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# Cross protection provides evidence for race-specific avirulence factors in *Fusarium oxysporum*

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Simultaneous inoculation with races 1 and 2 of the vascular wilt pathogen *Fusarium oxysporum* f.sp. *lycopersici* provided a high level of protection against race 2 in three tomato cultivars carrying resistance gene *I*, which confers resistance to race 1 but not race 2. However, simultaneous inoculation did not provide any protection in cultivars lacking this gene. Protection resulted in reduction and delay of wilt symptoms. Similarly, avirulent races of *F. oxysporum* f.sp. *melonis* protected muskmelon plants against virulent races of the same *forma specialis*. A ratio 10:1 between spore concentrations of inducer and challenger organism gave the highest cross protection, but ratio 0.1:1 still provided significant disease reduction. Cross protection was also obtained when inoculation with the inducer organism was performed 6 or 12 h before inoculation with the challenger organism. Autoclaved spores of the inducer did not have any protective effect, indicating that living propagules were required to initiate protection. The results suggest the presence of a gene-for-gene interaction between *F. oxysporum* f.sp. *lycopersici*-tomato and *F. oxysporum* f.sp. *melonis*-muskmelon, in which cross protection against a virulent race is mediated by recognition of a specific elicitor from the avirulent race by the plant resistance gene product and by subsequent induction of the plant defense reaction. 

## INTRODUCTION

*Fusarium oxysporum* Schlecht. is an economically important soilborne plant pathogen that causes vascular wilt disease in a wide variety of crops [*2*]. *F. oxysporum* includes over 120 different *formae speciales* classified on the basis of specificity towards their host species [*1*]. Isolates from a particular *forma specialis* can be further subdivided into physiological races with a characteristic pattern of virulence on differential host cultivars. The genetic mechanisms of race-cultivar specificity in *F. oxysporum* are largely unknown, mainly due to the lack of a sexual stage in this fungus which prevents genetic analysis. Two current hypotheses, based on the concept of gene-for-gene interactions [*7*] imply either the presence of avirulence gene products [*13*, *18*] or race-specific toxins [*21*, *22*].

New insights into the mechanisms of race-cv. specificity may come from the phenomenon of cross protection. It is well documented that previous or simultaneous inoculation with non-pathogenic or avirulent isolates of *F. oxysporum* can result in

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significant reduction in disease symptoms caused by highly virulent isolates of the same pathogen [*3*, *4*, *8*, *11*, *14–17*, *19*, *23*]. The present work has followed two main objectives: (i) to study cross protection between avirulent and virulent races of the same *forma specialis*, on plant cultivars either carrying or lacking the corresponding resistance gene; and (ii) to test the hypothesis that race-cv. specificity in *F. oxysporum* is controlled by the interaction of specific avirulence genes in the pathogen with their matching plant resistance genes. The systems *F. oxysporum* f.sp. *lycopersici*—tomato (*Lycopersicon esculentum* Mill.) and *F. oxysporum* f.sp. *melonis*-muskmelon (*Cucumis melo* L.) were chosen because the genetic bases of host resistance to races 1 and 2 of both *formae speciales* are well characterized [*2*, *18*, *24*]. We show that tomato plants carrying resistance gene *I* conferring resistance to race 1 of *F. oxysporum* f.sp. *lycopersici*, but not those lacking it are highly protected against virulent race 2 of the pathogen by co-inoculation with race 1. Similarly, avirulent races of *F. oxysporum* f.sp. *melonis* protect muskmelon plants carrying the corresponding resistance gene(s) against infection by virulent races. The results provide evidence for the existence of race-specific avirulence factors in these two *formae speciales* of *F. oxysporum*.

## MATERIALS AND METHODS

### *Isolates and inoculum production*

*F. oxysporum* f.sp. *lycopersici* strains 218 (race 1) and 42–87 (race 2), and *F. oxysporum* f.sp. *melonis* strains 23M (race 0), 18M (race 1) and 11–27 (race 2) were obtained from J. Tello, Universidad de Almería, Spain, and stored as a microconidial suspension in 30% glycerol at −80 °C. Fungi were grown in potato dextrose broth (Difco) on a rotary shaker at 28 °C for 4 days, and microconidia were harvested by filtration through autoclaved nylon mesh (10 μm pore size) and subsequent centrifugation at 8000 ***g*** for 10 min. Spore concentration was determined using a haemocytometer and adjusted to the appropriate density by diluting with sterile distilled water.

### *Inoculation of tomato and melon seedlings*

Seeds of tomato cvs. Moneymaker, Lorena and Vemar, as well as of muskmelon cvs. Gustal and Pancha were obtained from Sluis & Groot Semillas, El Ejido, Spain. Tomato cv. Hybrid 224-1 was from Servicio de Investigación y Tecnología Agroalimentaria, Junta de Extremadura, Badajoz, Spain. The tomato cvs. Vemar, Lorena and Hybrid 224-1 possess resistance gene *I* and are resistant to *Fusarium oxysporum* f.sp. *lycopersici* strain 218 (race 1) but not strain 42–87 (race 2). Tomato cv. Moneymaker does not possess resistance gene *I* and is susceptible to both races. Muskmelon cv. Gustal possesses single dominant genes conferring resistance against *F. oxysporum* f.sp. *melonis* strains 23M (race 0) and 18M (race 1) but it is susceptible to *F. oxysporum* f.sp. *melonis* strain 11–27 (race 2). Muskmelon cv. Pancha possesses resistance genes against strains 23M and 11–27 but is susceptible to strain 18M. Seeds were surface-sterilised by soaking for 30 min in a 0·7% sodium hypochlorite solution and washing three times for 5 min in sterile water, and germinated in moist vermiculite at 28 °C. Ten to fourteen-day-old seedlings (first true leaf stage) were inoculated by dipping the roots for 30 min in a suspension containing *F. oxysporum* microconidia in water at the appropriate concentration (usually $5 \times 10^6$ ml$^{-1}$ or otherwise as specified).

In co-inoculation experiments, spores of both isolates, each at the appropriate concentration, were applied simultaneously. In some experiments, a time interval of 6 or 12 h was introduced between the inoculation with the avirulent and the virulent isolate. For certain experiments, spores were autoclaved prior to diluting to the appropriate concentration. Control plants were immersed in water and five seedlings were used per treatment. Seedlings were planted in minipots containing moist vermiculite and maintained in a growth chamber at 28 °C in a 14 h photoperiod. Sterile water was added to the pots as needed. Disease severity was recorded after different time intervals using the following index: (1) no symptoms apparent; (2) beginning of wilt symptoms in leaves; (3), leaves heavily wilted; (4) all leaves completely wilted, stem standing; (5) dead plant (Fig. 1).

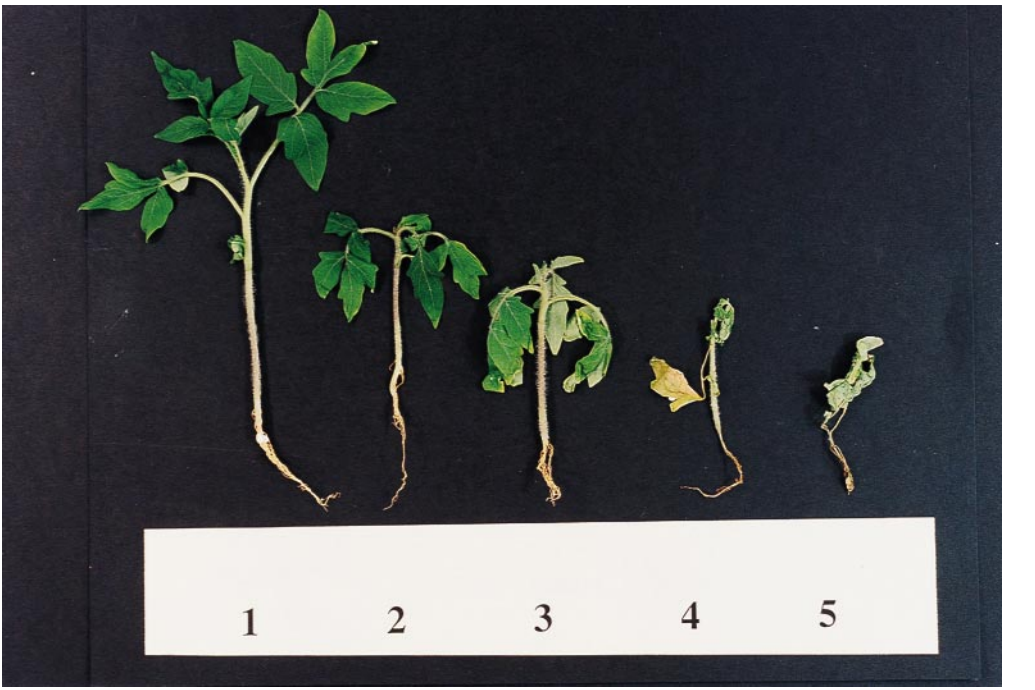


Fig. 1. Rating of progressive disease symptoms caused by *Fusarium oxysporum* f.sp. *lycopersici* on a susceptible tomato cultivar.

Experiments were arranged in a randomized block design and performed at least twice with similar results. Data were subjected to analysis of variance and least significant differences (LSD) were calculated at a significance level of $P = 0{\cdot}05$.

## RESULTS

*Cross-protection in tomato*

Simultaneous inoculation of tomato cvs. Vemar, Lorena and Hybrid 224-1, carrying resistance gene *I*, with microconidia of avirulent *F. oxysporum* f.sp. *lycopersici* race 1 provided significant protection against the virulent race (Fig. 2, Table 1). Levels of

Table 1

*Effect of simultaneous inoculation with* Fusarium oxysporum *f.sp.* lycopersici *isolate 218 (race 1) on fusarium wilt of tomato caused by* F. oxysporum *f.sp.* lycopersici *isolate 42–87 (race 2). Tomato cultivars Hybrid 224-1 and Lorena possess resistance gene* I *while cv. Moneymaker lacks this gene.*

| Fungal inoculation | | Disease Index* | | |
|---|---|---|---|---|
| Inducer | Challenger | Hybrid 224-1 | Lorena | Moneymaker |
| None | None | 1·0 a† | 1·0 a | 1·0 a |
| None | Race 1 | 1·0 a | 1·0 a | 4·0 bc |
| None | Race 2 | 5·0 c | 4·6 c | 4·8 c |
| Race 1 (10‡) | Race2 (1) | 1·2 ab | 1·0 a | 3·8 b |
| Race 1 (1) | Race2 (1) | 1·8 b | 2·0 b | 3·0 b |
| Race 1 (0·1) | Race2 (1) | 2·6 b | 2·8 b | 3·4 b |

* Disease severity was recorded 25 days after inoculation, using a scale from 1 to 5 (see Materials and Methods).

† Values are the means from 5 plants per treatment. Numbers in a column followed by the same letter are not significantly different ($P = 0{\cdot}05$). Experiments were repeated at least twice each giving similar results.

‡ The numbers in brackets refer to multiples or fractions of $5 \times 10^6$ microconidia ml$^{-1}$.

Table 2

*Effect of simultaneous inoculation with* Fusarium oxysporum *f.sp.* melonis *isolate 23M (race 0) on fusarium wilt of tomato caused by* F. oxysporum *f.sp.* lycopersici *isolate 42–87 (race 2) on cv. Vemar which possesses resistance gene* I.

| Fungal inoculation | | Disease Index* |
|---|---|---|
| Inducer | Challenger | Vemar |
| None | None | 1·0 a† |
| None | Race 0 | 1·0 a |
| None | Race 2 | 4·4 c |
| Race 0 (10‡) | Race2 (1) | 1·4 b |
| Race 0 (1) | Race 2 (1) | 2·0 b |
| Race 0 (0·1) | Race 2 (1) | 2·8 b |

* Disease severity was recorded 25 days after inoculation, using a scale from 1 to 5 (see Materials and Methods).

† Values are the means from 5 plants per treatment. Numbers in a column followed by the same letter are not significantly different ($P = 0{\cdot}05$). Experiments were repeated at least twice with similar results.

‡ The numbers in brackets refer to multiples or fractions of $5 \times 10^6$ microconidia ml$^{-1}$.

protection were similar in the three cvs. studied. Cross protection resulted both in a delay in symptom expression (see Fig. 2) and a reduction in the disease index. Disease incidence after 25 days was reduced by approximately 50 % when equal concentrations of macroconidia ($5 \times 10^6$ ml$^{-1}$) from both races were used for inoculation. When the ratio between race 1 and race 2 was 10 : 1 ($5 \times 10^7 : 5 \times 10^6$ ml$^{-1}$) protection was close to 100 %. When spores of race 2 were applied in 10-fold excess compared to race 1 ($5 \times 10^6 : 5 \times 10^5$ ml$^{-1}$), the latter still provided significant protection. Similar results

were obtained, when *F. oxysporum* f.sp. *melonis* race 0 was used as the inducer organism, although protection levels were slightly lower than with *F. oxysporum* f.sp. *lycopersici* race 1 as the inducer (Table 2). Conversely, only a very low level of protection was observed when tomato cv. Moneymaker, susceptible to both race 1 and 2 of *F. oxysporum* f.sp. *lycopersici*, was co-inoculated with these two races (Table 1). This suggests that the presence of resistance gene *I* was required for the induction of cross protection by race 1.

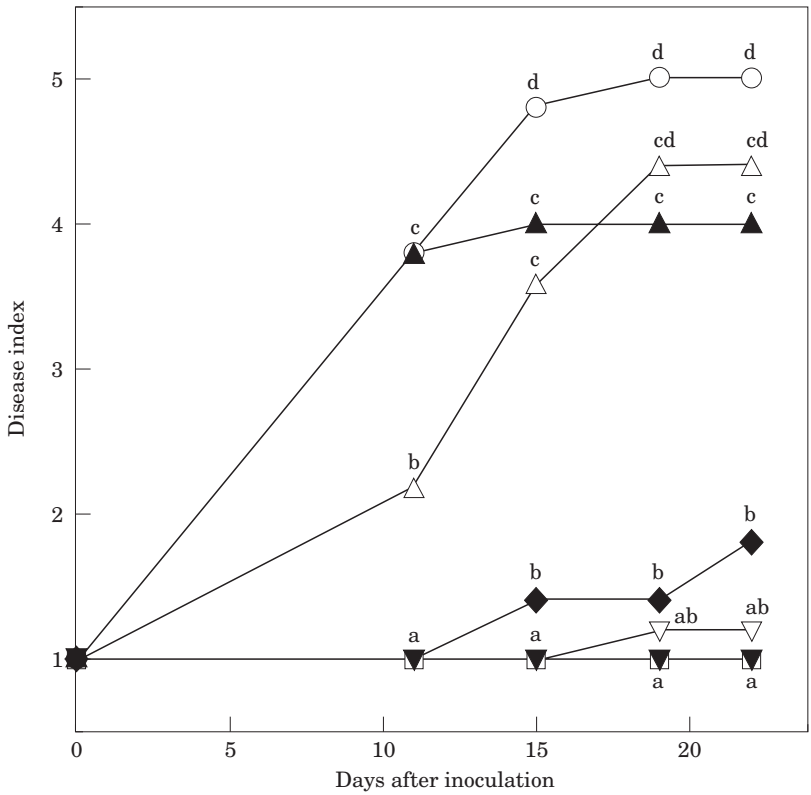


FIG. 2. Effect of simultaneous inoculation with *Fusarium oxysporum* f.sp. *lycopersici* isolate 218 (race 1) on fusarium wilt of tomato caused by *F. oxysporum* f.sp. *lycopersici* isolate 42–87 (race 2) on tomato cv. Vemar, carrying resistance gene *I*. Treatments were: uninoculated control (□); 218 (▼); 42–87 (○); 218+42-87, 10:1 (▽); 218+42-87, 1:1 (◆); 218+42-87, 0·1:1 (△); 218 autoclaved+42-87, 10:1 (▲). The proportion 1 refers to $5 \times 10^6$ microconidia $ml^{-1}$. Disease severity was recorded at the indicated time points, using a scale from 1 to 5 (see materials and methods). Values are the means from five plants per treatment. Data at a given time point with the same letter are not significantly different according to LSD ($P = 0{\cdot}05$). Experiments were repeated at least twice with similar results.

To determine whether cross protection was caused by physical or chemical interaction between spores of the avirulent and the virulent isolate during the inoculation process, a time interval of 6 or 12 h was introduced between inoculation with the inducer and the challenger organism. Under these conditions extensive disease reduction was still observed, indicating that the simultaneous presence of spores of the

two organisms was not required for cross protection (Table 3). To further check if cross protection was merely due to physical presence of conidia of the inducer organism on the root surface, microconidial suspensions of the inducer strains were autoclaved prior to simultaneous co-inoculation with *F. oxysporum* f.sp. *lycopersici* race 2. Even when applied at a 10-fold excess, autoclaved spores did not provide significant disease reduction (Fig. 2).

TABLE 3

*Effect of temporally separated inoculation with* Fusarium oxysporum *f.sp.* lycopersici *isolate 218 (race 1) on fusarium wilt of tomato caused by* F. oxysporum *f.sp.* lycopersici *isolate 42–87 (race 2) on cv. Vemar carrying resistance gene I*

| Fungal inoculation | | Disease Index* |
|---|---|---|
| Inducer | Challenger | Vemar |
| None | None | 1·0 a† |
| None | Race 1 | 1·0 a |
| None | Race 2 | 4·6 b |
| Race 1 (0 h‡) | Race 2 | 1·8 a |
| Race 1 (6 h) | Race 2 | 2·0 a |
| Race 1 (12 h) | Race 2 | 1·2 a |

* Disease severity was recorded 25 days after inoculation, using a scale from 1 to 5 (see Materials and Methods).

† Values are the means from 5 plants per treatment. Numbers in a column followed by the same letter are not significantly different ($P = 0{\cdot}05$). Experiments were repeated at least twice with similar results.

‡ The number of hours refers to the time interval between inoculation with race 1 and race 2.

TABLE 4

*Effect of simultaneous inoculation with* Fusarium oxysporum *f.sp.* melonis *isolates 23M (race 0), 18M* (race *1*) *or 11–27 (race 2) on fusarium wilt of muskmelon caused by* F. oxysporum *f.sp.* melonis *isolates 18M or 11–27. Muskmelon cultivars used were Gustal carrying resistance genes against* F. oxysporum *f.sp.* melonis *races 0 and 1, and Pancha resistant to* F. oxysporum *f.sp.* melonis *races 0 and 2*

| Fungal inoculation | | Disease index* | |
|---|---|---|---|
| Inducer | Challenger | Gustal | Pancha |
| None | None | 1·0 a† | 1·0 a |
| None | Race 0 | 1·0 a | 1·0 a |
| None | Race 1 | 1·0 a | 5·0 c |
| None | Race 2 | 5·0 b | 1·0 a |
| Race 0 | Race 1 | n.p.‡ | 3·6 b |
| Race 0 | Race 2 | 1·2 a | n.p. |
| Race 1 | Race 2 | 1·0 a | 3·2 b |

* Disease severity was recorded 25 days after inoculation, using a scale from 1 to 5 (see Materials and Methods).

† Values are the means from 5 plants per treatment. Numbers in a column followed by the same letter are not significantly different ($P = 0{\cdot}05$). Experiments were repeated at least twice with similar results.

‡ Not performed.

*Cross-protection in muskmelon*

To determine whether cross protection by avirulent races also occurs in other *formae speciales* of *F. oxysporum*, muskmelon cvs. Gustal and Pancha were co-inoculated with different combinations of races 0, 1 and 2 of *F. oxysporum* f.sp. *melonis*. Gustal carries single dominant genes conferring resistance against race 0 and race 1, whereas Pancha carries resistance genes against races 0 and 2. In Gustal, coinoculation with an equal proportion of microconidia of race 0 or race 1 provided almost complete protection against the virulent race 2 (Table 4). In cv. Pancha, cross protection against race 1 by avirulent races 0 or 2 was less pronounced, but still significant.

## DISCUSSION

Cross protection against *F. oxysporum* has been reported previously in several hosts, either induced by different *Verticillium* spp. [*12*], with saprophytic *F. oxysporum* isolates [*8*, *11*, *15*], or with pathogenic *formae speciales* of other hosts than those used in the studies [*3*, *14*, *16*, *23*]. Only in a single reported case, watermelon against *F. oxysporum* f.sp. *niveum*, was crossprotection obtained with avirulent races of the same *forma specialis* [*16*]. Several authors attributed cross protection to locally and/or systemically induced resistance in the host [*2*, *3*, *8*, *10*, *15*, *16*] and some authors reported that pathogens closely related to the challenge isolate, such as avirulent races of *formae speciales*, were better inducers of resistance than nonpathogens or unrelated pathogens [*3*, *16*].

Cross protection occurring within the same *forma specialis* may provide new insights into the genetic mechanisms that determine race-cultivar specificity in *F. oxysporum*. The present study has focused on two systems where the genetics of host resistance against different races of *F. oxysporum* is well known, *F. oxysporum* f.sp. *lycopersici* and tomato [*2*, *18*] and *F. oxysporum* f.sp. *melonis* and muskmelon [*24*]. In both systems, simultaneous or temporally separated co-inoculation with a race avirulent on a specific cultivar induced significant cross protection against the virulent race. Protection was highly reproducible and occurred under strong pressure, since in the non-protected pathogen control all plants usually died within 20 days. The degree of protection was dependent on the ratio between spore concentrations of the inducer and the challenger strain: levels of protection approaching 100% were provided by a 10-fold excess of inducer spores, but even with a 10-fold excess of challenger spores, protection was still significant. In contrast, in a previous report, equal or higher amounts of inducer compared to challenger inoculum were necessary to provide significant protection of tomato against *F. oxysporum* f.sp. *lycopersici* [*23*].

Possible mechanisms of cross protection include activation of the host defence reaction, as well as competition or antagonism between inducer and challenger organism. Two lines of evidence indicate that the protection observed in this study was due to induction of the plant defence rather than direct interaction between the different races: first, temporal separation of inoculation with the inducer and the challenger still provided significant protection, indicating that direct interaction between the two organisms at the site of infection was not required. In previous studies where time intervals had been introduced between inducer and challenger treatments,

cross protection was attributed to induction of the host defense [*3*, *11*, *16*, *23*]. More importantly, extensive protection of tomato cultivars by *F. oxysporum* f.sp. *lycopersici* race 1 against race 2 only occurred in cultivars carrying resistance gene *I* but not in a cv. lacking the gene. This supports the view that the main mechanism of cross protection is not competition between the two different races during infection, which would be expected to occur in the cultivar lacking resistance gene *I* but rather the induction of plant defense through the interaction between a specific avirulence factor of race 1 with the corresponding resistance gene. The low, but statistically significant level of protection observed in cv. Moneymaker suggests that some competition or antagonism may also occur between the two races.

The basis of susceptibility to *F. oxysporum* f.sp. *lycopersici* has been inferred as the consequence of the non-induction or suppression of an active resistance response [*2*, *6*, *14*]. Accordingly, the tomato resistance gene *I* which confers resistance against race 1 has been envisaged as a gene for recognition, whose absence leads to disease [*2*, *13*]. This hypothesis is supported by the recent molecular characterization of the I2 locus in tomato conferring resistance against *F. oxysporum lycopersici* race 2. Members of the I2 gene family show structural similarity to the group of leucine-rich repeat resistance genes involved in recognition of avirulence molecules [*5*, *18*]. Our results strongly support the hypothesis that race-cultivar specificity in *F. oxysporum* f.sp. *lycopersici* is controlled by a classical gene-for-gene system [*5*, *7*]. Genetic evidence for the presence of avirulence genes in this *forma specialis* has been provided previously by a study using race 1 mutants with altered virulence on plants carrying resistance gene *I*. The authors suggested that these strains carried mutations in loci involved in avirulence [*13*]. The same may be true in other *formae speciales* of *F. oxysporum*. We show here that *F. oxysporum* f.sp. *melonis* races 0, 1 and 2 induced cross protection in muskmelon cultivars carrying the correspondent resistance genes, against races that are normally virulent on these cultivars. Similar results have been obtained in the interaction between *F. oxysporum* f.sp. *niveum* and watermelon, suggesting that race-cultivar specificity in this host parasite system may be due to a gene-for-gene interaction [*3*].

The stage of infection at which recognition of the putative avirulence factors and induction of the defense response by the plan occurs is of considerable interest. In contrast to foliar pathogens, where an incompatible interaction is usually characterized by a necrotic hypersensitive reaction [*5*], in *F. oxysporum* it has been difficult to establish the temporal and spatial pattern of recognition and defence activation by the host [*2*]. In a recent study, the extent of vascular colonization by different races of *F. oxysporum* f.sp. *lycopersici* on susceptible and resistant tomato cultivars was determined in detail. The authors found that the degree of symptom expression was closely related to the extent of vascular colonization: avirulent races remained strictly localized, whereas virulent races progressively invaded the vascular tissue [*9*]. Since all the cultivars had equal inherent capacity to localize vascular infections, it was concluded that differences in colonization by the avirulent and the virulent race must be determined by the presence of the specific-resistance gene which controls the localization process. These results, together with our observation that cross protection is induced by simultaneous co-inoculation with the avirulent and the virulent race without the need of a time interval between induction and challenge, suggest that recognition and activation of

the plant's defenses are rapid events that occur shortly after the pathogen has entered the vascular tissue. At this stage, the putative avirulence factors must be present in the pathogen-plant interface in order to allow timely recognition by the resistance gene product.

In spite of the increasingly strong circumstantial evidence for the existence of race-specific avirulence factors in *F. oxysporum*, their nature and biochemical properties remain to be elucidated. Isolation of these compounds from a vascular root pathogen such as *F. oxysporum* may be technically challenging, especially if they are produced exclusively *in planta*. An alternative hypothesis to explain race-cultivar specificity in *F. oxysporum* f.sp. *lycopersici* is based on the finding that a 56 kDa protein purified from culture filtrates of race 1 was 400 times more toxic to protoplasts of tomato cultivars lacking resistance gene *I* than to cultivars carrying the gene [*22*]. The results of the present study than do not support the toxin hypothesis: if virulence of race 2 on cultivars carrying resistance gene *I* was due exclusively to the production of a highly toxic protein, the simultaneous presence of race 1 producing a protein with significantly less toxicity should not interfere with virulence. To reconcile these two views, one might claim that race 1 toxin acts simultaneously as an avirulence factor, and that its structural modification in race 2 simultaneously enhances toxicity to tomato/gene colours and abolishes avirulence function. A dual function as a phytotoxin and an avirulence factor has been reported for NIP1, a small cysteine-rich peptide from the barley pathogen *Rhynchosporium secalis* [*20*]. Future efforts directed at the molecular and biochemical characterization of these avirulence factors in *F. oxysporum* should further elucidate the mechanisms of race-cultivar specificity in this pathogen.

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