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Effects of *Alternaria alternata* f.sp. *lycopersici* toxins on pollen

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Summary. Effects of the phytotoxic compounds (AAL-toxins) isolated from cell-free culture filtrates of *Alternaria alternata* f.sp. *lycopersici* on in vitro pollen development were studied. AAL-toxins inhibited both germination and tube growth of pollen from several *Lycopersicon* genotypes. Pollen from susceptible genotypes, however, was more sensitive for AAL-toxins than pollen from resistant plants, while pollen of species not belonging to the host range of the fungus was not significantly affected by the tested toxin concentrations. AAL-toxins elicit symptoms in detached leaf bioassays indistinguishable from those observed on leaves of fungal infected tomato plants, and toxins play a major role in the pathogenesis. Apparently, pathogenesis-related processes and mechanisms involved in disease resistance are expressed in both vegetative and generative tissues. This overlap in gene expression between the sporophytic and gametophytic level of a plant may be advantageously utilized in plant breeding programmes. Pollen may be used to distinguish susceptible and resistant plants and to select for resistances and tolerances against phytotoxins and other selective agents.

Key words: *Alternaria alternata* f.sp. *lycopersici* – Pollen – Gametophytic-sporophytic gene expression – Pollen selection – Host-specific toxins

Introduction

The possibility of using large populations of haploid genomes makes pollen selection a potentially efficient selection system. Prospects of pollen selection are based on

observations that a selective pressure applied during pollen development in vivo alters the frequency of genes determining the desirable trait in the progeny. Pollen selection has been demonstrated by applying chilling stress during microsporogenesis or at the growth of pollen tubes in the styles of *Lycopersicon* species (Zamir et al. 1982; Zamir and Gadish 1987). Chilling stress favored the growth of low temperature tolerant *L. hirsutum* pollen and resulted in progenies better adapted to low temperature conditions. Application of pollen selection may be extended to other selective pressures. A prerequisite for pollen selection is that genes coding for the selectable traits are also expressed at the gametophytic level of the plant.

The gametophytic genome is first expressed after pollen meiosis. Prior to this stage, the diploid maternal information is eliminated by the cleansing process that characterizes the premeiotic cell (Dickinson and Heslop-Harrison 1970). Just after tetrad stage, both transcription and translation are initiated, resulting in the activity of various enzymes (Stinson and Mascarenhas 1985; Singh et al. 1985). Recently, Stinson et al. (1987) found indications that the pattern of initiation and accumulation of messenger RNAs (mRNAs) in pollen varies with time. Some mRNAs are only present during specific stages of microsporogenesis, while others are synthesized after microspore mitosis and accumulate in concentration up to pollen maturity. Proteins are synthesized during pollen germination and tube growth, but RNA transcription is absent, as concluded from the effects of transcription and translation inhibitors (for review see Knox 1984). Apparently, translation occurs from mRNAs presynthesized during microsporogenesis (Mascarenhas et al. 1985).

Although pollen has a unique function, most genes transcribed and translated during pollen development

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are also expressed in the vegetative parts of a plant. Using RNA saturation hybridizations, Willing and Mascarenhas (1984) reported that at least 64% of the pollen mRNA population of *Tradescantia paludosa* and *Zea mays* was also expressed in shoot tissues, while a maximum of about 60% of shoot mRNAs was present in the pollen of both species. Based on isozyme analyses, Tanksley et al. (1981), Sari Gorla et al. (1986) and Pedersen et al. (1987) found a similar percentage of genetic overlap between the sporophytic and gametophytic phases in *Lycopersicon esculentum*, *Zea mays* and *Hordeum vulgare*. Overlap in gene expression is also evident from similar responses in pollen and plants for tolerances and sensitivities for a wide range of agents: e.g. tolerances for zinc and copper (Searcy and Mulcahy 1985), salinity (Eisikowitch and Woodell 1975; Sacher et al. 1983), herbicides (Smith and Moser 1985), antibiotics (Bino et al. 1987), acidity and trace elements (Cox 1985), sensitivities for ozone (Feder 1985) and various other air pollutants (reviewed by Wolters and Martens 1987).

Comparing the effects of pathotoxins may give insight into a possible overlap between pollen and plants for reaction mechanisms corresponding with disease resistances. Phytotoxic compounds (AAL-toxins) isolated from cell-free culture filtrates of *Alternaria alternata* f.sp. *lycopersici* elicit symptoms in detached leaf bioassays that are indistinguishable from those observed on leaves of fungal infected tomato plants (Gilchrist and Grogan 1976). AAL-toxins are divided in T_A and T_B, each consisting of two closely related compounds with the same specific activity (Siler and Gilchrist 1983). AAL-toxins play a major role in the pathogenesis and exhibit the same host specificity as the pathogen, while toxins insensitivity is controlled by a single locus with two alleles expressing incomplete dominance when heterozygous (Grogan et al. 1975; Gilchrist and Grogan 1976; Clouse and Gilchrist 1987).

In another paper (Witsenboer et al. 1988), effects of AAL-toxins on leaves, shoots, leaf disks, calli, suspension cells and protoplasts are described. In the present paper we report effects of AAL-toxins on in vitro germination and tube growth of pollen from resistant and susceptible plants, and discuss the possibilities for using pollen selection in plant breeding programmes.

Materials and methods

Plant material

Plants were grown in glasshouses at 19°–24°C under daylight conditions. Tomato (*Lycopersicon esculentum* Mill.) genotypes used in this study were: LA 291, LA 1182 and LA 1164 (obtained from C. M. Rick, Tomato Genetics Stock Centre, Department of Vegetable Crops, University of California, Davis, USA); MsK 93, MsK 8 and MsK 9s (self-pollinated progeny of MsK 9; described by Koornneef et al. 1987); and the

cvs Moneymaker (supplied by Rijk Zwaan BV, De Lier, The Netherlands) and Floramerica (Petoseed Co., Saticoy/CA, USA). Pollen from *L. chilense* Dun. (IVT 721331), *L. glandulosum* Muller (IVT 731078), *L. hirsutum* Humb. & Bonpl. (IVT 73400) and *L. peruvianum* (L.) Mill. (IVT 731079) were collected from plants of the IVT-species collection. Host range specificity was tested on pollen from the solanaceous *Nicotiana tabacum* L. cv Petit Havana SR1 (Maliga et al. 1973), *Petunia hybrida* (Hook.) Vilm. cv Rosy Morn (Van Marrewijk 1969), *Solanum × berthaultii* Hawkes (BGRC 10063) (kindly provided by the Foundation for Agricultural Plant Breeding, Wageningen, The Netherlands) and a member of the Cucurbitaceae, *Cucumis sativus* L. (IVT K82273).

Purification and characterization of the AAL-toxins

Liquid cultures of *Alternaria alternata* (Fr.) Keissler f.sp. *lycopersici* for AAL-toxins production were grown as described by Clouse et al. (1985). Purification and quantification was carried out according to Witsenboer et al. (1988).

Pollen germination

Pollen was collected from flowers at anthesis and germinated in modified Brewbaker and Kwack (1963) media containing 12% sucrose and 0.8% agar and supplemented with 0, 15, 30, and 60 µg/ml AAL-toxins at pH 5.8. Pollen was incubated for 2 h at 24°C in the dark, and both stained and fixed with aceto-carmin (1%).

Germination and pollen tube length were determined for separate samples of 1–6 complete replicates; per flower and at each toxin concentration, germination of at least 200 pollen was scored and the length of 50 pollen tubes was measured. Since stress induces the extrusion of short pollen tubes (Stanley and Linskens 1974), pollen was recorded as germinated only when tube length was twice the diameter of the pollen grain.

Results were expressed as the mean ± standard error, and differences between means ($P < 0.05$) were analyzed using a two sample *t*-test.

Results

Pollen from the tomato genotypes Moneymaker and LA 291 and from *N. tabacum* were germinated on solidified media supplemented with different AAL-toxins concentrations. Effects of increasing AAL-toxins concentrations on germination percentages and pollen tube lengths are presented in Tables 1 and 2. In the controls (0 µg/ml), germination of LA 291 pollen was low compared to Moneymaker and *N. tabacum* pollen. However, mean tube lengths were similar for LA 291 and Moneymaker pollen. On the medium used, tobacco pollen germinated later and grew slower than pollen from tomato plants. Correspondingly, *N. tabacum* pollen tubes were shorter than those of *Lycopersicon* pollen. Addition of 15 µg/ml AAL-toxins to the medium reduced germination of LA 291 pollen to 47% and tube length to 30% of the control value. Compared with LA 291 pollen, germination (87%) and tube growth (69%) of Moneymaker pollen were less affected ($P < 0.05$), whereas germination and tube growth of tobacco pollen were not significantly

Table 1. Pollen germination percentages, absolute and as percentage of the control at different AAL-toxins concentrations for *Lycopersicon esculentum* cv Moneymaker, *L. esculentum* genotype LA 291 and *Nicotiana tabacum* cv Petit Havana (n = no. of replicates, values given are means \pm standard error)

	Moneymaker		LA 291		<i>N. tabacum</i>	
n	5		5		3	
0 $\mu\text{g/ml}$	59.3 \pm 9.7	100%	31.8 \pm 4.8	100%	48.8 \pm 11.6	100%
15 $\mu\text{g/ml}$	51.6 \pm 8.4	87%	14.9 \pm 2.9	47%	42.1 \pm 4.7	86%
30 $\mu\text{g/ml}$	13.2 \pm 7.2	22%	0.8 \pm 0.4	2%	38.4 \pm 4.8	79%
60 $\mu\text{g/ml}$	0.2 \pm 0.2	0.3%	0	0	21.1 \pm 3.9	43%

Table 2. Pollen tube lengths in 10^{-5} m and as percentage of the control at different AAL-toxins concentrations for the pollen populations used in Table 1

	Moneymaker		LA 291		<i>N. tabacum</i>	
0 $\mu\text{g/ml}$	23.6 \pm 6.2	100%	23.5 \pm 6.8	100%	8.6 \pm 0.5	100%
15 $\mu\text{g/ml}$	16.2 \pm 4.0	69%	6.9 \pm 1.1	30%	8.8 \pm 0.6	102%
30 $\mu\text{g/ml}$	4.9 \pm 1.6	21%	0.7 \pm 0.3	3%	8.5 \pm 0.7	99%
60 $\mu\text{g/ml}$	0.1 \pm 0.1	0.4%	0	0	7.3 \pm 0.5	85%

Table 3. Germination percentages of pollen from different plant species at 0 $\mu\text{g/ml}$ and 30 $\mu\text{g/ml}$ AAL-toxins. (n = no. of replicates, x_0 = percentage at 0 $\mu\text{g/ml}$, x_{30} = percentage at 30 $\mu\text{g/ml}$, % = difference between x_{30} and x_0 , values given are means \pm standard error)

Species	n	x_0	x_{30}	%
LA 291	5	31.8 \pm 4.8	0.8 \pm 0.4	2
LA 1182	2	55.7 \pm 16.9	2.4 \pm 2.4	4
LA 1164	1	20.0	2.0	10
MsK 8	4	39.5 \pm 7.2	0.4 \pm 0.3	1
MsK 9s	4	42.1 \pm 5.4	3.8 \pm 1.9	9
MsK 93	4	41.7 \pm 10.4	0.5 \pm 0.5	1
Moneymaker	5	59.3 \pm 9.7	13.2 \pm 7.2	22
Floramerica	5	62.2 \pm 17.4	23.0 \pm 4.4	37
<i>Lycopersicon chilense</i>	4	76.4 \pm 7.8	14.6 \pm 5.5	19
<i>L. glandulosum</i>	3	24.7 \pm 1.7	18.1 \pm 2.4	73
<i>L. hirsutum</i>	2	76.0 \pm 7.6	15.6 \pm 7.9	21
<i>L. peruvianum</i>	3	49.6 \pm 11.8	9.7 \pm 2.1	20
<i>Nicotiana tabacum</i>	3	48.8 \pm 11.6	38.4 \pm 4.8	79
<i>Cucumis sativus</i>	6	73.8 \pm 6.6	71.2 \pm 6.5	97

different from the control value. At 30 $\mu\text{g/ml}$, LA 291 pollen barely developed: both germination (2%) and tube length (3%) were severely reduced. At this AAL-toxins concentration pollen from Moneymaker was less affected ($P < 0.05$), while tobacco pollen only showed a small reduction in the germination percentage but no effect on tube length. The highest AAL-toxins concentration (60 $\mu\text{g/ml}$) totally inhibited LA 291 pollen development and almost completely restrained germination and tube growth of Moneymaker pollen. At 60 $\mu\text{g/ml}$, germi-

nation of *N. tabacum* pollen was reduced but pollen lengths were not affected.

Comparing the *Lycopersicon* pollen, largest differences in germination percentage and tube length were found at 30 $\mu\text{g/ml}$ AAL-toxins. At this concentration, Moneymaker pollen showed a tenfold higher germination percentage, while tube lengths were about seven times larger compared with LA 291 pollen.

As maximal divergence in pollen development was observed for germination percentages at 30 $\mu\text{g/ml}$ AAL-toxins, this concentration was used to compare toxin effects on pollen from several plant species. *Lycopersicon* plants and other species may be divided in three classes (Table 3). The first class showed a significant reduction in germination percentages as was demonstrated for pollen from LA 291 in Tables 1 and 2. Germination percentages at 30 $\mu\text{g/ml}$ ranged from 1%–10% of the control value. For the second class, germination was less affected by the AAL-toxins and ranged from 19%–73% of the control value. The last class included species not belonging to the host-range of *Alternaria alternata* f.sp. *lycopersici*. *N. tabacum* pollen germination at 30 $\mu\text{g/ml}$ was 79% of the control value; the ratio for *C. sativus* was 97%. Moreover, in a separate experiment using a different AAL-toxins isolate, pollen from *Petunia hybrida* and *Solanum \times berthaultii* were not significantly affected.

Discussion

AAL-toxins inhibited both in vitro germination and tube growth of pollen from tomato genotypes. Pollen from

LA 291, LA 1182, LA 1164, MsK 8, MsK 9s and MsK 93 was more sensitive than pollen from Money-maker and Floramerica. This is in agreement with results of a bioassay with detached leaves in which LA 291, LA 1182, LA 1164, MsK 93 and MsK 9s were selected as *Alternaria alternata* f.sp. *lycopersici* homozygous susceptible genotypes, while Money-maker and Floramerica plants were homozygous resistant (Witsenboer et al. 1988). Apparently, pollen from susceptible plants was more sensitive for AAL-toxins than pollen from resistant plants. The pollen response also indicated that all other tested *Lycopersicon* species were pathogen resistant. Hence, a sensitive pollen reaction was found for specific tomato genotypes, while a less sensitive reaction occurred in most cultivars and other *Lycopersicon* species.

AAL-toxins concentrations inhibiting *Lycopersicon* pollen development barely affected pollen growth of species not belonging to the host range of the stem cancer. Comparable effects were found in the leaflet bioassay, in which *N. tabacum*, *C. sativus* and various *Solanum* species were at least as tolerant to the toxins as resistant *Lycopersicon* genotypes (Gilchrist and Grogan 1976). Apparently, host-specific AAL-toxins activity was manifest in both vegetative and generative tissues.

AAL-toxins inhibited plant cell development at various levels of differentiation: protoplasts, calli, suspension cells, shoot induction on leaf disks, rooting of shoots and leaves (Witsenboer et al. 1988). In leaves, symptoms on susceptible plants were easily detectable from 12.5 ng/ml, while inhibition of root growth occurred at 60 ng/ml, and effects on callus growth and protoplasts plating efficiencies were evident at 100 ng/ml. AAL-toxins effects on pollen development of susceptible *Lycopersicon* genotypes, however, were evident only at much higher concentrations (15 µg/ml). This dissimilarity in sensitivity between pollen and other plant organs may be partly ascribed to differences in the experimental methods. Pollen development was followed for only 2 h and AAL-toxins effects at low concentrations were masked by the variation in each pollen sample for germination percentage and tube lengths. Assays with vegetative tissues were carried out over much longer periods, ranging from 3 days to 3 weeks for the different experimental methods. Dissimilarity in sensitivity of the various plant cell types may also be correlated with the action mechanism of the toxins. The role of AAL-toxins in pathogenesis, however, has still not been elucidated. AAL-toxins do not cause leakage of electrolytes (Kohmoto et al. 1982), but induce swelling of mitochondria (Park et al. 1981), and possibly inhibit aspartate carbamoyl-transferase (ACTase) activity (Gilchrist 1983; cited in Clouse et al. 1985). Since ACTase is located in chloroplasts (Shibata et al. 1986), the effects of AAL-toxins may be correlated with the presence of these organelles. Although mature pollen grains do contain plastids, the

organelles are not differentiated into chloroplasts, and this might explain the relative resistance found in pollen of susceptible tomato plants.

Resistance for AAL-toxins is controlled by a single locus with two alleles (*asc*-locus) exhibiting complete dominance for pathogen resistance and incomplete dominance for toxin insensitivity (Clouse and Gilchrist 1987). In pollen, resistance may be due to expression of the *asc*-gene by the male microgametophyte. During early tube growth in vitro, however, genes do not appear to be transcribed (Knox 1984). Probably, in pollen, mRNAs of the resistance gene are presynthesized during microsporogenesis, while AAL-toxins resistance is induced by translation during pollen germination. Effects of phytotoxins on pollen were also described for two other pathogen-plant systems. The phytotoxin isolated from *Alternaria brassicicola* (Schw.) Wilts. culture filtrates inhibited germination and tube growth of pollen from *Brassica* species (Hodgkin and MacDonald 1986). Resistance for the phytotoxin, however, was not reported. Extracts from leaves infected with *Helminthosporium maydis* Nisikado and Miyake race T were found to inhibit germination and tube growth of pollen from susceptible *Zea mays* L. plants, whereas pollen from pathogen resistant plants was insensitive for the diluted extracts (Laughnan and Gabay 1973). These results demonstrate that pathogenesis-related processes and mechanisms involved in disease resistances are both active in vegetative and generative tissues of several plant species.

The general overlap in genes expressed at the gametophytic and sporophytic level of a plant may be advantageously utilized in plant breeding programmes. Pollen may be used to distinguish susceptible and resistant plants. The pollen bioassay can be accomplished in 2 h, while leaf assays require several days. An additional advantage is the possibility to objectively quantify effects of phytotoxins by analyzing pollen germination and tube lengths. Laborious pollen length measurements may be omitted by using methods for the photometric quantification of in vitro pollen tube growth (Kappler and Kristen 1987). Another possibility to exploit the overlap in sporophytic-gametophytic gene expression in plant breeding is to select with pollen for resistances and tolerances against phytotoxins and other selective agents. The potential of this selection method, however, depends on the progress in some categories of methodology. As discussed by Bino and Stephenson (1988), selection conditions and methods for separation and concentration of selected from non-selected pollen have to be optimized, while techniques for the application (pollination) of manipulated microgametophytes on pistils insuring fertilization need to be improved.

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