

The Role of Phytoalexins in the Seedling Resistance to *Leptosphaeria maculans* in Some Crucifers

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Brassica juncea, *Brassica carinata* and *Sinapis arvensis* resistant lines to *Leptosphaeria maculans* and four *Brassica napus* cultivars susceptible to this pathogen in seedling stage were analyzed in relation to the accumulation of phytoalexins after inoculation with *L. maculans*. Cotyledon inoculations with spore suspensions of an aggressive and a non-aggressive isolate of *L. maculans* were performed on seedlings of these lines. The quantity of accumulated phytoalexins in the cotyledons was determined at different time intervals after inoculation. The content and composition of phytoalexins differed considerably among the tested species and according to the fungal isolate used. In the tested *Brassica* species seedling resistance against *L. maculans* could not be related to phytoalexin accumulation. However, in *Sinapis arvensis* phytoalexins might contribute to the resistance reaction to this pathogen.

Introduction

Leptosphaeria maculans (Desm.) Ces et de Not., the causal agent of the blackleg disease, is the most important pathogen of the cruciferous crops. Cruciferous plants react against the infection with *L. maculans* by synthesizing sulphur-containing indole phytoalexins (Dahiya and Rimmer, 1989; Rouxel *et al.*, 1989; Pedras and Seguin-Swartz, 1992). These compounds have in fact an inhibitory effect to *L. maculans* *in vitro* (Dahiya and Rimmer, 1988; Rouxel *et al.*, 1989; Pedras and Taylor, 1991), but the exact function of these phytoalexins in the resistance to *L. maculans* is still unclear. Rouxel *et al.* (1989) demonstrated that the accumulation of the phytoalexin brassilexin in detached leaves of a resistant *Brassica juncea* line infected with *L. maculans* was higher and more rapid than in leaves of a susceptible *B. napus* line. Later Rouxel *et al.* (1991) looked at the correlation between resistance and the accumulation of phytoalexins in leaves of plants which had been sprayed with the unspecific abiotic elicitor cupric chloride. The results indicated that there was a relationship between the accumulation of brassi-

lexin and resistance, since the resistant *Brassica* species with the B-genome (*B. carinata*, *B. juncea*, *B. nigra*) showed a higher brassilexin accumulation than the most susceptible lines of the species *B. napus*, *B. oleracea* and *B. rapa*. However, a resistant *B. nigra* and a resistant *B. rapa* line accumulated only small amounts of brassilexin. On the other hand, the accumulation of significant amounts of brassilexin was not observed in any of the susceptible plants.

Dahiya and Rimmer (1989) investigated the accumulation of the two phytoalexins methoxybrassinin and cyclobrassinin in *Brassica* spp. after the infection with the blackleg fungus *in vitro*. They found that both phytoalexins accumulated in *Brassica* tissue after inoculation with a non-aggressive isolate of *L. maculans*, but only cyclobrassinin was detected in tissue which had been inoculated with an aggressive isolate. Furthermore they could show that detached leaves or stem segments of a resistant *B. juncea* line accumulated more methoxybrassinin and cyclobrassinin than susceptible lines of *B. napus* and *B. campestris* after inoculation with a non-aggressive isolate of *L. maculans*.

We report here results concerning qualitative and quantitative differences in phytoalexin accumulation in cotyledons of some crucifers when challenged with *L. maculans*.

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Materials and Methods

Plant material

Four cultivars of rapeseed (*B. napus* ssp. *oleifera*) were used: Lesira, Lirajet, Liropa and Olymp. These cultivars are susceptible to *L. maculans* in the seedling stage. Also *B. carinata*, *B. juncea* and *Sinapis arvensis*, which are resistant to *L. maculans*, were analyzed. The resistance to *L. maculans* had previously been assessed in a cotyledon inoculation test on the basis of lesions obtained.

The seeds were germinated on moist filter-paper in Petri dishes and the seedlings transferred into soil two days after germination. The plants were grown at 22 °C with constant light and transferred after inoculation to a culture chamber at 20 °C and a photon flux density of 100 $\mu\text{mol s}^{-1} \text{m}^{-2}$ under a 16 h photoperiod with 70–90% relative humidity.

Inoculation with *Leptosphaeria maculans*

A highly aggressive isolate of *L. maculans* (W4) and a non-aggressive one (SV1) (Hassan *et al.*, 1991) were cultured on V8 agar at 22 °C under near ultraviolet illumination for 12 days. Spore suspensions were obtained by flooding the surface of the culture plates with sterile water. The spore suspensions were filtered through a 60 μm sieve and the concentration was adjusted to 5×10^7 spores ml^{-1} . Six days after transferring to soil, the seedlings were inoculated by wounding the cotyledons at two sites with a needle and placing a 10 μl drop of the spore suspension over each wound. In the case of the smaller cotyledons of *Sinapis arvensis*, only 5 μl were applied. Controls were inoculated with sterile distilled water. Cotyledon inoculations were repeated for each of the independent experiments described in the results.

Phytoalexin analyses

After inoculation, samples consisting of 15 to 40 seedlings were taken every two days until the 16th day after inoculation. The cotyledons were maintained at –18 °C. Afterwards they were freeze-dried and stored under dry conditions in a desiccator until use.

The extraction and purification of the samples was performed according to Kollmann *et al.* (1989) with some modifications. The freeze-dried plant material was milled with a pestle, suspended in

acetone (10 ml mg^{-1}) and set aside for 5 min. 20 μg internal standard (methiocarb) were added. After centrifugation (15 min, 15,700 $\times g$) the supernatant was evaporated to dryness at 40 °C and reduced pressure. The residue was taken up in 4 ml *n*-hexane and passed through a Chromabond C18 reversed phase cartridge (Macherey-Nagel). The column was washed with 3 \times 1 ml *n*-hexane. The phytoalexins were eluted with 3 \times 1 ml 60% (v/v) aqueous methanol. Following removal of the methanol by rotary evaporation at 40 °C, the residue was resuspended in 100 μl ethanol (absolute). After filtration (0.45 μm), 20 μl of the filtrate were taken for HPLC analysis.

The HPLC analysis of phytoalexins was performed on a HPLC system equipped with two pumps (Knauer pump 64), a controller (Barspec Chrom-A-Set) and a detector (Barspec Chrom-A-Scope). The BDS (Barspec Data System) software was used for measuring peak area. The analytical column was Nucleosil C18 (5 μm) (250 \times 5 mm). The column was eluted with a successive linear gradient from 50% methanol in water to 35% over 3 min; to 15% over 8 min; to 100% over 3 min. The eluent was held for 6 min and then brought back to 50% methanol over 5 min and allowed to equilibrate for 5 min. The flow rate was 1 ml min^{-1} at room temperature. Detection took place at 218 nm. Brassinin, brassilexin, cyclobraassinin, methoxybrassinin and spirobrassinin were identified by direct comparison with purified phytoalexins. Cyclobraassinin sulphoxide was identified by comparison of chromatograms of *B. juncea* and *B. napus* leaves treated with cupric chloride with those of controls.

Results

Accumulation of phytoalexins in cotyledons after infection with an aggressive isolate of *Leptosphaeria maculans*

The content and composition of phytoalexins which were synthesized in cotyledons after inoculation with *L. maculans* differed considerably among the tested species. The four *B. napus* cultivars presented a similar reaction. Phytoalexin accumulation for the cultivar Olymp is shown in Fig. 1a. Four different phytoalexins were found in *B. napus*, namely spirobrassinin, cyclobraassinin sulphoxide, brassilexin and cyclobraassinin. Of

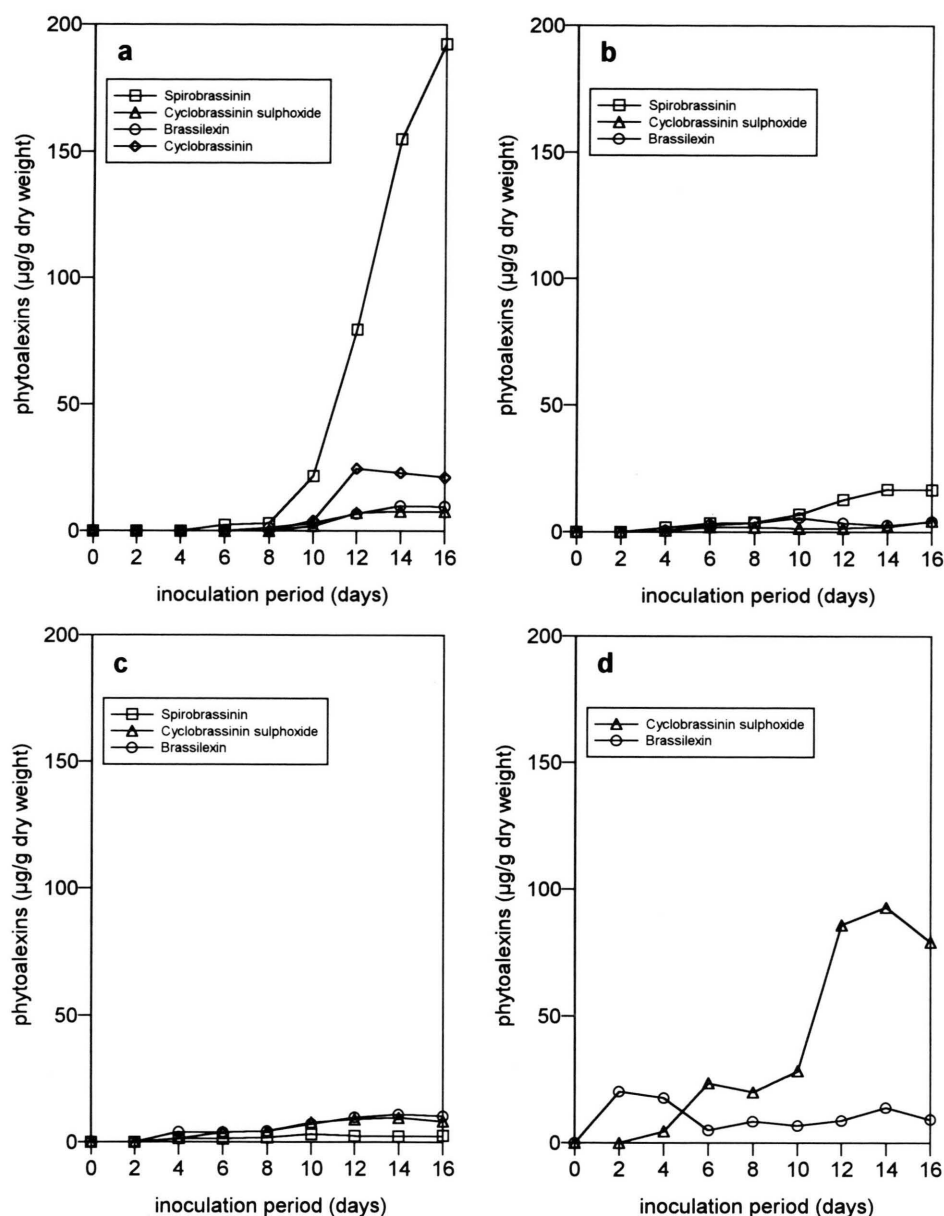


Fig. 1. A time-course study of phytoalexin accumulation in cotyledons of *Brassica napus* (a), *Brassica juncea* (b), *Brassica carinata* (c), and *Sinapis arvensis* (d) as response to inoculation with an aggressive isolate of *Leptosphaeria maculans*.

these phytoalexins, spirobrassinin accumulated in the largest amounts. Spirobrassinin was detected already on the 6th day after inoculation, whereas the others were not yet detectable at this stage. Between the 10th and 12th day the phytoalexins accumulated in large amounts. Until the end of the

observation time, *i.e.* on the 16th day, the content of spirobrassinin increased remarkably while the content of the other three phytoalexins remained constant. The experiment with *B. napus* cultivars was repeated three times and in each case essentially the same results were obtained.

In the analyzed *B. juncea* line (Fig. 1b) three phytoalexins were basically detected: spiobrassinin, brassilexin and cyclobassinin sulphoxide. Cyclobassinin was only present in small traces (data not shown). All three phytoalexins were first detected four days after inoculation. Until the 8th day after inoculation the phytoalexin content of *B. juncea* was greater than that of *B. napus*; however, the latter was higher after the 10th day. In *B. juncea* spiobrassinin was the main phytoalexin; it increased until the 14th day and then remained constant. Cyclobassinin sulphoxide increased until the 6th day and afterwards presented only small variations. Brassilexin content increased until the 10th day after inoculation and declined again. This experiment was performed four times and the results differed only slightly.

In *B. carinata* (Fig. 1c) only a slight accumulation of phytoalexins was recorded. The three phytoalexins spiobrassinin, cyclobassinin sulphoxide and brassilexin were first detectable on the 4th day after inoculation. Cyclobassinin sulphoxide and brassilexin were present in approximately equal amounts. The content of them increased continuously until the 14th day and after that no further increase was recorded. Spiobrassinin was only present in small amounts and the level of it did not change significantly during the observation period. This experiment was performed three times and the reproducibility was good.

In the case of *Sinapis arvensis* (Fig. 1d) only brassilexin and cyclobassinin sulphoxide accumulated in relevant quantities. Spiobrassinin was present in only small amounts from the 8th day of observation time on (data not shown). Brassilexin was detected already two days after inoculation and its amount increased and decreased during the observation period at irregular intervals. Cyclobassinin sulphoxide appeared at the 4th day and between the 6th and 8th day it became the main phytoalexin. During the observation time the content of cyclobassinin sulphoxide tended to increase. Among the investigated species *Sinapis arvensis* was the only one in which phytoalexins were detectable two days after inoculation. Until the 10th day after inoculation *Sinapis arvensis* showed the highest phytoalexin content among all four species. This experiment was performed three times and the results were essentially the same.

Accumulation of phytoalexins in cotyledons after infection with a non-aggressive isolate of Leptosphaeria maculans

The experiment using the non-aggressive isolate SV 1 was only performed with the species *B. napus* cv. Olymp and *B. juncea*.

In *B. napus* (Fig. 2a) three different phytoalexins were detected, namely spiobrassinin, cyclobassinin sulphoxide and brassilexin. The first phytoalexins appeared on the 6th day after inoculation. Spiobrassinin was found to be the main phytoalexin and its content was always greater than that of the other phytoalexins. The amount of spiobrassinin showed an increasing tendency with some variations. Brassilexin and cyclobassinin sulphoxide content increased until the 10th day and afterwards it declined again. By comparing the accumulation of phytoalexins in *B. napus* after inoculation with the aggressive isolate W 4 to that with the non-aggressive isolate SV 1 the following differences can be found. In the case of SV 1 the phytoalexin content on the 6th and 8th day was slightly higher than in the case of W 4. The phytoalexins brassilexin and cyclobassinin sulphoxide appeared earlier as well. On the 10th day the contents were similar in both cases and from the 12th day onwards the content of phytoalexins in *B. napus* inoculated with W 4 was much higher. Another difference is that cyclobassinin was not accumulated after inoculation with SV 1, while after inoculation with W 4 it was detected from the 10th day on and was then the second important phytoalexin. This experiment was repeated twice and nearly identical results were obtained.

In *B. juncea* inoculated with SV 1 (Fig. 2b) spiobrassinin, cyclobassinin sulphoxide and brassilexin were detected four days after inoculation. The accumulation of brassilexin and spiobrassinin showed a bell-shaped course, while the accumulation of spiobrassinin took a zigzag course. The amount of phytoalexins was similar to that found after inoculation with the aggressive isolate W 4, but there was a greater proportion of cyclobassinin sulphoxide than brassilexin. Another difference was the remarkable decrease of spiobrassinin after the 12th day. This experiment was repeated twice and the results differed slightly.

In non-inoculated controls phytoalexins were not detectable.

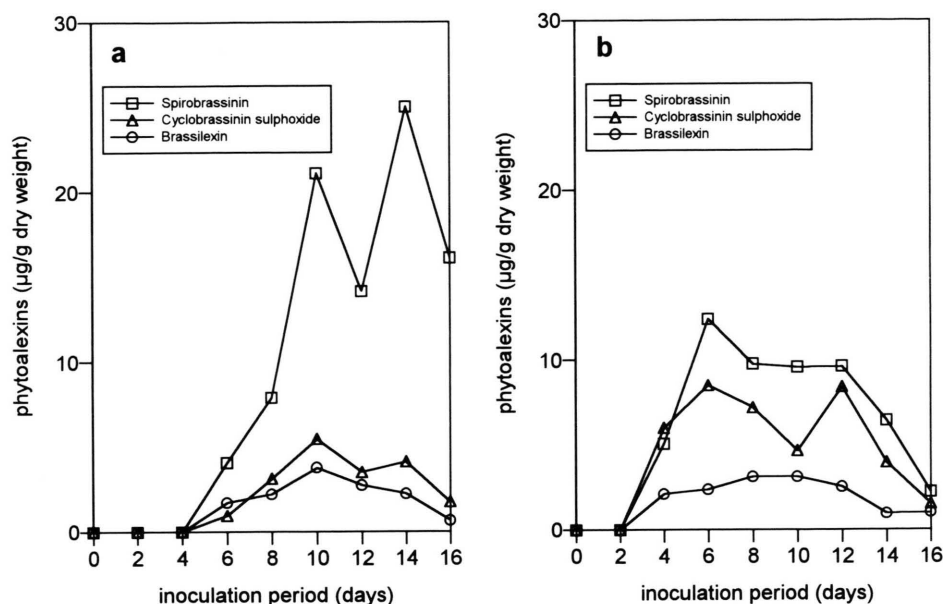


Fig. 2. A time-course study of phytoalexin accumulation in cotyledons of *Brassica napus* (a) and *Brassica juncea* (b) as response to inoculation with a non-aggressive isolate of *Leptosphaeria maculans*.

Discussion

Although phytoalexins contribute to the development of a resistance reaction, only in a few plant-parasite systems has it been proven that race-specific resistance is correlated with the accumulation of phytoalexins. The system *Glycine max*–*Phytophthora megasperma* represents an example. In this instance a quick accumulation of the phytoalexin glyceollin I in the interaction with an incompatible race of the pathogen and only a slight accumulation with a compatible race could be found (Hahn *et al.*, 1985).

In the investigated interaction between *Brassica* species and the pathogen *L. maculans*, phytoalexins do not seem to be determinant in the expression of resistance. In the tested resistant species *B. juncea* and *B. carinata*, phytoalexins did not appear shortly after inoculation and accumulated in only small quantities from the 4th day onwards. On the other hand, *B. juncea* and *B. carinata* were able to synthesize large amounts of brassilexin and cyclobrassinin sulphoxide within a few hours, if the cotyledons were sprayed with the unspecific elicitor cupric chloride (data not shown).

In the susceptible species *B. napus*, the phytoalexin content within the first eight days after inoculation differed slightly from that of the species *B. juncea* and *B. carinata*. The accumulation of phytoalexins within the first ten days was similar in the compatible (W4) and the incompatible (SV1) reaction. In the compatible reaction the quantity of synthesized phytoalexins is positively correlated with the fungal expansion in the tissue, *i.e.* first after symptoms are seen an intensive accumulation of phytoalexins occurs.

Pedras and Taylor (1993) showed that indole phytoalexins possibly favour fungal invasion. These phytoalexins inhibit the formation of sirodesmin PL, a fungal metabolic product which suppresses the synthesis of the specific toxin phomalide.

Of interest is the phytoalexin accumulation in *Sinapis arvensis*. In this case phytoalexins could play a more important role in the defence against the blackleg fungus as is the case for the tested *Brassica* species. The phytoalexins of *Sinapis arvensis* appeared earlier after inoculation and they accumulated in higher amounts than in the other analyzed cases.

Our results are different from those described in previous studies on phytoalexin accumulation in *Brassica* species. In the study of Rouxel *et al.* (1991) the differences in quantities and composition of the accumulated phytoalexins were evident, due to the different method of elicitation. They used cupric chloride as an elicitor, which was much more effective than elicitation by the pathogen but also unspecific. This led to the synthesis of all phytoalexins that the plant is able to synthesize.

Dahiya and Rimmer (1989) found large amounts of cyclobrassinin in detached leaves or stem segments of *B. juncea* after infection with an aggressive isolate of *L. maculans*, and large quantities of cyclobrassinin and methoxybrassinin after infection with a non-aggressive isolate. In the present investigation methoxybrassinin was not detected in *B. juncea* cotyledons and cyclobrassinin was detected only in traces after inoculation with

an aggressive isolate of *L. maculans*. The reason for these differences could be tissue-specific differences in phytoalexin synthesis, but more probably the differences depend on the fact that *Brassica* tissue *in vitro* shows no resistance against the blackleg fungus (Badawy *et al.*, 1992) and therefore the fungus is able to grow on detached leaves as well as on medium.

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