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Pollen selection for *Alternaria* resistance in oilseed brassicas: responses of pollen grains and leaves to a toxin of *A. brassicae*

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Summary. The effects of destruxin B, a host-specific toxin of *Alternaria brassicae* that causes black spot disease in oilseed brassicas, were studied on in vitro pollen germination and pollen-tube growth of *Brassica campestris* var "brown sarson", *B. juncea, B. napus* cvs "Westar" and "Cresor", *B. nigra* and *Sinapis alba*. Pollen grains of *B. nigra, B. juncea* and *B. campestris* were the most sensitive and those of *S. alba* the least sensitive to the toxin. Effects of the toxin were also studied on the leaves of these species, and the degree of sensitivity of leaves of different species was comparable to that of their pollen grains. The results on the responses of pollen grains as well as leaves to the toxin are in agreement with the degree of susceptibility/resistance of these species to *A. brassicae* reported in the literature, indicating that the genes imparting susceptibility/restistance are expressed in the pollen, a prerequisite for pollen selection. Results are also presented which show that the toxin fed to the cut end of isolated inflorescence axis is readily taken up by the developing pollen and results in the inhibition of germination of susceptible pollen. This technique offers a simple and effective method for application of selection pressure to eliminate pollen grains susceptible to the toxin from effecting fertilization.

Key words: *Alternaria brassicae* – Black spot disease – *Brassica* spp. – Destruxin – Pollen selection

Introduction

The transfer of genes imparting resistance/tolerance to biotic and abiotic stresses into cultivars is one of the major goals of plant breeding programmes (Goodman et al. 1987). Many of the emerging biotechnologies have shown considerable promise in reducing the time and cost of achieving these objectives (Vasil 1990). Pollen selection is one such technique (Ottaviano and Mulcahy 1989; Evans et al. 1990; Hormaza and Herrero 1992). Pollen as the means for application of selection pressure is considered to be more effective and economical when compared to that of the sporophytes (Mulcahy 1983). One of the prerequisites for application of pollen selection is the expression of the specific gene(s) in the pollen. In recent years many investigations have shown a positive correlation between the responses of the pollen and those of the sporophyte to a range of biotic and abiotic stresses (Evans et al. 1990; Hormaza and Herrero 1992). Studies on these lines against fungal toxins are, however, limited (Laughnan and Gabay 1973; Bino et al. 1988; Hodgkin 1990).

The black spot disease caused by *Alternaria brassicae* is a major disease of oilseed brassicas in many parts of the world and causes significant yield losses (Degenhardt et al. 1974; Petrie 1975; Kolte 1985, 1991). *Alternaria brassicae* produces a major host-specific toxin, destruxin B (Bains and Tewari 1987; Buchwaldt and Jensen 1991). Pollen grains of *Camelina sativa* (resistant host) and *Brassica campestris* var "yellow sarson" (susceptible host) cultured in vitro showed differential sensitivity to destruxin B (Bains 1989); pollen of the latter species were inhibited to a greater degree than those of the former. To obtain detailed information on the expression of toxin tolerance we have compared the responses of pollen grains and leaves of the major oilseed brassicas to destruxin B. Since, in *Brassica*, pollen treated with a toxin solution can not be used effectively for pollination (Hodgkin 1987), we also report on an alternate method of incorporating toxin through excised inflorescence axes.

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Materials and methods

Table 1 lists the taxa used in the present investigation. Seeds of various lines were obtained from Drs. S. R. Rimmer of University of Manitoba, Winnipeg, and G. Seguin-Swartz of Agriculture Canada, Saskatoon. Plants of all the species were grown under glasshouse conditions. Natural lighting was supplemented with fluorescent tubes and incandescent bulbs for 16 h per day. Plants were fertilized once a week with a fertilizer, 20-20-20 (Plant Products Co, Bramalae, Ontario). Destruxin B was supplied by Dr. J. P. Tewari of University of Alberta, Edmonton.

In vitro pollen germination

Pollen grains from freshly dehisced anthers or from those dehisced the previous day were used for in vitro germination. Pollen grains were prehydrated by maintaining the dehisced anthers (on a dry slide) in petri plates lined with moist filter paper (RH > 90%) for 1 h; prehydration was necessary for obtaining consistent in vitro germination, particularly in pollen from anthers dehisced the previous day. Pollen grains were cultured in sitting drops (Shivanna and Rangaswamy 1992) using Hodgkin and Lyon's medium (Hodgkin and Lyon 1986). The cultures were maintained under laboratory conditions (20 ± 2 °C) and scored for pollen germination and pollen-tube length after 2 h. Two pollen cultures were raised for each treatment, and each experiment was repeated a minimum of 3 times. In each replicate over 200 pollen grains were scored for pollen germination using six to ten microscopic fields from each of the replicate cultures. A pollen grain was considered to be germinated when the length of the tube was at least equal to the diameter of the pollen grain. Pollen-tube length was measured in over 50 samples in each replicate of a treatment.

Destruxin B was dissolved in 60 µl 100% methanol and made up to 1 ml with distilled water (stock solution); this solution was incorporated in the germination medium at 10^{-6}–10^{-3} *M* concentration. Methanol at this concentration had no effect on the pollen germination or pollen-tube growth of any of the species tested. Duncan's multiple range test was used to analyse the data.

Responses of leaves to destruxin

The effects of destruxin were studied on the fourth or fifth leaf from 1-month-old seedlings, which were placed adaxial surface upwards in petri plates lined with moist filter paper. Small areas of 2–3 mm^2 were gently scratched on the upper leaf surface with the tip of a Pasteur pipette, and a 10 µl drop of distilled water or destruxin dissolved in distilled water was applied to each scratched area (Bains and Tewari 1987). Two spots were made on each side of the midrib; distilled water was applied on the lower left spot, and destruxin at 10^{-5}, 10^{-4} and 10^{-3} *M* on the top left, top right and lower right spots, respectively. The leaves were maintained under laboratory conditions under continuous fluorescent light at 150 µE m^{-2} s^{-1} and observed for chlorotic/necrotic symptoms after 72 h.

Incorporation of toxin through inflorescence axis

The experiments involving the use of inflorescences to incorporate toxin were confined to *B. napus* cv "Cresor". Fresh inflorescences from the main branches were used; all developing fruits, opened flowers and those which would open on the day of the treatment were removed. Inflorescences with 5–6 cm of the axis were excised, and the cut ends were dipped in 0.5 ml of distilled water or destruxin B solution in Eppendorf tubes for 8 h. After the treatment, the inflorescences were transferred to a liquid medium used for *Brassica* flower bud culture (Polowick and Sawhney 1991) with 2% sucrose in culture vials (25 ml). The mouth of the vial was covered with parafilm, and the inflorescence axis was inserted through a hole made in the parafilm. The medium was replaced each day. The inflorescences were maintained at $25° \pm 2$ °C under continuous illumination provided by fluorescent tubes at 150 µE m^{-2} s^{-1}.

Opened flowers or buds with dehisced anthers were excised each day, sequentially numbered for each inflorescence and their pollen grains tested for viability by the fluorescein diacetate test (Heslop-Harrison and Heslop-Harrison 1970) and in vitro germinability as described above. Pollen grains were also used to pollinate excised pistils implanted in 0.8% agar set in petri plates (Shivanna and Rangaswamy 1992). The pistils were maintained under laboratory conditions and fixed in acetic alcohol (glacial acetic acid: absolute ethanol 1:3) after 24 h of pollination. The pistils were then cleared overnight in 6 *N* NaOH and observed under the fluorescence microscope for pollen germination and pollen-tube growth (Linskens and Esser 1957).

Table 1. Plant species used in this study

Brassica campestris L. ssp. *oleifera* var brown sarson cv pusa Kalyani
B. juncea (L.) Czern. cv pusa hold
B. napus L. cv Cresor
B. napus L. cv Westar
B. nigra (L.) Koch Population 2 (Saskatoon Research Station Selection)
Sinapis alba L. cv Ochre

Results

Effects of toxin on in vitro pollen germination and pollen-tube growth

All of the species tested showed good pollen germination (> 80%); however, they showed differences in the length of their pollen tubes. The tube length was maximum (ca. 150 µm) in both cultivars of *B. napus* and minimum (ca. 75 µm) in *S. alba*. Figure 1 shows the results on pollen germination and pollen-tube growth in all six taxa studied at broad concentration ranges of destruxin B. The data presented are percentages of control values set at 100%. The toxin had no effect on pollen germination at the lowest concentration (10^{-6} *M*) tested in any of the species; however, pollen-tube length was significantly reduced in *B. napus* cv "Westar" (Fig. 1 C), *B. juncea* (Fig. 1 D), *B. campestris* (Fig. 1 E) and *B. nigra* (Fig. 1 F) at this concentration. The toxin inhibited pollen germination completely at 10^{-5} *M* and higher concentrations in all species except *B. napus* cv "Cresor" (Fig. 1 B) and *S. alba* (Fig. 1 A). In *B. napus* cv "Cresor" (Fig. 1 B) less than 5% of the pollen germinated at 10^{-5} *M*, and in *S. alba* (Fig. 1 A) some pollen grains germinated even at 10^{-4} *M* destruxin. In all the taxa examined many pollen grains showed small protuberances at inhibitory concentrations of the toxin i.e. 10^{-5} *M*–10^{-3} *M*.

In another experiment, the effects of destruxin were studied at narrow concentration ranges in three species,

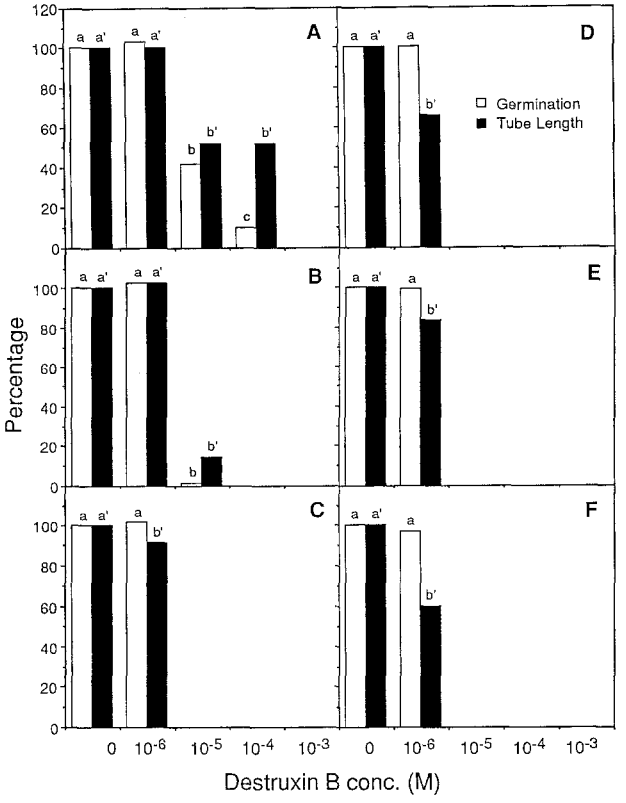

Fig. 1 A–F. Effects of destruxin B on in vitro pollen germination and pollen-tube growth in different taxa. *Sinapis alba* (**A**), *Brassica napus* cv "Cresor" (**B**), *B. napus* cv "Westar" (**C**), *B. juncea* (**D**), *B. campestris* (**E**) and *B. nigra* (**F**). The data are expressed as percentages over the control. Columns with *different letters* within a species are significantly different at $P=0.05$, using Duncan's multiple range test

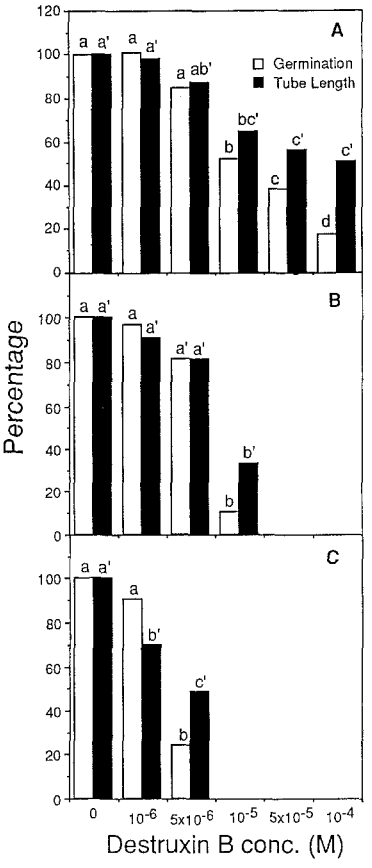

Fig. 2 A–C. Effects of destruxin B, at narrow concentration ranges, on in vitro pollen germination and pollen-tube growth of *S. alba* (**A**), *B. napus* cv "Cresor" (**B**) and *B. nigra* (**C**). The data are expressed as percentages over the control. Columns with *different letters* within a species are significantly different at $P=0.05$, using Duncan's multiple range test

S. alba, B. napus cv "Cresor" and *B. nigra,* all of which exhibited different levels of toxin sensitivity (Fig. 1). These results (Fig. 2) were basically similar to the earlier results but brought out the differences more clearly between *B. napus* and *B. nigra*. In *B. napus* cv "Cresor" (Fig. 2B), neither pollen germination nor pollen-tube length was reduced at $5\times10^{-6}\,M$, while in *B. nigra* (Fig. 2C), a significant reduction in pollen-tube length was apparent at $10^{-6}\,M$, and in pollen germination, at $5\times10^{-6}\,M$. In *S. alba* (Fig. 2A) pollen germination and tube elongation was evident at all of the concentrations tested.

Effects of destruxin on leaves

No chlorotic areas developed around the spots applied with distilled water and $10^{-5}\,M$ destruxin in leaves of any of the species (Fig. 3). However, at higher concentrations of the toxin diffuse chlorotic areas without a clear boundary developed around the spots (Fig. 3). There were clear differences between species in the intensity and the size of the chlorotic areas. Such areas were much wider and more intense in *B. napus* cv "Westar" (Fig. 3C), *B. campestris* (Fig. 3D), *B. juncea* (Fig. 3E) and *B. nigra* (not shown) than in other species. In these four species the chlorotic areas often extended to the other side of the midrib (e.g. Fig. 3D). The chlorotic areas were less intense in *B. napus* cv "Cresor" (Fig. 3B) and smaller and much less intense in *S. alba* (Fig. 3A) than in the other species. Thus, in decreasing order of sensitivity the symptoms of the toxin were the most severe in *B. nigra, B. juncea* and *B. campesteris,* followed by cvs "Westar" and "Cresor" of *B. napus* and *S. alba.*

Effects of toxin fed through the inflorescence axis

The inflorescence explants were monitored for 4 days in liquid culture by which time most of the flower buds measuring 2–3 mm and showing microspore at the time of excision had matured. During this period, eight to ten

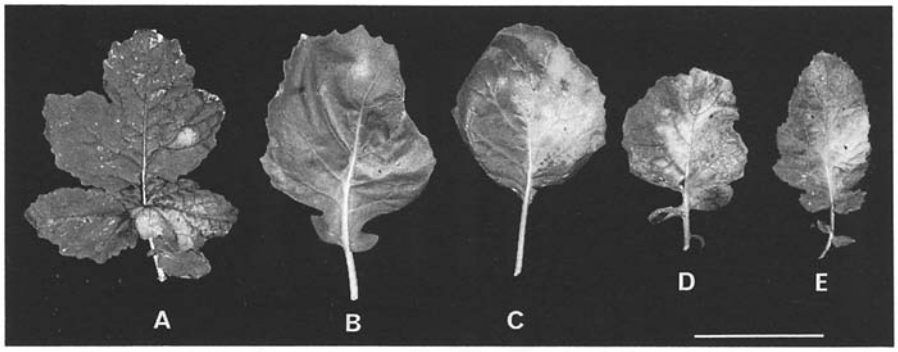

Fig. 3. Effects of destruxin B on leaves of different species 72 h after the treatment: **A** *Sinapis alba*, **B** *Brassica napus* cv "Cresor", **C** *B. napus* cv "Westar", **D** *B. campestris*, **E** *B. juncea*. In each leaf chlorotic areas are visible around the spots treated with 10^{-4} *M* (*upper right of midrib*) and 10^{-3} *M* (*lower right of midrib*) destruxin. No chlorotic areas developed around the spots treated with distilled water (*lower left*) and 10^{-5} *M* destruxin (*upper left of midrib*). In *B*, 10^{-4} *M* destruxin was applied onto top left of midrib. *Bar:* 5 cm

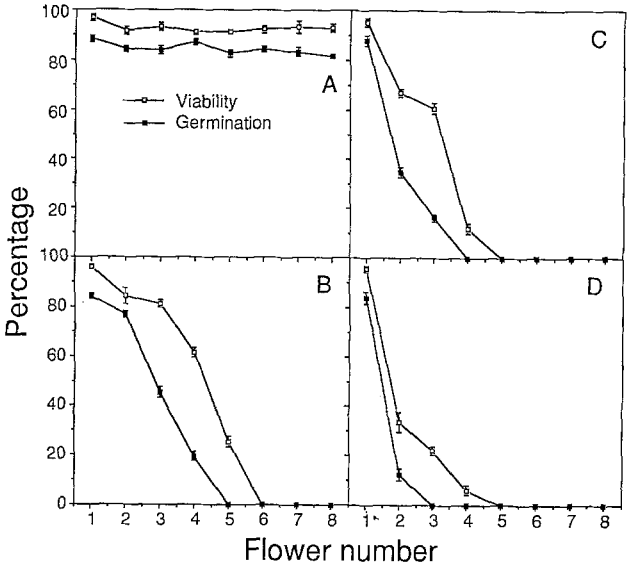

Fig. 4A–D. Percentage viability (FDA test) and in vitro germination of pollen grains from flowers numbered sequentially, beginning with the oldest, in the inflorescences treated with distilled water (**A**), and 10^{-4} *M* (**B**), 5×10^{-4} *M* (**C**) and 10^{-3} *M* (**D**) destruxin B (mean ± SE)

flowers opened, and their anthers dehisced normally in the control and in 10^{-5} *M* destruxin. At higher concentrations of the toxin, however, flowers failed to open, although the anthers dehisced in six to eight older flower buds during the 4 days. Concentrations of 10^{-4}, 5×10^{-4} and 10^{-3} *M* of the toxin also affected elongation of the flower buds. Unlike in the control in which about-to-open flower buds measured 11–13 mm, the buds at high toxin concentrations (10^{-4}–10^{-3} *M*) reached only 7–9 mm in length. Also, under the latter conditions the flower buds turned greyish particularly at 10^{-3} *M* destruxin, and most of the younger buds in the inflorescences died.

Figure 4 shows the pollen viability and germinability of pollen in flowers of inflorescence explants. Flowers are numbered sequentially starting with the oldest. In the control (Fig. 4A) as well as in 10^{-5} *M* destruxin (data not presented), pollen from all of the flowers showed a high level of viability (>90%) and in vitro germinability (>80%), which were comparable to those of the pollen from freshly opened flowers in vivo. The stigmas pollinated with these pollen also showed good pollen germination and pollen-tube entry into the stigma. Higher concentrations of destruxin significantly reduced both pollen viability and germinability in the second or third flower onwards, depending on the concentration (Fig. 4B–D). The reduction in germinability was more marked than that of viability. At all of the concentrations in which there were marked differences between pollen viability and in vitro germination, many of the pollen grains showed small protuberances that were similar to those observed at inhibitory concentrations of the toxin in the germination medium. Also, pollen samples which did not show in vitro germination did not germinate on the stigma.

Discussion

This study demonstrates the differential sensitivity of pollen grains of different *Brassica* species to the fungal toxin, destruxin B. Pollen grains of *B. nigra*, *B. juncea* and *B. campestris* are highly sensitive; the toxin inhibits pollen-tube growth significantly at concentrations as low as 10^{-6} *M*. Pollen of *B. napus* cv "Cresor" are less sensitive as they are not affected by the toxin up to 5×10^{-6} *M*. *B. napus* cv "Westar" seems to fall between cv "Cresor" and highly sensitive species. Pollen grains of *S. alba* are the least sensitive; large numbers of them germinate even when the concentration of the toxin is

increased tenfold over the concentration inhibitory to highly sensitive species. Our results on the sensitivity of the leaves of these species to destruxin B correspond to those on the sensitivity of pollen grains, and are in agreement with the susceptibility of these species reported for *Alternaria brassicae* (Bains and Tewari 1987; Kolte 1991).

Present studies as well as those of Bains (1989) on the sensitivity of pollen and leaves of different species of *Brassica* and related genera establish a strong correlation between the responses of the pollen and the sporophyte, indicating that the genes imparting resistance/susceptibility to *A. brassicae* are expressed in the pollen. The results clearly shows the feasibility of using pollen grains in selection for disease resistance. Studies of Hodgkin (1990) on pollen selection for resistance to another species of *Alternaria, A. brassicicola,* have also indicated this feasibility.

None of the cultivars of oilseed brassicas show a resistance to *A. brassicae* (Kolte 1991) strong enough for application of selection pressure to pollen. Many of the wild and allied species of *Brassica,* such as *Camelina sativa* and *Sinapis alba,* show a high degree of resistance to *A. brassicae* but are strongly incompatible with crop species. However, in recent years many intergeneric hybrids between some of the resistant species and the cultivars have been produced through embryo rescue (Ripley and Arnison 1990; Shivanna 1991; Gundimeda et al. 1992). Studies are in progress to transfer resistance genes from the wild species to the cultivated varieties (Chevre et al. 1991). Pollen selection should be a promising approach in handling backcross progeny of such hybrids. A pollen bioassay can be used to analyze backcross progenies for disease resistance and would be much more rapid and efficient when compared to conventional screening or to the leaf bioassay (Laughman and Gabay 1973). The application of effective selection pressure on the pollen grains would result in the selection of pollen grains with resistant genes for fertilization.

One of the most convenient methods of applying selection pressure to pollen is to first incubate them in a culture medium containing the toxin and then to use them for pollination. This method, however, is not effective in *Brassica*, as pollen grains incubated in the culture medium are unable to penetrate the stigmatic papillae (Hodgkin 1987). Limited success has been achieved with such pollen by pollinating decapitated styles; however the extent of seed set with such pollen was very low in both toxin-treated and control pollen (Hodgkin 1990). Our attempts to achieve seed set with such pollinations were not consistent (data unpublished). Therefore, for an effective application of selection pressure to pollen of *Brassica,* an alternative method is needed.

The procedure of feeding toxin through excised inflorescence axes incubated in a culture medium, as reported here, is a simple and efficient method for the incorporation of the toxin into pollen grains. This procedure does not affect the ability of the resistant pollen to germinate on the stigma or the ability of the pollen tubes to enter the stigma. Depending on the concentration, the toxin is taken up by the pollen of the second to fourth flower onwards and results in a significant increase in the frequency of pollen grains that are viable but fail to germinate. Such pollen grains show the typical protuberances that are shown by pollen grains incubated in germination medium containing inhibitory concentrations of the toxin. Thus, by manipulating the concentration of the toxin fed into different flowers and by monitoring the responses of the pollen in flowers, the incorporation of an effective concentration of the toxin into pollen samples for pollen selection is feasible.

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