

Differentiation Between Two Formae Speciales of *Fusarium oxysporum* by Antisera Produced in Mice Immunologically Tolerized at Birth Through Lactation

G. Del Sorbo, F. Scala, R. Capparelli, D. Iannelli, and C. Noviello

First, second, and fifth authors: Institute of Plant Pathology, Via Università 100, 80055 Portici (NA), ITALY; third and fourth authors: Department of Animal Production, Via Università 100, 80055 Portici (NA), ITALY.

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ABSTRACT

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The immunological tolerance technique was used to produce antisera able to differentiate between two formae speciales (*lycopersici* and *dianthi*) of *Fusarium oxysporum*. Induction of tolerance was obtained in C57BL/6 mice that at birth received soluble mycelial extract through lactation. Offspring of mothers injected intravenously within 12 h after delivery with 1 mg of protein of *F. oxysporum* f. sp. *lycopersici* extract, isolate 1110, were immunized with the extract (100 µg of protein) of *F. oxysporum* f. sp. *dianthi*, isolate 1111, 28, 35, 42, and 49 days after birth. Similarly, offspring of mothers injected with *F. o. dianthi*, isolate 1111, were immunized with *F. o. lycopersici*, isolate 1110. In both cases, antisera collected at 56 days of age clearly distinguished between the two formae by immunoradiometric assay and Western blotting. Differentiation was not possible

with antisera collected from mice not exposed to the antigen at birth. Tolerization was short-lived because antisera collected from tolerized mice at 88 days could no longer discriminate between the two formae. Further, it was shown that the strain of mice, route of antigen administration, and form and doses of antigen all influence the induction of tolerance. Tolerance was induced in the C57BL/6 strain of mice but not in the BALB/c strain. No tolerizing effect was observed when the antigen was administered intraperitoneally to newborn mice or when a particulate antigen (spores) replaced the soluble extract. The technique proved useful for targeting the immune response toward fungal antigens of interest and for revealing fine antigenic differences between serologically and taxonomically related entities.

Fusarium oxysporum Schlechtend.:Fr. emend. W.C. Snyder & H.N. Hans. is the causal agent of wilt disease of numerous crops. More than 120 formae speciales and races have been described within this species (1). Identification of these morphologically indistinguishable entities is currently performed by pathogenicity tests, which are often time-consuming and difficult to carry out. Therefore, several analytical techniques, either alternative or complementary to pathogenicity tests, have been sought to study diversity at the subspecific level (16-18,22,23,25,26).

Immunological methods have been used successfully in the identification and differentiation of many plant pathogens (5,12,24,27). However, the application of antibodies for this purpose is not without problems. One limiting factor is the presence of extensive cross-reactions with antigens other than the desired ones. This problem has been particularly serious with pathogens that are difficult to purify, for example mycoplasmas and certain viruses and fungi, which have a very complex antigenic structure. In these cases, even the use of highly specific reagents, such as monoclonal antibodies, may not be effective. In our previous work (17), monoclonal antibodies produced against spores could not recognize epitopes specific for one forma specialis or physiological race of *F. oxysporum*.

To improve the discriminating power of antibodies, some authors have applied the immunological tolerance technique. When immunized with an antigen slightly different from the one to which it was exposed during the neonatal period, an animal will form antibodies only against the epitopes not present in the antigen used for tolerance. This approach has been used successfully, first in transplantation immunology to avoid allograft rejection (3) and, recently, in neuroscience (13) to recognize subsets of cortical neurons. In plant pathology, the method has been used to identify mycoplasma-like organisms (MLOs) and tomato spotted wilt virus (14,15). These pathogens are difficult to purify, and, consequently, the corresponding antibodies cross-react exten-

sively with the plant extract. Hsu et al (14,15) obtained hybridomas secreting monoclonal antibodies specific for these pathogens, using healthy plant tissue extracts to tolerize newborn mice and, then, infected plant tissue extracts to immunize them.

In this paper, we explored the potential of immunological tolerance to obtain specific antisera against the complex antigenic structure of fungi. In particular, our goal was to obtain antisera able to distinguish between the taxonomically and serologically related entities *F. oxysporum* Schlechtend.:Fr. f. sp. *dianthi* (Prill. & Delacr.) W.C. Snyder & H.N. Hans. and *F. oxysporum* Schlechtend.:Fr. f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hans.

MATERIALS AND METHODS

Fungal cultures. The isolates used in this study, together with their sources, are listed in Table 1. They were grown in liquid culture shaken at 26 C for 8 days in 2-L flasks containing 600 ml of Czapek Dox broth (Difco Laboratories, Detroit, MI).

Preparation of spores. Mycelium and spores were centrifuged (5,000 g for 15 min), and the supernatant was discarded. The pellet was washed three times with phosphate buffer, 0.15 M, pH 7.2, containing 0.85% NaCl (PBS) and filtered through MN 67 filter paper (Macherey Nagel, Duren, Germany). The filtrate contained spores almost completely free of mycelium fragments. Spores were pelleted by centrifugation (5,000 g for 15 min) and stored at -20 C.

Preparation of fungal extracts. Mycelium and spores were suspended in a minimum amount of extraction buffer (PBS containing 1 mM EDTA, 1 mM PMSF, 0.5% [w/v] sodium cholate, 0.5% [w/v] sodium deoxycholate) and quartz sand (Sigma Chemical Co., St. Louis, MO) in a mortar and homogenized while kept on ice. The homogenate was centrifuged (100,000 g for 60 min). The supernatant (representing the fungal extract) was adjusted at a protein concentration of 5 mg/ml and stored at -20 C. Before use, protein aggregates were removed by ultracentrifugation (100,000 g for 120 min). Only the upper third of the preparation was used. Protein was assayed according to the method of Bradford (4).

Animals. BALB/c and C57BL/6 mice, originally obtained from C. R. Italia (Calco, Como, Italy), were raised in our laboratory. Parturition was checked approximately every 12 h. The day the mice were born was the day the litter was found.

Induction of immunologic tolerization by the intraperitoneal route. Within 12 h after birth, mice received, at 2-day intervals, 10 intraperitoneal injections of 10^5 , 5×10^5 , or 10^6 spores of isolate 1111 of *F. o. dianthi* or of isolate 1110 of *F. o. lycopersici*. Spores were suspended in 30 μ l of PBS. Two BALB/c and two C57BL/6 mice were used per dose of spores of isolate 1111 and per dose of spores of 1110. At the age of 28 days, mice treated at birth with spores of isolate 1111 were immunized with spores of isolate 1110 and vice versa. The immunization consisted of four injections given at 1-wk intervals. Each injection consisted of 10^7 spores suspended in 100 μ l of PBS.

The same protocol was followed for tolerization with fungal extracts. Tolerizing doses were 5, 30, or 100 μ g of protein of fungal extract diluted in 30 μ l of extraction buffer, and the immunizing dose was 100 μ g of protein in 100 μ l of extraction buffer. Extracts were prepared from isolates 1111 and 1110. In both experiments, the controls were mice (of the same age and strain as the treated ones) that did not receive the early series of injections.

Induction of immunologic tolerization through lactation. BALB/c and C57BL/6 lactating mice intravenously received 1 mg of protein of fungal extract (isolate 1111 or 1110) diluted in 200 μ l of extraction buffer within 12 h of postparturition. Starting at the age of 28 days, the offspring of mothers treated with extract from isolate 1111 were immunized with extract from isolate 1110 and vice versa. The immunization consisted of four injections given at 1-wk intervals. At each injection, the animal received 100 μ g of extract in 100 μ l of extraction buffer.

In the case of tolerization with spores, groups of two C57BL/6 lactating mice received 10^5 , 5×10^5 , or 10^6 spores of isolate 1110 intravenously in 30 μ l of PBS within 12 h of parturition. Starting at 28 days, offspring were immunized at weekly intervals with 10^6 spores of isolate 1111 in 100 μ l of PBS. Control mice did not receive the tolerizing regimen during lactation.

Serological and biochemical methods. The immunoradiometric assay was performed as already described (17). Spores were attached to the wells of the plastic plate according to Stocker and Heusser (30).

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed with a Mini Protean II slab cell (BioRad, Richmond, CA) in a discontinuous buffer system according to Laemmli (20). Acrylamide concentrations of the stacking and running gels were 4 and 14%, respectively. Electrophoresis was conducted at 150 V for 60 min.

Western blotting was performed in a mini trans blot electrophoretic transfer cell (BioRad) according to Towbin et al (31). Mouse antibodies and goat antimouse IgG-alkaline phosphatase conjugate (BioRad) were diluted 1:500 and 1:4,000, respectively, in TTBS (20 mM Tris, 0.5 M NaCl, 0.05% [v/v] Tween 20, pH 7.5) containing 1% (w/v) gelatin.

Determination of the immune status. Blood samples were collected from the retroorbital vein of mice at 28, 56, and 88 days of age. On day 28, bleedings were performed before immunizations. Antisera were analyzed by immunoradiometric assay and, in some cases, also by Western blotting.

TABLE 1. Isolates of *Fusarium oxysporum* used in this study

Forma specialis	Isolate number	Race	Source
<i>lycopersici</i>	567	1	OC ^a (Latina)
	842	1	OC (Montfavet)
	1110	1	M. Cirulli (Bari)
<i>dianthi</i>	587	1	A. Garibaldi (Torino)
	1111	1	A. Garibaldi (Torino)
	588	2	A. Garibaldi (Torino)
	1112	2	A. Garibaldi (Torino)

^aOC = own collection.

RESULTS

Tolerization by the intraperitoneal route. Figure 1A shows the results obtained with C57BL/6 mice injected at birth with 10^5 spores of isolate 1110 and at the age of 4 wk with 10^7 spores of isolate 1111. Sera collected from these mice at 28 days reacted similarly with spores of both formae speciales. In addition, the intensity of these reactions was lower than that of preimmune sera obtained from control mice. At 56 and 88 days, the mice gave reactions not significantly different with either kind of spores but with an intensity higher than that of control mice. Thus, the spores to which the animal was exposed immediately after birth contributed late in life to induce an immune response rather than tolerance.

The same results were observed with C57BL/6 mice tolerized with 10^5 spores of isolate 1111 and immunized at the age of 4 wk with 10^7 spores of isolate 1110 (Fig. 1B).

The remaining groups of mice treated with spores by the intraperitoneal route displayed results very similar to those shown in Figure 1A and B. Thus, the strain of mice employed (BALB/c or C57BL/6), the number of spores injected at birth (10^5 , 5×10^5 , or 10^6), and the forma specialis of the spores (i.e., *lycopersici* or *dianthi*) had no determinative effect.

Tolerization was also attempted with fungal extracts. Administration of a soluble antigen instead of a particulate antigen did not make a difference (Fig. 1C and D). Again, the dose of extract given to an animal at birth (5, 30, or 100 μ g of protein), the forma specialis from which the extract was obtained (*lycopersici* or *dianthi*), and the strain of the animal (BALB/c or C57BL/6) did not influence the results.

Tolerization by the maternal route. In mice, antigen exposure during suckling causes specific unresponsiveness (19,28). Therefore, it was decided that this approach also should be tried. Figure

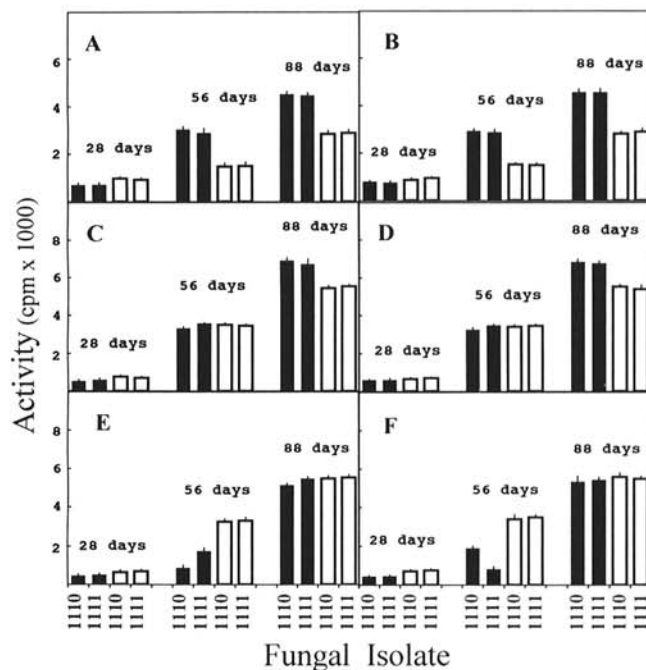


Fig. 1. Immunoradiometric analysis of sera from C57BL/6 mice subjected to tolerization. Sera from tolerized and control mice (two for each group) were tested individually. Bars are means of three independent determinations, each in triplicate, with one standard error. A, Tolerization with 10^5 spores of *Fusarium oxysporum* f. sp. *lycopersici* isolate 1110 by the intraperitoneal route; B, tolerization with 10^5 spores of *Fusarium oxysporum* f. sp. *dianthi* isolate 1111 by the intraperitoneal route; C, tolerization with 5 μ g of protein of extract from *F. o. lycopersici* isolate 1110 by the intraperitoneal route; D, tolerization with 5 μ g of protein of extract from *F. o. dianthi* isolate 1111 by the intraperitoneal route; E, tolerization with extract from *F. o. lycopersici* isolate 1110 by the lactation route; and F, tolerization with extract from *F. o. dianthi* isolate 1111 by the lactation route. ■ indicates tolerized mice; □ indicates control mice.

IE shows the antibody profile of C57BL/6 mice exposed during suckling to the extract from isolate 1111. At the age of 56 days, the tolerizing effect of early exposure to extract of isolate 1110 was evident. The activity shown against this antigen was clearly lower than that against isolate 1111, especially when compared to the activity developed by the mice of the control group. By day 88, the tolerance was lost, and no differences could be observed between the antibody response of treated and control mice.

Tolerance induction also was observed in both C57BL/6 mice exposed during suckling to the extract from isolate 1111 and immunized with isolate 1110. Again, the unresponsiveness toward the antigen encountered during suckling was marked at 56 days of age but disappeared by the age of 88 days (Fig. 1F).

Tolerance was not observed in any of the BALB/c mice, whether first exposed to isolate 1110 and immunized with isolate 1111 or vice versa (data not shown).

Next we investigated whether tolerance could be obtained in C57BL/6 mice with a particulate antigen. As already observed in the case of the intraperitoneal route, spores were unable to induce tolerance. The effect of the mice strains, tolerization routes, and forms and doses of antigen on the induction of tolerance are summarized in Table 2.

Serum samples collected at different dates from mice tolerized with isolate 1110 and immunized with isolate 1111 also were analyzed by Western blotting. The results confirmed those of the immunoradiometric assay. The antisera collected at the age of 28 days did not react with either of the two extracts. The antisera collected at 56 days formed only one faint band with extracts from three isolates of *F. o. lycopersici* but formed several

TABLE 2. Results of tolerization experiments

Mouse strain	Tolerization route	Antigen	Dose	Tolerizing effect
C57BL/6	intraperitoneal	spores	10^5 , 5×10^5 , 10^{6a}	absent
BALB/c	intraperitoneal	spores	10^5 , 5×10^5 , 10^{6a}	absent
C57BL/6	intraperitoneal	extract	5,30,100 ^b	absent
BALB/c	intraperitoneal	extract	5,30,100 ^b	absent
C57BL/6	maternal	extract	1,000 ^b	present
BALB/c	maternal	extract	1,000 ^b	absent
C57BL/6	maternal	spores	10^{7a}	absent

^aNumber of spores administered per injection.

^bMicrograms of protein administered per injection.

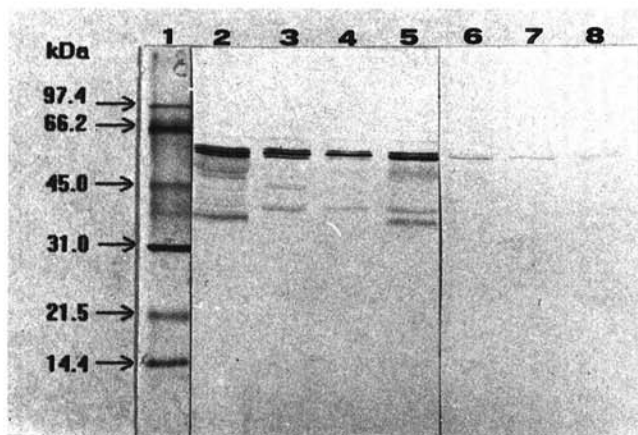


Fig. 2. Western blotting pattern displayed by extracts of four isolates of *Fusarium oxysporum* f. sp. *dianthi* and three isolates of *Fusarium oxysporum* f. sp. *lycopersici* with antiserum collected at 56 days of age from one of the mice tolerized by the maternal route with extract from *F. o. lycopersici* isolate 1110 and immunized with *F. o. dianthi* isolate 1111. Lane 1: Biotinylated molecular standards. Lanes 2-3: Isolates 1111 and 587 of *F. o. dianthi* race 1, respectively. Lanes 4-5: Isolates 1112 and 588 of *F. o. dianthi* race 2, respectively. Lanes 6-8: Isolates 1110, 567, and 842 of *F. o. lycopersici* race 1, respectively.

bands with extracts from four isolates of *F. o. dianthi* (Fig. 2). However, the response was not selective enough to discriminate between isolates of races 1 and 2 of *F. o. dianthi*. The sera of control mice, collected at 56 days of age, did not discriminate between the two formae speciales and gave a much more complex reaction pattern than that observed with tolerized mice (Fig. 3). It was evident that the extract from isolate 1110 presented to the mice neonatally suppressed the ability of the immune system to recognize the molecules that the two extracts have in common. At 88 days, the tolerizing effect was no longer observed; antisera from tolerized animals at this date did not distinguish between isolates of the two formae speciales (Fig. 4). The comparison of Figures 2 and 4 shows the change occurring between days 56 and 88 of the immune response of tolerized mice.

DISCUSSION

Techniques able to detect differences among morphologically indistinguishable formae and races of pathogenic fungi are very desirable for taxonomic, phylogenetic, and diagnostic purposes.

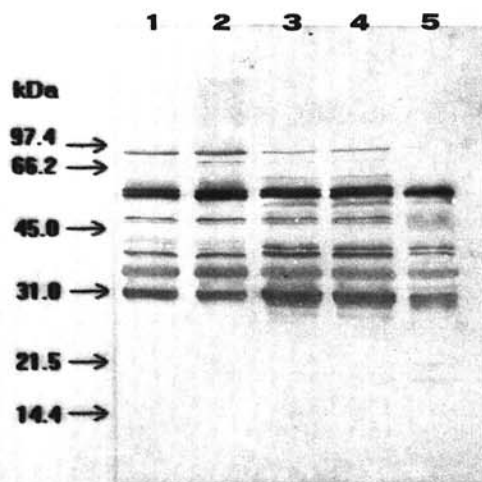


Fig. 3. Western blotting pattern displayed by extracts of three isolates of *Fusarium oxysporum* f. sp. *dianthi* and two isolates of *Fusarium oxysporum* f. sp. *lycopersici* with antiserum collected at 56 days of age from one of the control mice (i.e., a nontolerized mouse immunized with extract from *F. o. dianthi* isolate 1111). Lanes 1-2: Isolates 1111 and 587 of *F. o. dianthi* race 1, respectively. Lane 3: Isolate 1112 of *F. o. dianthi* race 2. Lanes 4-5: Isolates 1110 and 567 of *F. o. lycopersici* race 1, respectively.

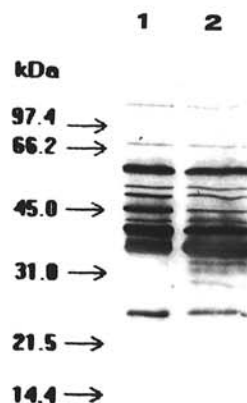


Fig. 4. Western blotting pattern displayed by extracts of isolate 1111 of *Fusarium oxysporum* f. sp. *dianthi* (lane 1) and isolate 1110 of *Fusarium oxysporum* f. sp. *lycopersici* (lane 2) with antiserum from the same mouse used in Figure 2 but collected at the age of 88 days.

Furthermore, quick and standardized systems to monitor formae and races may be useful for a rational control of diseases and for breeding programs. For this reason, many efforts are constantly devoted to the improvement and development of these techniques. Hsu et al (14,15) used the immunological tolerance method to produce monoclonal antibodies specific for MLOs and tomato spotted wilt virus.

In this paper, for the first time, we explored the possibility of using the immunological tolerization technique to distinguish between formae speciales of *F. oxysporum*. However, much work also has been conducted to identify the effects a number of factors may have on the induction of tolerance, i.e., the strain of mice, route of antigen exposure, and form (soluble mycelial extract or spores) and dose of the antigen.

The experiments described in this paper established that tolerance can be induced in C57BL/6 mice but not in BALB/c mice. Golub and Weigle (11), using the same two animal strains and human gamma globulin to induce tolerance, reached the same conclusion. Thus, two independent studies, in which different antigens and different protocols and routes of administration were used, indicate that the C57BL/6 strain can easily be made unresponsive. However, this state of unresponsiveness is relative and not absolute. Komatsu et al (19) found that tolerance to human gamma globulin could be induced in BALB/c mice, although not as effectively as in C57BL/6. Hockfield (13) also was able to induce tolerance to rat brain gray matter in BALB/c mice. Differences in antigen, mode of immunization, and methods of assaying the immune response may account for the above results. The use of a potentially less immunogenic material for the mouse (rat brain gray matter versus fungal extract) might have induced tolerance in the experiments described by Hockfield (13) and immunity in the experiments described in this paper.

Not all individuals within a species have the same genetic potential for T cell receptor diversity. In different mouse strains, the T cell receptor repertoire has been contracted by extended deletions within the T cell receptor V β locus (29). Theoretically, the unresponsiveness to fungal extracts observed in C57BL/6 mice could be due to the absence in the T cell receptor repertoire of this strain of clones, which is able to recognize these antigens efficiently. However, in this strain, unresponsiveness has been induced against several antigenically complex antigens such as human gamma globulin (11,19,28), bovine gamma globulin (7-9), and fungal extracts (as demonstrated in this study). Therefore, we do not find this explanation convincing.

In mice, susceptibility to induction of tolerance with heterologous gamma globulin is controlled by the Tol-1 locus. C57BL/6 mice are susceptible, whereas BALB/c mice are resistant (21). The genes at the Tol-1 locus act on the way the antigen is processed by macrophages (21). We propose that C57BL/6 mice may inefficiently process not only the gamma globulin but also fungal extracts. Thus, tolerance, as evidenced in this study, would be another effect of the Tol-1 genes. At this stage, it would be premature to invoke additional tolerance genes in the C57BL/6 strain.

In this study, tolerance could be induced only when the antigen was administered to suckling C57BL/6 mice via the colostrum. As proposed by Komatsu et al (19), presumably this route is more efficient in promoting tolerance because of the slow catabolism of the fed antigen and the long exposure of the animal to the antigen obtained by lactation. Further, Komatsu et al also have suggested (19) that through this route small aggregates present in the antigen preparation may be removed during the passage of the antigen, first in the mammary gland of the mothers and then in the liver of the suckling animal. Removal of aggregated material from the antigen preparation (6,21) predisposes the immune system to tolerance.

Our results also show that the form of the antigen is important for determining whether early antigen exposure can lead to unresponsiveness. In suckling C57BL/6 mice, fungal extracts induced tolerance, whereas spores were invariably immunogenic. A likely explanation for this finding is that spores are readily phagocytized by macrophages and consequently prone to cause immunity rather than tolerance (2). In the case of fungal extracts

thought to be highly immunogenic for the mouse, tolerance can be achieved only when a soluble antigen and the lactation route combine with the use of a strain of mice (C57BL/6) predisposed to unresponsiveness.

Using neonatal tolerization, we have generated antisera that discriminate between two formae speciales of *F. oxysporum*. We also have defined the general conditions required for extending the technique to mycology. In the near future, by tolerizing C57BL/6 mice at birth with the extract from one physiological race of *F. oxysporum* and priming the animals with the extract from another physiological race, we plan to obtain antisera that can differentiate between the two races. Having established the time when mice become unresponsive, immortalization of antibodies through the hybridoma technique also will become a feasible approach. Studies on the vegetative compatibility within various formae speciales of *F. oxysporum* have shown that a high level of diversity may exist among isolates of the same race (10). In this paper, using an entirely different technique, we confirmed this result. Patterns of isolates belonging to the same race were different in the number and size of bands (Fig. 2). The number of bands did not change, and the diversity was maintained even when a fivefold higher quantity of extract was employed. Tolerization with one isolate and immunization with a different isolate of the same race may restrict the range of antibodies formed, so the antiserum may be sufficiently specific to allow detection of differences among isolates of the same race of *F. oxysporum*. This would contribute to tracing the evolution of this organism. Immunological tolerization is perhaps the only means of obtaining antisera with such a narrow specificity.

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