Short communication

Responses of *Asparagus officinalis* pollen to the culture filtrate of *Fusarium oxysporum* f. sp. *asparagi*

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Accepted 23 August 1999

Abstract

*Fusarium* crown and root rot in asparagus is the main cause of crop decline in the world. Because chemical treatments are not effective, disease control efforts should focus on the development of genetically resistant cultivars. This is a long process when traditional methods are used, because of the complexity of this pathosystem. In several species, the application of selective agents to gametophytes has been found to increase the efficiency of selection; however, there is no information on either pollen response to selective agents or gametophytic selection in asparagus. Therefore, and as a preliminary study, the effect of a toxic culture filtrate of *Fusarium oxysporum* f. sp. *asparagi* on in vitro and in vivo pollen germination was evaluated on two susceptible pistillate genotypes and one tolerant and one susceptible staminate genotypes. In vitro, the toxic culture filtrate did not affect either pollen germination or tube growth of the tolerant genotype, but diminished the percentage of pollen germination and increased the percentage of germinated grains with long tubes of the susceptible genotype with respect to the control. Although no useful selection for resistance was achieved, these results suggest that the percentage of pollen germination in a toxic medium might be correlated with the plant response to the pathogen. In vivo, the toxic culture filtrate negatively affected pollen germination and tube growth in all combinations; furthermore, it induced several abnormalities in pollen tube growth, that varied with the genotypic combination. The correlation between pollen reaction in vivo and the plant response was not studied, but it was observed that pollen was not insensitive to the culture filtrate of the fungus in vivo. These results
suggest that the feasibility of application of gametophytic selection in asparagus merits further investigation. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Asparagus; *Fusarium oxysporum* f. sp. *asparagi*; Pollen; Toxic culture filtrate

Garden asparagus, *Asparagus officinalis* L. is a perennial dioecious species cultivated in temperate and subtropical regions of the world. The main cause of its decline is the disease known as “crown and root rot”, caused by a complex of soil-borne fungi of the genus *Fusarium* (Endo and Burkholder, 1971; Ridao, 1992). It is difficult to control this disease successfully with cultural or chemical methods due to the perennial nature of the crop and the high cost of the treatments (Lacy, 1979). Dissemination of these pathogens by air and water further hinders the achievement of adequate long-term control. This problem could be overcome by obtaining asparagus cultivars with genetic resistance to *Fusarium* spp. (Tu et al., 1985). However, and despite the fact that this objective constitutes a priority in breeding programs of several countries, only cultivars described as tolerant have been obtained so far. The difficulty of obtaining resistant cultivars stems from the fact that disease resistance is, apparently, controlled by polygenes (Ellison, 1986) and that the crop is a perennial. Furthermore, asparagus breeding is a long process when traditional selection methods are used because the species is an obligate outcroasser and field evaluations have to be conducted for several years to obtain reliable data for genetic materials that would be under production for 15–20 years (Reuther, 1984). Therefore, the development of efficient selection methods would be highly beneficial for shortening the breeding process.

Traditionally, plant selection has been carried out only in the sporophytic generation. However, several authors have suggested that a high proportion of genes are expressed in both, the sporophytic and the gametopropic generations (for a review, see Ottaviano and Mulcahy, 1989). Furthermore, Ottaviano et al. (1988), among others, pointed out that male gametophytic selection could be actually more intense than sporophytic selection because of some particular features of pollen populations: large size, direct expression of recessive characters in diploid organisms, direct exposure to environmental stresses and pollen competition. Provided that some adaptive traits are determined by genes that are expressed in both ontogenic phases, the utilization of a selective agent on male gametophytes is likely to be positively correlated with changes in the next sporophytic generation; consequently, the selection process in plant breeding could be accelerated (Hormaza and Herrero, 1996).

There are many references to the application of this selection method for several characters in various genera, i.e. *Lycopersicon*, *Brassica*, *Silene*, *Juglans* and *Mimulus*, among others (see Ottaviano and Mulcahy, 1989, and Hormaza and Herrero, 1996). Pollen selection in the presence of fungal toxins has yielded
contradictory results regarding the disease resistance of the next sporophytic generation in relation to the parental one. For example, in the tomato – *Alternaria solani* pathosystem, an increment in plant resistance was observed in the offspring derived from the treatment (Darakov, 1995) whereas in the pathosystem *Nicotiana lansdorffii-Fusarium*, the offspring was more susceptible than the parents (Simon and Sanford, 1986). There is no information about either pollen response to selective agents or gametophytic selection in asparagus. Thus, as a preliminary study, the effect of a toxic culture filtrate of *F. oxysporum* f. sp. *asparagi* on pollen germination and pollen tube growth was evaluated in vitro and in vivo.

Cut roots and crowns of staminated and pistillated three-year-old plants of open pollinated cv. Argenteuil, grown in an experimental field in Balcarce, were visually evaluated for crown and root rot symptoms, according to Stephens et al. (1989). Those that exhibited either none or many typical *F. oxysporum* f. sp. *asparagi* (FOA) lesions were identified; hereinafter, they will be referred to as ‘tolerant’ and ‘susceptible’ genotypes, respectively. A pathogenic isolate of FOA (‘Fox 32’) was provided by the Laboratorio de Fitopatología, EEA Balcarce-FCA. It was incubated in 1.7% liquid malt extract broth (MEB) at 23 ± 1°C in the dark and in constant agitation at 100 rpm, for 35–40 days. A culture filtrate was obtained by vacuum filtration through Whatman 41 and Micron 0.22 μm filters.

To determine its in vitro toxicity, 10–15 four to five-weeks-old seedlings grown in vitro, derived from a susceptible population, were inoculated with 0.5 or 1 ml of pure culture filtrate by pouring it over the surface of the solid culture medium, and ten uninoculated seedlings remained as checks. One week after inoculation, roots became translucid and water-soaked; reddish brown elliptical lesions were detected on primary and secondary roots. On the other hand, no root lesions or decoloration were observed in the control seedlings.

Two susceptible pistillate (female) genotypes and one susceptible and one tolerant staminate (male) genotypes were used in the pollen germination studies. For the in vitro study, pollen was collected from open flowers of both male genotypes. A 28% sucrose solution was used as the control germination medium; four other germination media were generated by replacing either 3% or 6% of the volume of distilled water of the control germination medium with 1.7% MEB solution (3MEB and 6MEB media, respectively), or with culture filtrate (3CF and 6CF media, respectively). Four samples (replicates) of pollen per treatment (genotype × germination medium) were incubated in hanging drops at 37°C for 3 h (Marcellán and Camadro, 1996). Then, a drop of acetocarmine was added to the cover slip, and 200 grains per replicate were microscopically observed, to determine the percentage of germination and the percentage of grains that developed tubes longer than their diameters (hereinafter referred to as ‘pollen grains with long tubes’). The data were analyzed by means of the GLM procedure (SAS Institute, 1988).

For the in vivo study, pollen from either the tolerant or the susceptible genotype was applied to the stigmas of open flowers of two susceptible female genotypes,
after placing a small drop of control germination medium, 3CF or 6CF as pollination vehicle. Four to ten stigmas were pollinated per treatment (male genotype × female genotype × pollination vehicle). Forty-eight hours after pollination, pistils were fixed in FAA (v/v/v 1 formaldehyde: 8 glacial acetic acid:1 ethanol) for a minimum of 24 h; then they were rinsed with tap water, placed in an 8 N NaOH solution for 3 h for softening, abundantly rinsed with tap water and stained with aniline blue in 0.1 N monobasic potassium phosphate (Martin, 1958). Pistils were observed by fluorescence microscopy, and pollen germination and tube growth were evaluated by using a subjective scale (1 = most grains did not germinate or, if they did, their tubes reached only the stigma and/or the first third of the style; most tubes: 2 = reached the second third of the style; 3 = reached the last third of the style and/or the entrance to the ovary; 4 = grew among the ovules). Data were analyzed by means of the FREQ and the CATMOD procedures (SAS Institute, 1988).

The results of the in vitro study are shown in Table 1. Mean comparisons between media were carried out for each of the two genotypes separately because the genotype × medium interactions for both percentage of germination and percentage of grains with long tubes were significant (p < 0.05). The addition of either MEB or 3% of culture filtrate to the germination medium had no effect on the percentage of pollen germination of any genotype in comparison with its control (p > 0.05). On the contrary, pollen germination in the medium with 6% of culture filtrate was not significantly affected in the tolerant genotype but was reduced in the susceptible one. These results are similar to those reported for Helianthus annuus with Alternaria helianthi toxins (Ravikumar and Chikkodi,

Table 1

Percentage of germinated pollen grains and percentage of germinated pollen grains with long tubes of two genotypes (tolerant and susceptible to FOA), in five culture media (note: within each genotype, same letters indicate nonsignificant differences at α = 0.05; MEB = malt extract broth; CF = culture filtrate; 3MEB and 3CF = 3%; 6MEB and 6CF = 6%)

<table>
<thead>
<tr>
<th>Male genotype</th>
<th>Germination medium</th>
<th>% germinated grains</th>
<th>% grains with long tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolerant</td>
<td>Control</td>
<td>67.63a</td>
<td>79.50a</td>
</tr>
<tr>
<td></td>
<td>3MEB</td>
<td>64.88a</td>
<td>54.50bc</td>
</tr>
<tr>
<td></td>
<td>6MEB</td>
<td>49.38a</td>
<td>49.00c</td>
</tr>
<tr>
<td></td>
<td>3CF</td>
<td>49.38a</td>
<td>70.00abc</td>
</tr>
<tr>
<td></td>
<td>6CF</td>
<td>62.38a</td>
<td>77.20ab</td>
</tr>
<tr>
<td>Susceptible</td>
<td>Control</td>
<td>58.25a</td>
<td>18.00b</td>
</tr>
<tr>
<td></td>
<td>3MEB</td>
<td>58.87a</td>
<td>31.50b</td>
</tr>
<tr>
<td></td>
<td>6MEB</td>
<td>54.87a</td>
<td>24.50b</td>
</tr>
<tr>
<td></td>
<td>3CF</td>
<td>55.25a</td>
<td>60.50a</td>
</tr>
<tr>
<td></td>
<td>6CF</td>
<td>47.25b</td>
<td>52.50a</td>
</tr>
</tbody>
</table>
1998), *Brassica* with *Alternaria* toxins (Shivanna and Shawney, 1993) and *Lycopersicon* with *Alternaria alternata* f. sp. *lycopersici* toxins (Bino et al., 1988), among others. If this positive relation were due to the expression of the same genes in both ontogenic phases, the efficiency of selection for this trait might be improved if it were carried out at the pollen level, because large numbers of genotypes could be readily screened. This possibility would be particularly meaningful in asparagus, because visual evaluations are commonly carried out in cut, bare crowns and roots, an approach that is complex and time-consuming for the breeder and stressful for the plants. However, a large number of genotypes has to be evaluated to ascertain the applicability of in vitro pollen screening.

Pollen tube elongation did not follow the pattern of pollen germination: (1) pollen tube growth in the control medium was poorer in the susceptible genotype than in the tolerant one, (2) the addition of MEB to the germination medium altered pollen tube growth in the tolerant genotype with respect to the control, but not in the susceptible one, and (3) the addition of culture filtrate to the germination medium had no effect on pollen tube growth in the tolerant genotype, but significantly increased it in the susceptible one in comparison with the control. Since *Fusarium* spp. are known to produce plant growth regulators or analogous compounds (McLean, 1996), it is possible that some factor required for pollen tube growth of the susceptible genotype was supplied by the culture filtrate. In the presence of culture filtrate, the response observed on in vitro pollen tube growth appears to be unrelated to the percentage of in vitro pollen germination. This has been also reported by Rowe and Stortz-Lintz (1993) in alfalfa with culture filtrate of *F. oxysporum*.

For the in vivo study (data not shown), and averaged across the genotypes, the culture filtrate concentration was linearly and inversely associated with in vivo pollen germination and tube growth (*p* = 0.018); no differences were detected due to the genotypic combination. Simon and Sanford (1986) obtained similar results studying in vivo pollen tube growth of *Nicotiana langsdorfii* in the presence of fusaric acid, a toxin produced by *Fusarium* spp. However, several abnormalities in pollen tube growth were observed almost exclusively when culture filtrate was applied prior to pollination, whose type and frequency varied with the genotypic combination (Table 2). Interestingly, these abnormalities, not reported so far in the literature on pollen reaction to pathotoxins, are identical to some of the previously described in interspecific incompatible crosses in the genus *Rhododendron* (Williams et al., 1982).

The basis of the in vivo pollen reaction to the culture filtrate in asparagus is unknown. Although the correlation between the plant response to the pathogen and the pollen reaction to the culture filtrate in vivo was not studied, the feasibility of application of gametophytic selection should not be discarded. Further studies on the disease response of the offspring derived from the
Table 2
Abnormalities of in vivo pollen tube growth in crosses of two male genotypes (tolerant and susceptible to FOA), with two susceptible female genotypes ‘1’ and ‘2’ (note: dash: normal tube growth; (a) abnormal callose deposition; (b) erratic tube growth into the ovary; (c) zig-zagging growth, (d) helicoidal growth of short tubes; (e) variable tube diameter, and (f) protuberant tube extremes; CF = culture filtrate; 3CF = 3%; 6CF = 6%; N = number of replicates)

<table>
<thead>
<tr>
<th>Male genotype</th>
<th>Pollin vehicle</th>
<th>Female genotype (susceptible)</th>
<th>Replicate number</th>
<th>Frequency</th>
<th>Replicate number</th>
<th>Frequency</th>
<th>Replicate number</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolerant</td>
<td>Control</td>
<td>1</td>
<td>N 1 2 3 4</td>
<td></td>
<td>N 1 2 3 4 5 6 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3CF</td>
<td>2</td>
<td>a –</td>
<td>0.50</td>
<td>6</td>
<td>b – – – –</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6CF</td>
<td>3</td>
<td>a,c d a,c</td>
<td>1.00</td>
<td>7</td>
<td>– – – – a,c b,e</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Susceptible</td>
<td>Control</td>
<td>2</td>
<td>– –</td>
<td>0.00</td>
<td>4</td>
<td>a – – – –</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3CF</td>
<td>4</td>
<td>c,d a c</td>
<td>0.75</td>
<td>4</td>
<td>f d,f – –</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6CF</td>
<td>3</td>
<td>a – –</td>
<td>0.33</td>
<td>3</td>
<td>– – f</td>
<td></td>
<td>0.33</td>
</tr>
</tbody>
</table>
treatment are needed to ascertain whether a positive change in disease resistance has actually taken place. This aspect of the study is currently under way.

Acknowledgements

This research was partially financed by the UNMdP and INTA.

References