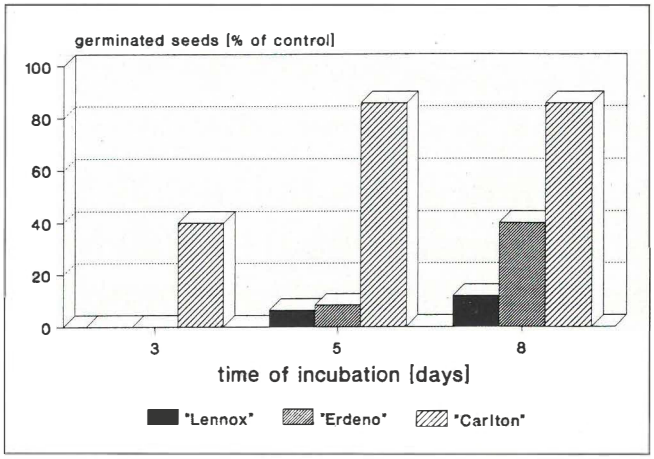

Figure 1. Inhibition of germination of white cabbage seeds having different grades of susceptibility.
Abb. 1. Hemmung der Keimung von Weißkohlsamen unterschiedlicher Anfälligkeit.

Solubility tests: To characterize the toxic substances the toxins' solubility in organic solvents of varied polarity were tested. N-hexane, toluene and acetoacetate were used to extract the filtrates.

Thin-layer chromatography: Studies were done using thin-layer chromatography. 0,2 ml of the extracts of filtrate and mycelium were applied to thin-layer chromatography foils (silica gel, layer thickness 0,2 mm). The foils were developed twice in a mixture of benzene and diethylether (40:9, v/v). The indicator organisms *Chlorella pyrenoidosa, Eurotium repens* and *Cladosporium cucumerinum* were suspended in media. After spraying with the suspensions, the foils were incubated in a humid chamber at 20 °C with a 12:12h light:dark period. After 1–9 days of incubation the zones of inhibition were detected and the R_f-values of the antibiotically active substances were determined.

Table 1. Evidence of antibiotically active metabolites produced by *M. brassicicola* on different solid and liquid cultures after 41 days of incubation at 20 °C in the dark.
Tab. 1. Hinweise auf die Produktion antibiotisch aktiver Metaboliten durch *M. brassicicola* auf unterschiedlichen Fest- und Flüssigmedien nach 41tägiger Inkubation bei 20 °C im Dunkeln.

indicator organism	solid media			broth culture*	
	PDA	SNA	V8	PDA	SNA8
Bacillus megaterium	(+)	–	–	–	–
Mycotypha microspora	+++	–	+++	+	–
Eurotium repens	+++	++	+++	+	+
Ustilago violacea	–	–	–	+	+
Chlorella pyrenoidosa	++	+	+	+	+

Radius of inhibition of the indicator organisms measured from the margin of *M. brassicicola* colony respective testplate: – no inhibition, (+) sporadically observed inhibition, + up to 10 mm, ++ 10–20 mm, +++ more than 20 mm
* all + enumerated for liquid cultures measured less than 6 mm.

Table 2. R_f-values detected by thin-layer chromatography (benzene/diethylether; 40:9, v:v) of the antibiotically active metabolites and inhibition of the indicator organisms *C. pyrenoidosa, E. repens* and *C. cucumerinum*.
Tab. 2. R_f-Werte der dünnschichtchromatographisch aufgetrennten antibiotisch aktiven Metaboliten (Benzol/Diethylether; 40:9, v:v) und Hemmung der Indikatororganismen *C. pyrenoidosa, E. repens* und *C. cucumerinum*.

	R_f-value	*Chlorella pyrenoidosa*	*Eurotium repens*	*Cladosporium cucumerinum*
substance I	0,70	++	++	+/–
substance II	0,75	++	+	+/–
substance III	0,78	+	–	–
substance IV	0,91	++	+	+/–

– no inhibition, +/– temporary inhibition, + inhibition, ++ strong inhibition.

Results

Both the generative compact (V8) and the vegetative white mycelium (PDA, SNA) produced toxic metabolites which affected the growth of *C. pyrenoidosa, E. repens* and *Mycotypha microspora*. *Bacillus megaterium* was inhibited sporadically only when the fungus was grown on PDA solid medium. Extracts of the filtrates also inhibited the growth of *Ustilago violacea* (Table 1), no effects could be detected on *Escherichia coli* and *Fusarium oxysporum*. The inhibitory effect increased with time of incubation. The most effective inhibition of *U. violacea* resulted by extracting the filtrate with acetoacetate, whereas *C. pyrenoidosa, E. repens,* and *M. microspora* were most effectively inhibited using n-hexane for the extraction. Extracts of the sterile culture media never affected the growth of the indicator organisms.

Bioassays with thin-layer chromatography proved the presence of at least four antibiotic substances (R_f-values are shown in Table 2).

The germination test showed a conspicuous inhibition of the brassica seeds by the extracts of the filtrate und mycelium, whereas the seeds of *M. sativa,* which does not belong to the

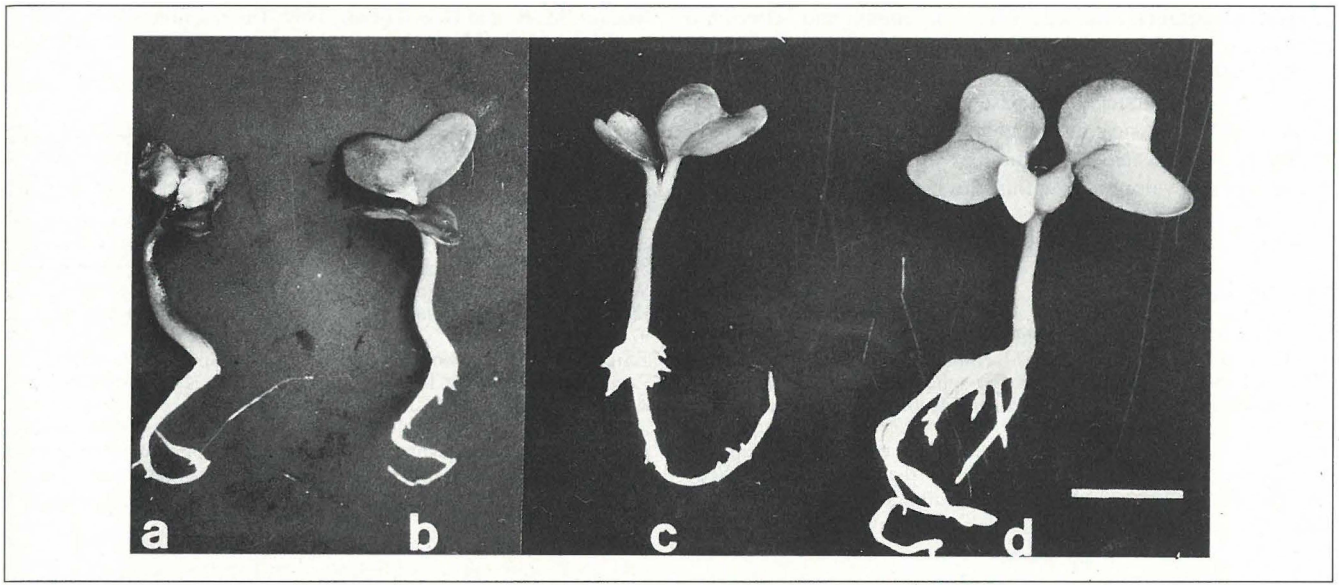

Figure 2. Influence of the aqueous filtrate extract on the development of seven day old seedlings of the cultivar 'Lennox', seven days after application of the test substances. Shown are a) the shortest and b) the longest seedling of the test with extract containing the active substances and c) the shortest and d) the longest seedling of the methanol control. The scale bar corresponds to 8.5 mm.

Abb. 2. Einfluß wäßriger Filtratextrakte auf die Entwicklung sieben Tage alter Sämlinge der Sorte 'Lennox', nach siebentägiger Einwirkung das Extraktes. Abgebildet sind a) der kürzeste und b) der längste Keimling des aktive Substanzen enthaltenden Extraktes und c) der kürzeste und d) der längste Keimling der methanolischen Kontrolle. Der Balken entspricht 8.5 mm.

host spectrum of *M. brassicicola*, were hardly affected. Tests with the seeds of white cabbage demonstrated a clear correlation between the resistance of the cultivar to the pathogen based on field observations over several years and the inhibition of seed germination caused by the extracts of the mycelium and filtrate (Figure 1). Initial tests using injured and non-injured primary leaves of 20 day old seedlings dabbed with extracts of the filtrate showed necrotic lesions and which spreaded correspondingly to the plant resistance level as mentioned above. This observation indicated a certain host specificity of the antibiotically active metabolites. However, due to the algicidal and fungicidal effects this is not a host specificity in the sense of PRINGLE & SCHEFFER (1964).

Aqueous extracts of medium filtrate caused growth-retardation of the main roots and inhibition of the side root development in seven day old seedlings (Figure 2). The cotyledons of the toxin-treated seedlings were smaller than those of the controls, perhaps a consequence of the lack of nutrient supply due to the underdevelopment of the roots. A significant difference between susceptible and less susceptible cultivars was not detectable, it might be a consequence of the high toxin concentration.

Discussion

Antibiotically active metabolites produced by *M. brassicicola* on solid medium and in liquid culture have been detected. The inhibition of the indicator organisms by this metabolites increased with the time of incubation of *M. brassicicola*. This observation can be explained by a higher concentration of the toxins which can be achieved by raising production, accumulation or reducing decomposition of the antibiotically active substances. The degree of inhibition varied according to the incubation time and the nature of the indicator organisms used. This suggested the production of more than one antibiotically active metabolite. This presumption was supported by the results from solubility tests, which showed differing solubilities of the toxic compounds in various polar organic solvents, and thin-layer chromatography, which proved the presence of at least four antibiotically active metabolites.

These toxins could be important tools in evaluating a screening for cabbage cultivars which are highly resistant to ring spot disease. ZORNBACH (1989) examined the cotyledons of some white cabbage cultivars after inoculating them whith mycelium of *M. brassicicola*. This test, however, was not suitable for calculating the degree of resistance. HARTILL (1977) studied the inhibition of the infection of young white cabbage plants with *M. brassicicola* by allylisothiocyanate, which is incrusted in the wax layer of the leaves. This inhibitor is catabolized in the course of aging at different rates by the various cultivars. Because of the differing physiological ages of the cotyledons a comparison between the various cultivars as to their grades of susceptibility could not be made.

One advantage of using extracts containing the phytotoxic metabolites in a screening test for resistance of white cabbage cultivars is that the activity of the extracts is not dependent on allylisothiocyanate previously having been degraded. A further advantage is the ability to precisely apply the toxins and to control and adjust the concentration of the toxic compounds, if necessary. Screening for resistant cultivars using toxin containing extracts would, therefore, be the method of choice against a screening using the pathogen itself.

As the resistance of plants against the pathogen correlates with the seedlings' and leafs' reaction to the toxins it should be possible to develop a screening for *M. brassicicola* resistant white cabbage cultivars in combination with germination tests.

It is probable that the antibiotics are involved in the infection process, as seeds of resistant cultivars reacted less to the toxins than susceptible ones did, and as non-host seeds were not affected to a significant extent by the toxins. A further function of the fungicidal and sporadically produced bactericidal metabolites could, in addition to the weakening of the host, be the decrease or exclusion of competitive fungi and bacteria from the common habitat. RUDNICK (1986) often

found *Leptosphaeria maculans, Phoma lingam* and *Alternaria brassicae* on *M. brassicicola* infected white cabbage leaves and DIXON (1981) observed bacterial infections on leaf spots caused by *M. brassicicola*. These organisms are strong competitors of *M. brassicicola,* especially due to the longer growth period of *M. brassicicola* on the leaf surface and its very slow intercellular development within the host.

References

DIXON, G. R., 1981: Mycosphaeralla brassicicola (ringspot). In Vegetable Crop Diseases, pp. 126–128, Macmillan Publishers CID: London.
DRING, D. M., 1961: Studies on Mycosphaerella brassicicola (Duby) Oudem, Transactions of the British Mycological Society **44** (2), 253–264.
HARTILL, W. F. T., 1977: Epidemiology and control of ringspot in cabbages and cauliflowers, Proceedings of the New Zealand Weed Pest Control Conference **30,** 91–95.
NELSON, M. R. und G. S. POUND, 1959: The reaction of environment to the ringspot (Mycosphaerella brassicicola) disease of crucifers, Phytopathology **49,** 633–640.
PRINGLE, R. B. und R. P. SCHEFFER, 1964: Host-specific plant toxins, Annual Review of Phytopathology **2,** 133–156.
RUDNICK, M., 1986: Epidemisches Auftreten der Ringfleckenkrankheit an Kohl in Schleswig-Holstein, verursacht durch Mycosphaerella brassicicola (Duby) Lindau, Nachrichtenblatt des Deutschen Pflanzenschutzdienstes **38** (6), 83–85.
SHERF, A. F. und MACNAB, 1986: Ringspot (Mycosphaerella brassicicola). In Vegetable Diseases and their Control, p. 728, Wilson and Sons, New York, Chichester, Brisbane, Toronto, Singapore.
WEIMER, J. L., 1926: Ringspot on crucifers caused by Mycosphaerella brassicicola (Fr.) Lindau, Journal of Agricultural Research **32,** 97–132.
ZÄHNER, H., 1965: Biologie der Antibiotika. Springer: Berlin.
ZORNBACH, W., 1988: Ringfleckenkrankheit an Kohl, Gb+Gw 1988, **8,** 325–327.
ZORNBACH, W., 1989: Untersuchungen zur Pathogenese, Epidemiologie und Bekämpfbarkeit von Mycosphaerella brassicicola (Duby) Lindau, dem Erreger der Ringfleckenkrankheit an Cruciferen, Dissertation: Hamburg.