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Differences in response to the toxin sirodesmin PL produced by *Phoma lingam* (Tode ex Fr.) Desm. on protoplasts, cell aggregates and intact plants of resistant and susceptible *Brassica* accessions

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Summary. The selective property of sirodesmin PL, a toxin produced by *Phoma lingam,* was studied on protoplasts, cell aggregates, leaves and roots. Directly after isolation, protoplasts from all the different *Brassica* accessions were sensitive when treated with toxin in a concentration higher than 1 μM. When more differentiated plant tissue. i.e. cell aggregates, leaves or roots, were investigated, insensitivity to the toxin was found in the plant material resistant to *P. lingam,* while the plant material susceptible to *P. lingam* was sensitive. The results reveal that a clear correlation between resistance to *P. lingam* and insensitivity to sirodesmin PL is present, and that the toxin can be used to distinguish resistant plant material from susceptible both in vivo and in vitro.

Key words: *Phoma lingam* – *Brassicaceae* – Sirodesmin PL-Toxin – Selective

Introduction

Recovery of disease resistance by selection of cells which are resistant to a toxic compound produced by a plant pathogen represents a direct application of cell culture techniques for crop improvement. Selection for pathotoxin resistance in cell culture has been done in several plant-pathogen systems with successful results. By performing in vitro selection, disease-resistant plants have been obtained in the following combinations: *Nicotiana tabacum* to *Pseudomonas syringae* and *Alternaria alternaria* (Thanutong et al. 1983), *Zea mays* to *Helminthosporium maydis* race T (Gengenbach et al. 1977), *Saccharum* sp. to *H. sacchari* (Heinz et al. 1977; Larkin and Scowcroft 1983), *Oryza sativa* to *H. oryzae* (Ling et al. 1985) and *Avena sativa* to *H. victoriae* (Rines and Luke 1985).

Besides purified or partially purified toxins, crude fungal culture filtrates have also been used for selection. Sacristan (1982, 1985) used culture filtrate from *Phoma lingam,* a fungal disease in cruciferous crops, in the selection of resistant calli and stem embryos of *Brassica napus* after mutagen treatment. From these experiments, resistant plants were regenerated. However, variation in resistance among the plants was obtained and a segregation was found in the progenies (Sacristan 1985), making further studies in the search for resistance necessary.

Sjödin et al. (1988) purified toxic compounds from the culture filtrate of *P. lingam*, and the molecular structures of sirodesmin PL and deacetylsirodesmin PL were determined. Furthermore, studies of the effects caused by the toxic compound sirodesmin PL on protoplasts of susceptible hosts and non-hosts to the pathogen were peformed, revealing that sirodesmin PL was lethal to protoplasts from all the material investigated. In contrast, a host-specific property was found when the protoplasts from the non-hosts had formed cell walls and had regenerated cell aggregates. This host-specificity was also found when the toxin was added to intact plants of these species (Sjödin et al. 1988). The fact that cells of non-hosts to *P. lingam* became less sensitive to sirodesmin PL the longer the delay between the start of the culture and the addition of the toxin was applied encouraged us to make comparative studies on cells of susceptible and resistant *Brassica* accessions with regard to their response to the toxin.

Resistance to *P. lingam* has been identified in species related to *B. napus* after screening tests with pycnospores of the pathogen (Sjödin and Glimelius 1988). In the pres-

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ent study, the resistant plant material obtained by Sjödin and Glimelius (1988) was investigated with the purpose of evaluating whether the toxin sirodesmin PL was capable of distinguishing between resistant and susceptible *Brassica* accessions both on the cell level and on intact plant tissues.

Materials and methods

Plant material

Brassica napus L. spp. oleifera cv. Elvira, a winter rape cultivar, and two spring rape cultivars, Hanna and Niklas, all susceptible to *P. lingam*, were investigated together with resistant and susceptible accessions of *B. juncea*, *B. nigra* and *B. carinata*. Seeds *B. napus* of were kindly provided by W. Weibull AB, Landskrona, and Svalöf AB, Svalöv, Sweden. The different origins of *B. juncea*, *B. nigra* and *B. carinata* accessions were described by Sjödin and Glimelius (1988).

Preparation of toxin

The *P. lingam* isolate 275.63, kindly provided by Centraal Bureau voor Schimmelcultures, Baarn. The Netherlands, was selected for high toxin production in in vitro culture (Sjödin et al. 1988). The fungal isolate was grown under optimal conditions and the toxin sirodesmin PL was purified from the filtrate according to Sjödin et al. (1988). Sirodesmin PL was stored in dry condition under nitrogen at −70 °C. The toxin was dissolved in methanol and dilutions with water were made from that stock solution for the different bioassays.

Isolation, culture and toxin treatment of protoplasts

Mesophyll protoplasts were isolated and cultured according to Sjödin et al. (1988). Sirodesmin PL was added to the freshly isolated protoplasts in a range of 0.5–10 µM. Viability was determined using 0.5% Evans blue (Chroma-Gesellschaft, Schmied) dissolved in 0.4 M sorbitol (Kanai and Edwards 1973). To analyse cell wall formation, 0.1% calcofluor white (American Cyanamid) disolved in 0.4 M sorbitol was used.

Toxin treatment of cell aggregates

The toxic effects of sirodesmin PL on cell aggregates of the different material were investigated using a bead-type culture method (Shillito et al. 1983). Protoplasts were isolated as described above and cultured in 5 cm Petri dishes. When the first cell divisions were visible, four to five days after isolation, 1 ml of the protoplast suspension was plated in 3 ml 0.4% agarose dissolved in a modified 8p medium (Glimelius et al. 1986) with 0.4 M glucose, but without hormones. This media addition results in a dilution of the hormone concentrations to 1.13 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.55 µM 6-benzylaminopurine (BAP) and 0.13 µM 1-naphtylacetic acid (NAA). After gelling, beads with the embedded protoplasts were placed in 5 cm Petri dishes with 7 ml fresh 8pm medium containing 1.13 µM 2,4-D, 0,55µM BAP and 0.13 µM NAA, which were the same hormone concentrations as in the beads. After 14 to 20 days, when small star-shaped aggregates had formed, sirodesmin PL was added in various concentrations (1.0–15 µM) to the cultures. The cultures were kept under cool white light 20 W/m^2 (215W General Electric fluorescent tubes), with a day lenght of 16 h and a temperature of 25 °C. The cultures were continuously observed and three weeks after toxin addition, cell survival was analysed by fluorescein diacetate (FDA) staining (Sundberg et al. 1987), using an inverted UV and light microscope (Olympus IMT).

Leaf assay

Seeds were sown in 7 cm pots and seedlings were grown in the greenhouse with a day length period of 16 h and a temperature of 20 °C during the day and 18 °C at night. Sirodesmin PL was added in a dilution sequence of 1–80 µM to fully expanded cotyledons and to adult leaves in the three- to six-leaved stage. The toxin droplet was either added to a punctured leaf or deposited on the leaf. The development of lesions was continuously observed and measured for ten days.

Root assay

Surface-sterilized *Brassica* seeds (Glimelius 1984) were used in a modified root growth bioassay (Walton and Earle 1983). The seeds were soaked in water for 1 h, rolled in wet paper towels and germinated in darkness at 25 °C for 60 h. Seedlings with primary roots of about 15 mm were selected, measured and placed on filter papers (Whatman no. 1) in Petri dishes soaked with 5 ml sterile distilled water containing the toxin (1–50 µM). Ten seedlings per plate and three plates per treatment were kept for 48 h in darkness. The net elongation of the primary roots was measured in order to determine the effects of the toxin.

Results

Effects of the toxin on protoplasts

Viability of protoplasts isolated from 9 resistant and 14 susceptible *Brassica* accessions was analysed 1–10 days after toxin treatment. A drastic reduction in viability was observed during the first two days in all cultures. About 80% of the protoplasts were dead compared to control cultures 48 h after initiation of the cultures in the concentrations of 1–4 µM sirodesmin PL, whereas higher toxin concentration caused an immediate death of the protoplasts. According to calcofluor white staining, protoplasts cultured in 1–4 µM sirodesmin Pl were unable to rebuild a complete cell wall and no cell divisions occured during this time. All protoplasts were dead after ten days' culture.

In concentrations lower than 1.0 µM, the cells survived and formed cell aggregates. In a concentration of 0.5 µM, cell aggregates were formed in a range of 46%–61% of the control cultures, whereas in 0.75 µM a decrease to 17%–26% was obtained. The toxic effects caused by sirodesmin PL were obtained in all accessions tested independent of the resistance level expressed in intact plants.

Effects of the toxin on cell aggregates

When 9 resistant and 14 susceptible *Brassica* accessions were treated with sirodesmin PL after 15–20 cells divisions when star-shaped cell aggregates had formed, a significant difference between resistant and susceptible material was obtained. Particularly at low concentra-

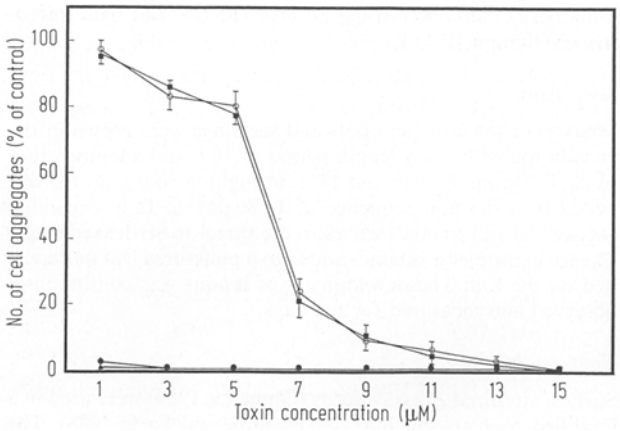

Fig. 1. Survival of cell aggregates in bead-type cultures treated with various concentrations of sirodesmin PL. *B. napus* cv. Hanna (●) and *B. juncea* cv. Shigatse (▲) are susceptible, whereas *B. juncea* 1 (○) and *B. carinata* Etiopia (■) are resistant to the pathogen. The values are means of five independent experiments and the *bars* indicate standard deviation

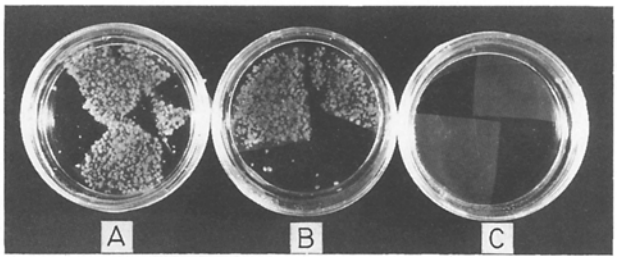

Fig. 2 A–C. Cell aggregates in bead cultures. **A** *B. napus* cv. Hanna susceptible to *P. lingam*, **B** *B. juncea* 1 resistant to *P. lingam* treated with 3 μM sirodesmin PL, **C** Culture of *B. napus* cv. Hanna treated with 3 μM sirodesmin PL

tions, 1–5 μM sirodesmin PL, differences in sensitivity to the toxin were clearly expressed (Figs. 1 and 2).

The in vivo response to the toxin

When 1–3 μM sirodesmin PL was added to punctured leaves of the 14 susceptible *Brassica* accessions tested, small lesions were obtained, while no symptoms were detected on leaves from the nine resistant accessions treated in the same way (Fig. 3). When higher toxin concentrations were added to punctured leaves of resistant *Brassica* species, a delay before the symptoms appeared was observed, whereas the susceptible plant material developed symptoms within 24 h (Fig. 4). In concentrations higher than 10 μM no difference between the various *Brassica* representatives tested were obtained. The same results were obtained on the diverse *Brassica* accessions when the toxin was applied to intact leaf surfaces without wounding, independent of differences in leaf morphology. However, the symptoms occurred more rapidly

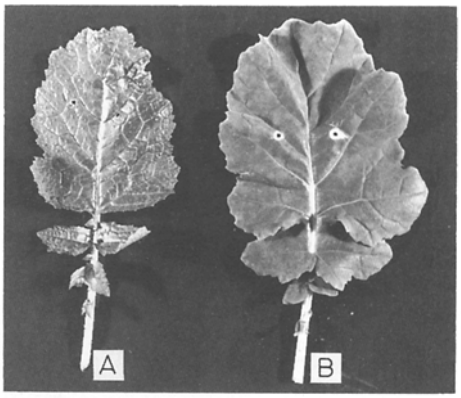

Fig. 3 A and B. Adult leaves treated with 2 μM sirodesmin PL on the left side and 3 μM sirodesmin PL on the right side of leaf, respectively. **A** *B. juncea* 3, resistant to *P. lingam*, **B** *B. napus* cv. Hanna susceptible to *P. lingam*

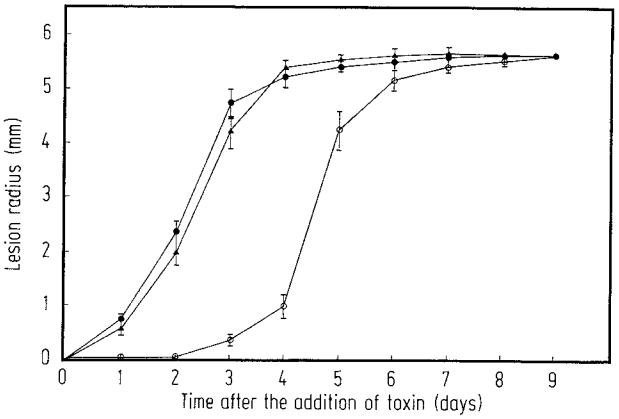

Fig. 4. Lesion size measured as the radius, in relation to treatment time, of the susceptible *B. napus* cv. Hanna (●), *B. juncea* cv. Shigatse (▲), and the resistant *B. juncea* 1 (○) treated with 6 μM sirodesmin PL. The values are means of four independent experiments and the *bars* indicate standard deviation

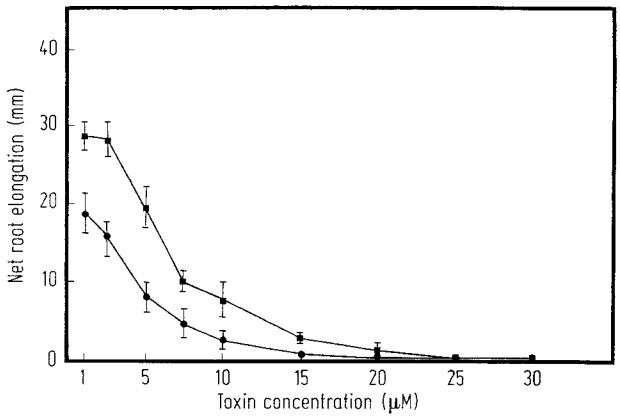

Fig. 5. Root elongation of *B. napus* cv. Hanna (●) and *B. juncea* 1 (■) cultures in different concentrations of sirodesmin PL. The measurements are means of five independent experiments and the *bars* indicate standard deviation

when the leaves were punctured compared to treatment of intact leaves.

In the root growth assay, the toxic effects of six resistant and ten susceptible *Brassica* accessions were studied. The roots of the resistant plant material showed higher tolerance to sirodesmin PL and the net elongation of the roots was larger compared to the susceptible accessions (Fig. 5).

Discussion

Several investigations have shown that protoplasts isolated from resistant and susceptible cultivars can differ in response to treatments with the pathotoxins victorin, phytoalternarin, *Helminthosporium maydis* race T-toxin, helminthosporiside and *Phyllosticta maydis*-toxin. In those cases, sensitive reactions were detected on protoplasts from the susceptible plant material (Strobel 1975; Kasai et al. 1975; Rancillac et al. 1976; Earle et al. 1978).

Cell suspensions and protoplasts are usually much more sensitive to the pathotoxins than other types of plant material and are thus very suitable as bioassays (Behnke and Lönnendonker 1977; Yoder et al. 1977; Earle et al. 1978). When comparing the response to *Helminthosporium maydis* race T-toxin, the protoplasts were 900 times more sensitive than a leaf puncture assay (Yoder 1981). In our investigation the protoplasts were also the most sensitive system, although the sensitivity was only 5 times higher than the response of the regenerated cell aggregates to the toxin. The cell aggregate assay was almost as sensitive as the leaf assay.

In this study, however, all protoplasts were sensitive to sirodesmin PL, independent of the resistance level expressed in the intact plant material. In contrast, cell aggregates of resistant *Brassica* accessions showed a clear tolerance to the toxin when added in low concentrations (1–5 μM). When cell aggregates derived from susceptible accessions of *B. carinata*, *B. juncea* and *B. nigra* were treated with the toxin, the same sensitivity as in the susceptible *B. napus* was found, demonstrating that the difference obtained in response to the toxin was not species-specific but related to the resistance. A similar response was obtained when leaves and roots were treated with the toxin.

When comparing the sensitivity to the toxin of non-host plants (tobacco and potato) and the resistant *Brassica* accessions, a slightly higher tolerance to the toxin was found in the cell aggregate cultures of the non-hosts (data not shown). This difference was even more pronounced when the toxin was applied on leaves of greenhouse grwon plants.The leaves of the non-hosts showed tolerance to the toxin at least ten times higher than the resistant *Brassica* accessions (data to shown).

The results obtained revealed that the cell wall or a more organized tissue is necessary for the recognition or action of sirodesmin PL. This was also found for *Periconia circinata* and *Helminthosporium carbonum* race 1 toxins, where intact leaves of the susceptible cultivars were damaged by the toxins. However, in these cases no toxic effects were detected on protoplasts (Earle 1978).

Selective toxic effects have also been found in studies by Sacristan (1982) when applying the crude culture filtrate of *P. lingam* on wheat, rye and clover seedlings. Sacristan found that an inhibition of growth in the presence of the culture filtrate was about the same in rapeseed and clover seedlings, while wheat and rye seedlings showed a much higher tolerance to the fungal filtrate. This indicates that the fungal filtrate had a partial host-selective effect. However, contradictory results have also been reported. Boudart (1981) compared the effects of sirodesmin PL on hypocotyls of a rapeseed cultivar susceptible to *P. lingam* with a rapeseed cultivar more tolerant to the pathogen and found no differences in sensitivity to the toxin. Similarly, Pedras et al. (1987) reported no specificity to sirodesmin PL in cotyledon tests of two *B. napus* cultivars and one *B. juncea* cultivar, the latter determined as resistant to *P. lingam*. In both these cases, however, the concentrations of sirodesmin PL used were much higher than those used in this investigation, resulting in differences in response to sirodesmin PL. This clearly shows the importance of careful screening, using a wide range in the concentrations of the toxin and a thorough evaluation of the toxic effects. In conclusion a clear correlation between sensitivity to the toxin and susceptibility to the pathogen has been found and it is possible to distinguish resistant material from susceptible in vitro as well as in vivo, using sirodesmin PL as the selective agent.

This selective property is utilized in experiments where transfer of genes for resistance to the pathogen via somatic hybridization is being performed. The resistance analysis of the hybrid material from these experiments will further reveal the selective capacity of sirodesmin PL.

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