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PCR-based analysis of disease in tomato singly or mixed inoculated with *Fusarium oxysporum* f. sp. *lycopersici* races 1 and 2

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Summary. The pathogenic response of two tomato cultivars to races of *Fusarium oxysporum* f. sp.. *lycopersici* (cv. Momotaro, insensitive to race 1 of the pathogen, and cv. Ponderosa sensitive to race 1), was studied in greenhouse and laboratory experiments by inoculating the cultivars singly with race 1 or race 2, and in mixed inoculation with the two races of the pathogen. A pre-symptom PCR assay two weeks after inoculation showed that a fragment of the intergenic spacer region (IGS) of ribosomal DNA was amplified by DNA templates from leaf samples of cv. Momotaro tomato plants inoculated with only race 2, or with race 1+2, but in the cv. Ponderosa the fragment was amplified only in plants inoculated with race 1+2. Race-specific analysis using the sp13 and sp23 primers confirmed that the amplified fragment was from race 2 in cv. Momotaro and from races 1+2 in cv. Ponderosa. Later wilt symptoms mirrored the pre-symptom and post-symptom molecular analytical results: cv. Momotaro plants inoculated with only race 1 remained symptomless, while the 'Momotaro' plants inoculated with both races (1+2) did not manifest more severe wilt symptoms than plants inoculated with race 2 alone; cv. Ponderosa plants that were mixed-inoculated with race 1+2 manifested more severe symptoms, and at an earlier date than plants inoculated with only race 2. Growth parameters such as number of leaves and plant height showed the race 1+2 infected cv. Ponderosa were significantly retarded in growth, suggesting that significant synergism between the fungal races in tomato pathosystem can occur only when the host cultivar is sensitive to both races. An additional important finding is that pre-symptom leaf sampling of apparently healthy plants is useful in PCR diagnostic analysis to predict impending fusarial wilt outbreaks in tomato especially in infested soil.

Key words: tomato wilt, *Fusarium oxysporum*, races, PCR, rDNA IGS.

Introduction

The vascular wilt fungus *Fusarium oxysporum* is a filamentous ascomycete, various forms of which attack different plant species. Many of these forms are implicated in diseases of economic importance all over the world (Agrios, 2005). Within the *formae speciales*, races are known to occur and these must be identified because such races behave differently in different pathosystems.

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Three physiologic races of *F. oxysporum* f. sp. *lycopersici*, named 1, 2 and 3 in order of their discovery (Alexander and Tucker, 1945; Booth, 1971; Grattidge and O'Brien, 1982), are traditionally distinguished by each having a specific pathogenicity to tomato cultivars. Species of *Fusarium* are traditionally differentiated by their morphological characteristics on selective media (Nelson, 1983, Burgess *et al.*, 1994). It is almost impossible, however, to identify pathogenic types, or *formae speciales*, and races of *Fusarium oxysporum*, using morphological features.

Arie *et al.* (1995, 1997) proposed immunoassays as an alternative method to identify species and

subspecies of *F. oxysporum*, while more recently molecular markers have become popular for this purpose. Some of the techniques that have been reported are: DNA fingerprinting with nuclear repetitive DNA sequences (Namiki *et al*., 1994), amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995), random amplified polymorphic DNA (RAPD) (Kalc *et al*., 1996), restriction fragment length polymorphisms (RFLP) (Baayen *et al*., 1997) and direct amplification of length polymorphism (Desmarais *et al*., 1998), among others.

Plant pathogens and the diseases they cause have an enormous capacity to affect the well-being of man through their adverse effects on plants that serve useful socio-economic purposes. Understanding the mechanisms by which these pathogens effect such changes is therefore a necessary albeit daunting task.

The PCR-based differentiation of the various races of the vascular wilt fungi of tomato, *Fusarium oxysporum* f. sp. *lycopersici* (*FOL*) and f. sp. *radicis lycopersici* using specific primer sets (Hirano and Arie, 2006) is an example of how the molecular approach can be used to facilitate identification and hence to manage the disease.

In this study the hypothesis was tested that mixed inoculation with races 1+2 of *F. oxysporum* f. sp. *lycopersici* would lead to synergistic interaction between these two races, so that disease severity would be greater in mixed infected plants that in plants infected with either race 1 or race 2 alone. The main aim of the experiments was to evaluate whether leaf and root tissues before and after symptom manifestation could be used in the PCR-based diagnosis of fusarial disease in tomato

Materials and methods

Planting operation

The tomato cv. Momotaro (*I i-2 i-3*; Takii Seed, Kyoto, Japan), resistant to *FOL* race 1 but susceptible to race 2, and the cv. Ponderosa, which is susceptible to both *FOL* races 1 and race 2, were used. Tomato seeds were sown in 250-ml experimental pots containing steam-sterilized soil supplemented with 3 g fertilizer per pot at seeding. The pots were arranged in trays and kept on stands in a glasshouse where the temperature ranged between 25 and 30°C. Upon germination, two plants were left in each pot and were watered regularly to prevent water stress.

Inoculum preparation and inoculation procedure

Fusarium oxysporum f. sp.. *lycopersici* race 1 (MN-59, C. Kistler, USDA-ARS, Minneapolis, MN, USA) and race 2 (JCM 12575, Japan Collection of Microorganisms, RIKEN, Wako, Saitama, Japan) were maintained on potato dextrose agar at 25°C. Mycelial blocks about 1 cm in diameter were cut from cultures of *FOL* race 1 and 2 growing on potato dextrose agar and transferred to 100 ml potato dextrose broth in 200 ml flasks. They were incubated in shake culture (ca. 120 rpm) at room temperature for 5 days to produce bud cells (spores) for use as inocula. At the end of the incubation period, the spores were harvested by pelleting through centrifugation at 3000 rpm for 15 min. The spores were re-suspended in water and the concentration was adjusted to 1×10^7 spores ml^{-1} with the aid of a haemacytometer, under a microscope.

At 3 weeks from seeding, the tomato cultivars were inoculated with either race 1 or race 2, or a mix of races 1+2. After stabbing the soil around the plants with a peg, the inoculum (2 ml/pot) was poured on the soil. The purpose of the stabbing was to soften the soil to facilitate the entry of the inoculum. For inoculation with races 1+2, 2 ml each of the spore suspension was mixed together and the mixed suspension was poured on the soil. Plants inoculated with water served as control. Each treatment was replicated in eight separate pots. The treatment design was a 2×4 factorial fitted into a completely randomized design (CRD) and replicated 4 times.

Extraction and purification of genomic DNAs from fungal mycelium

The method employed was essentially the mini-preparation protocol reported by Saitoh *et al.* (2006). A mycelium block about 10 mm in diameter was cut from 5-day old fungal cultures of each of the four fungal isolates and placed in separate 1.5 ml Eppendorf tubes. Five hundred μl of lysis buffer (200 mM Tris HCl, 50 mM ethylene diamine tetra acetic acid [EDTA], 200 mM NaCl, 1% n-lauroyl sarcosine-Na; pH 8.0) was added. The mixture was kept at room temperature for 10 min before being dispersed with toothpick. Potassium acetate (150 μl) was then added and the mixture vortexed for 10 seconds. DNA was pelleted with 99.5% ethanol, washed with 70% ethanol by centrifugation for 5 min at 15,000 rpm, 4°C. DNA pellets were air-dried and further re-suspended in water for storage.

Extraction of total genomic DNA from plant tissues

The roots and lower leaves of one tomato plant per replicate pot were sampled at 16 and 24 days post inoculation (DPI). Roots were washed thoroughly with running water to remove adhering soil particles and any fungal component. The roots were dried on tissue paper. For purposes of comparison, a subset of the roots was further surface-sterilized with 0.05% sodium hypochlorite, rinsed in water and air-dried before DNA extraction. Total DNA was extracted from the roots and lower leaves following Hirano and Arie (2006). Briefly, the procedure involved homogenization with extraction buffer (200 mM Tris–HCl, pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5% SDS), centrifugation at 9,000 rpm for 5 min at 4°C, purification with phenol- chloroform-isoamyl (25:24:1; PCI), and precipitation with isopropanol. Harvested DNA pellets were air-dried for 15 min and re-suspended in milliQ water at 50 ng μl^{-1}.

Polymerase chain reaction

The PCR assay was carried out at least 4 times using the total DNA from the root and leaf samples as templates to amplify the ribosomal DNA intergenic spacer (IGS) region in the *FOL* genome. Genomic DNA extracted from pure fungal cultures, as described (race 1 and 2), was co-analyzed as a positive control. For this purpose, primers FIGS11 (5'-GTAAGCCGTCCTTCGCCTCG) and FIGS12 (GCCATACTATTGAATTTTGC) (Kawabe *et al.*, 2005) were used. This primer set amplifies a ca. 600-bp fragment from *F. oxysporum* and *F. moniliforme* (=*Gibberella fujikuroi*) in the *Fusarium* spp. The PCR reaction mixture (10 μl) consisted of 1 μl of 10× Taq polymerase buffer (New England Biolabs, Ipswich, MA, USA), 0.2 μl of 10 mM dNTP mix (New England Biolabs), 0.05 μl of *Taq* DNA polymerase (New England Biolabs), 4 pM (each) of the primers and 1.0 μl of the template DNA. The PCR reaction was carried out using the Gene Amp PCR system 9700 series (Applied Biosystems, Foster City, USA). The thermal conditions were set as follows: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and elongation at 72°C for 1 min; final extension at 72°C for 7 min.

For the specific determination of the *FOL* race, PCR was performed with two specific primer sets sp13 and sp23 described by Hirano and Arie (2006). The sp13 primer set amplifies a ca. 450-bp fragment from *FOL* races 1 or 3, while the sp23 primer set amplifies a ca. 520-bp fragment from races 2 and 3 (Hirano and Arie, 2006). In this study, *FOL* races 1 and 2 were used, so that amplifications with sp13 and sp23 primer sets would reveal the presence of race 1 and race 2, respectively. The reaction mixture (30 μl) contained 3 μl of 10× Taq polymerase buffer, 0.9 μl of 10 mM dNTP mix, 0.15 μl *Taq* DNA polymerase, 20 pM of each of the primers and 1 μl of template DNA. Samples were cycled in a TaKaRa Gradient PCR cycler (Takara-Bio, Shiga, Japan). The annealing temperature was graduated from 58 to 65°C and eventually the PCR tubes were arranged so that the cycling (50 cycles) conditions for the sp13 primer set was 94°C for 1 min, 59.4°C for 1 min, and 72°C for 2 min, and for the sp23 primer set 94°C for 1 min, 61.9°C for 1 min and 72°C for 2 min. The initial denaturation temperature was 94°C for 5 min while the final extension at 72°C was for 7 min.

Results and discussion

Disease symptoms and plant growth

The most common symptom observed in inoculated susceptible plants was wilting. At 16 DPI none of the inoculated plants had yet manifested any wilt symptoms or significant growth differences as compared with the control. The number of leaves and the height of the plants measured at that time was similar in both cultivars (Table 1).

At 24 DPI, however, wilt symptoms and growth differences had become apparent, and, as expected, the plant reaction varied with the inoculation treatment. 'Momotaro' tomato plants inoculated with only race 1 remained apparently healthy without any symptoms, whereas those inoculated with only race 2 were smaller and severely wilted (Table 1, Fig. 1). 'Momotaro' plants inoculated with races 1+2 showed symptoms and growth similar to plants inoculated with only race 2; indicating there was no difference between the effect produced by race 2 and race 1+2 in this cultivar.

At 24 DPI, cv. Ponderosa plants inoculated singly with race 1, or race 1+2, showed considerable wilting and were significantly smaller in size than the healthy control plants or than the race-2-only inoculated plants. Plants inoculated with only race 2 had delayed symptom manifestation as only mild wilting was apparent at 24 DPI (Fig. 2). At 30 DPI, however, such plants had become severely wilted (data not shown) showing that cv. Ponderosa was indeed susceptible to race 2.

Table 1. Growth attributes of two tomato cultivars at the pre-symptom and the symptom manifestation stage after inoculation with *Fusarium oxysporum* f. sp *lycopersici* races 1 and 2.

Treatments		Number of leaves[a]			Plant height (cm)		
		At inoculation	DPI		At inoculation	DPI	
			16	24		16	24
Cultivar							
	Momotaro	4.2	7.3	7.9a	8.2	17.2	27.1a
	Ponderosa	4.1	7.4	7.2b	7.7	16.6	25.4b
	SEM	0.099 ns	0.128 ns	0.132	0.263 ns	0.291 ns	0.321
Fungal treatment							
	Control	4.1	7.4	8.6a	7.5	16.5	32.8a
	Race 1	4.1	7.5	7.6b	8.0	17.5	27.1b
	Race 2	4.2	7.0	7.1bc	7.8	16.6	24.5c
	Race1+2	4.3	7.4	6.9c	8.5	17.0	20.6d
	SEM	0.140 ns	0.180 ns	0.191	0.372 ns	0.411 ns	0.454
Cultivar × Fungal treatment							
Cv. Momotaro	Control	4.3	7.3	8.8a	8.0	16.5	31.5b
	Race 1	4	7.4	8.8a	8.1	17.8	32.8ab
	Race 2	4.3	7.3	6.8bc	8.1	16.8	22.7d
	Race1+2	4.2	7.5	7.5b	8.8	17.5	22.1d
Cv. Ponderosa	Control	4.0	7.8	8.5a	7.5	16.5	34.0a
	Race 1	4.3	7.8	6.5c	8.3	17.0	21.5d
	Race 2	4.0	6.8	7.5b	7.8	16.5	27.0c
	Race1+2	4.1	7.3	6.3c	8.2	16.6	19.0e
	SEM	0.198 ns	0.255 ns	0.271	0.525 ns	0.582 ns	0.641

[a] Means within a section of a column followed by the same letter are not significantly different, at $P<0.05$, using the New Duncan's multiple range test.
ns, not significant.

Fig. 1. Symptoms on the tomato cv. Momotaro inoculated with *Fusarium oxysporum* f. sp. *lycopersici* 24 days after inoculation. Plants were inoculated with race 1, and race 2, and jointly with races 1+2 by pouring the spore suspension (2.0×10^7 spores ml^{-1}) on the soil around the plants. Control plants (control) were inoculated with water.

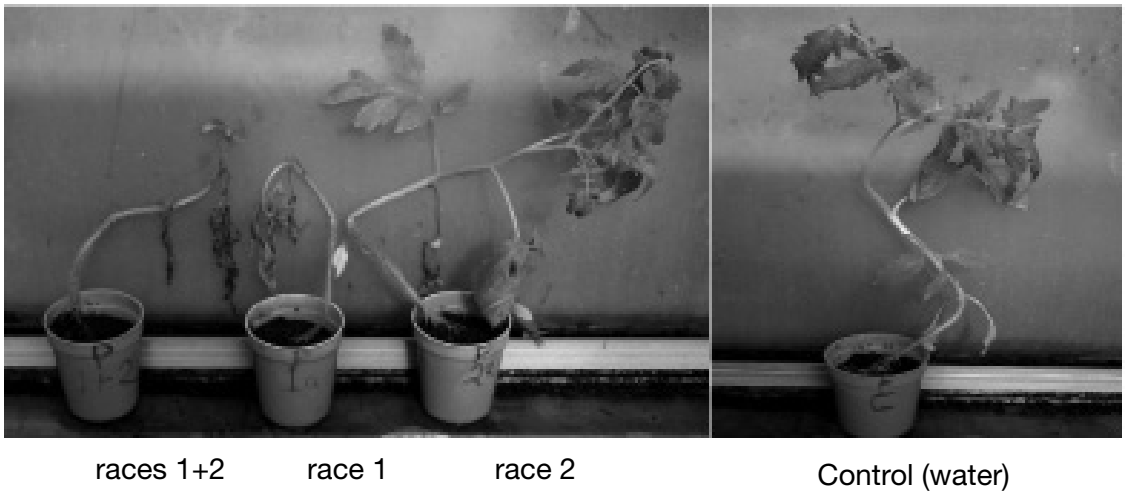

Fig. 2. Symptoms on the tomato cv. Ponderosa inoculated (as described in Figure 1) with various races of *Fusarium oxysporum* f. sp. *lycopersici* 24 days after inoculation.

Detection of *Fusarium oxysporum* in tomato tissues by amplification of rDNA IGS region fragment

Detection at 16 DPI before wilt symptom manifestation.

In the cv. Momotaro, there was clear amplification in the leaf samples from plants inoculated with only race 2, and with races 1+2. However, there was no amplification with DNA templates from 'Momotaro' root samples treated with water only or from root samples surface-sterilized before DNA extraction (Fig. 3). In the cv. Ponderosa, on the other hand, there was clear amplification only with templates from the leaves of plants inoculated with races 1+2, but not with template from plants infected with only one race. DNA templates from the root samples were however not amplified in any of the repeat trials (Fig. 4). The results seemed to indicate that in the tissues of the cv. Ponderosa, the movement of the fungal DNA was enhanced by co-inoculation of race 1+2, while this was not the case in the cv. Momotaro.

Because of the unexpected nature of the amplification of DNA templates from the leaf samples in both cultivars, an attempt was made to isolate the fungus from the same leaf samples using a Fusarium-selective medium (Komada's medium; Komada, 1975) but this was unsuccessful (data not shown) suggesting that the amplified fungal DNA in the leaves had migrated there, probably through the vessels, ahead of the invading fungal mycelium. The determination of the precise route was not however an objective of the study and it remained unclear. An attempt to ascertain the route will be undertaken at a later date.

Detection after symptom manifestation at 24 dpi

With the cv. Momotaro, amplification was achieved with the leaf samples from plants inoculated with only race 2 and those inoculated with race 1+2. No amplification occurred with leaf samples from plants inoculated with race 1 (Fig. 5). These results mirrored the pre-symptom analysis as well as the symptoms described above i.e. 'Momotaro' plants infected with race 1 alone were symptomless, while the others became wilted.

An interesting observation was that the fragment of the IGS region was amplified from all 'Momotaro' root samples, whether inoculated with one or with two races. This of course indicated that enough fungal material was embedded in the root samples at this time. However, since *FOL* was not detected in the leaf samples from cv. Momotaro tomatoes inoculated with race 1, it strongly suggested that while *FOL* race 1 became established in the root (perhaps in the cortex) of the race 1-resistant tomato cultivar, it could neither reside in the vessels of that cultivar, nor extend to the upper part of the plants, nor cause the symptoms on the cultivar that were observed there (Fig. 1).

PCR analysis of cv. Ponderosa root samples at 24 DPI, mirrored those of the cv. Momotaro, but the DNA templates from all the leaf samples did not amplify the fragment in all replicates (Fig. 6). This result is probably a further reflection of the genetic differences between the two tomato cultivars, which cause them to react differently to infection with race 1 or race 2. While we are not oblivious of the possible pitfalls of PCR analysis, which may derive from the manner of sample preparation, excesses of enzymes, machine fluctuations and other factors, the findings here presented seem valid as proved by the repeat experiment.

Although the detection of the IGS region fragment in the samples was not sufficient to discriminate between races, it could nevertheless be very useful as confirming fungal occurrence, especially when coupled with preliminary symptom observation on the field. Detection of the IGS region in the leaf tissues of both cultivars has an important implication: namely that leaf tissues may be better for fungal detection than other portions of the host, especially when the symptoms are not yet manifest.

Detection of *FOL* races 1 and 2 in tomato tissues using the race-specific primer sets sp13 and sp23

Detection before wilt-symptom manifestation at 16 DPI.

As mentioned above (Hirano and Arie, 2006), the sp13 primer set specifically amplifies a ca. 460 bp DNA fragment in *FOL* races 1 and 3, while the sp23 primer set amplifies a ca. 520 bp fragment in *FOL* races 2 and 3. Although those authors stated that these primer sets reliably diagnose different isolates of the races of *FOL* in tomato tissues, they did not mention infection involving multiple races. By using these race-specific primers as diagnostics, it was expected that an answer would be found to the question how well races jointly inoculated in tissue samples (leaves and roots) of tomato plants multiplied in them.

Figure 7 shows the amplification of the fragments from the various samples. Leaf samples from the 'Momotaro' plants inoculated with races 1+2 generated amplicons with the sp23 primer set.

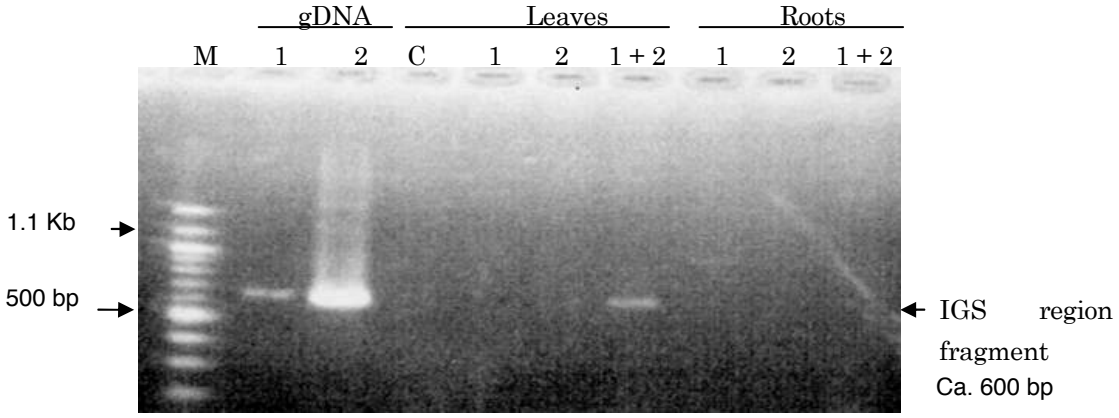

Fig. 3. PCR products with the IGS region primers using as template DNA extracted from the roots and leaves of tomato cv. Momotaro 16 days after inoculation. Lanes, M, 100-bp DNA marker; gDNA, fungal genomic DNA extracted from *Fusarium oxysporum* f. sp. *lycopersici* races 1, and race 2 was used as a template; roots, total DNA extracted from tomato cv. Momotaro roots inoculated singly with race 1, and with race 2, and jointly with races 1+2, was used as a template; leaves, total DNA extracted from tomato cv. Momotaro roots inoculated singly with race 1, and with race 2, and jointly with races 1+2, was used as a template.

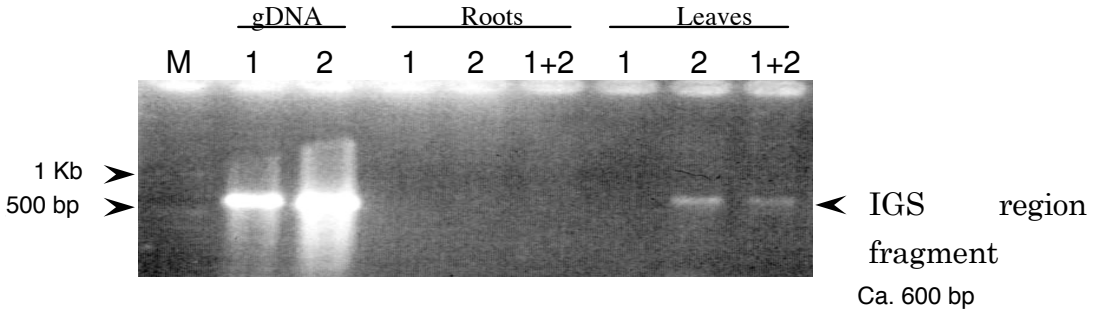

Fig. 4. PCR products with the IGS region primers using as templates DNA extracted from the roots and leaves of tomato cv. Ponderosa 16 days after inoculation. Lanes: M, 100-bp DNA marker; gDNA, fungal genomic DNA extracted from *Fusarium oxysporum* f. sp. *lycopersici* race 1, and race 2 was used as a template; leaves, total DNA extracted from tomato cv. Ponderosa leaves inoculated with water (C), singly with race 1, and with race 2, and jointly with races 1+2, was used as a template; roots, total DNA extracted from tomato cv. Ponderosa roots inoculated singly with race 1, and with race 2, and jointly with races 1+2 was used as a template.

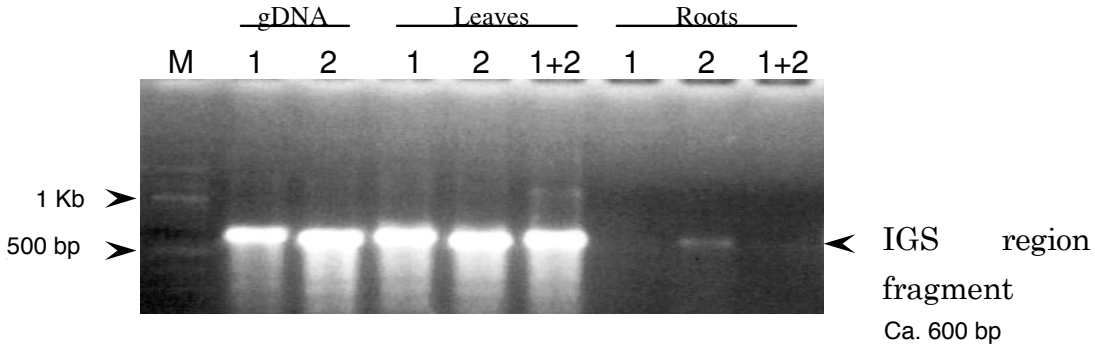

Fig. 5. PCR products with the IGS region primers using as templates DNA extracted from the roots and leaves of tomato cv. Momotaro 24 days after inoculation. Lanes, M, 100-bp DNA marker; gDNA, fungal genomic DNA extracted from *Fusarium oxysporum* f. sp. *lycopersici* race 1, and race 2 was used as a template; roots, total DNA extracted from tomato cv. Momotaro roots inoculated singly with race 1, and with race 2, and jointly with races 1+2, was used as a template; leaves, total DNA extracted from tomato cv. Momotaro roots inoculated singly with race 1, and with race 2, and jointly with races 1+2 was used as a template.

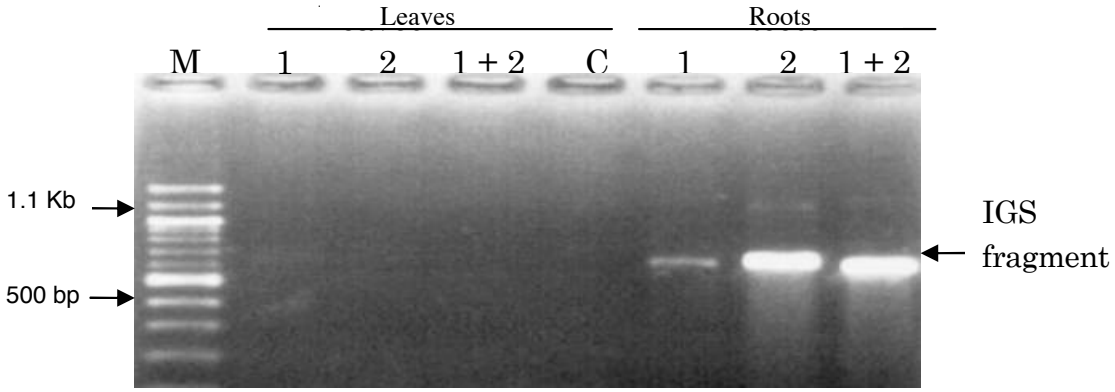

Fig. 6. PCR products with the IGS region primers using as templates DNA extracted from the roots and leaves of tomato cv. Ponderosa 24 days after inoculation. Lanes: M, 100-bp DNA marker; leaves, total DNA extracted from tomato cv. Ponderosa roots inoculated singly with race 1, and with race 2, and jointly with races 1+2 was used as a template; roots, total DNA extracted from tomato cv. Ponderosa roots inoculated with water (C), and singly with race 1, and with race 2, and jointly with races 1+2 was used as a template.

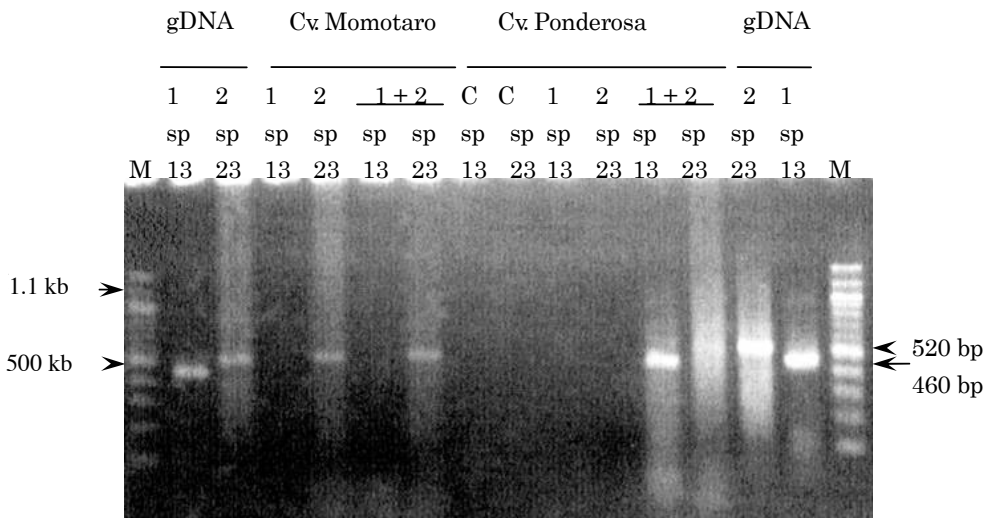

Fig. 7. Detection of *Fusarium oxysporum* f. sp. *lycopersici* race 1 and 2 from tomato (cv. Momotaro and cv. Ponderosa) leaf tissues 16 days after inoculation.
Lanes, M, 100-bp DNA marker; gDNA, fungal genomic DNA extracted from *Fusarium oxysporum* f. sp. *lycopersici* race 1 and race 2 was used as a template; cv. Momotaro, total DNA extracted from the leaves of tomato cv. Momotaro inoculated singly with race 1, and with race 2, and jointly with races 1+2 was used as a template; cv. Ponderosa, total DNA extracted from the leaves of tomato cv. Ponderosa inoculated with water (C), and singly with race 1, and with race 2, and jointly with races 1+2 was used as a template; sp13, the primer set sp13 amplifies a ca. 460-bp fragment specifically from race 1; sp23, the primer set sp23 amplifies a ca. 520-bp fragment specifically from race 2.

However, no amplification was obtained with the sp13 primer set confirming that only race 2 occurred in the leaf tissues, while race 1 was absent. This is an indication of the lack of synergism between races 1 and 2 in the cv. Momotaro.

Leaf samples from the cv. Ponderosa inoculated with races 1+2 generated strong signals with sp13, and a weak, rather diffuse signal with sp23. No amplicons were generated by leaf samples from plants inoculated with only one race. This revealed that the fragment of the IGS region in the samples was more from race 1 than race 2. However, the fact that the amplicons were detected in plants with mixed inoculation before they appeared in plants with a single-race inoculation suggests that there was a synergistic relationship between race 1 and race 2 in the cv. Ponderosa.

Detection after wilt symptom manifestation at 24 DPI.

Figure 8 shows the amplification signals of PCR products using the total DNA from samples obtained 24 DPI.. The amplification clearly showed that race 1 was present in amplifiable quantities in the roots of the cv. Momotaro inoculated with only race 1, and with races 1+2. In the leaf samples, however, as with the IGS region fragment, race 1 was not detected. Figure 9 indicates that in the cv. Ponderosa race 1 and race 2 were both present in root samples inoculated with race 1, or race 1+2, but that they were absent in the leaf samples, as was earlier indicated by the detection of the IGS region fragments.

Conclusions

The molecular analysis and the symptoms found in the study suggested that in the cv. Momotaro the colonization of race 1 was restricted to the roots, probably the root cortex. This restriction may be one of the mechanisms by which this tomato cultivar maintains its insensitivity to *FOL* race 1. This is in

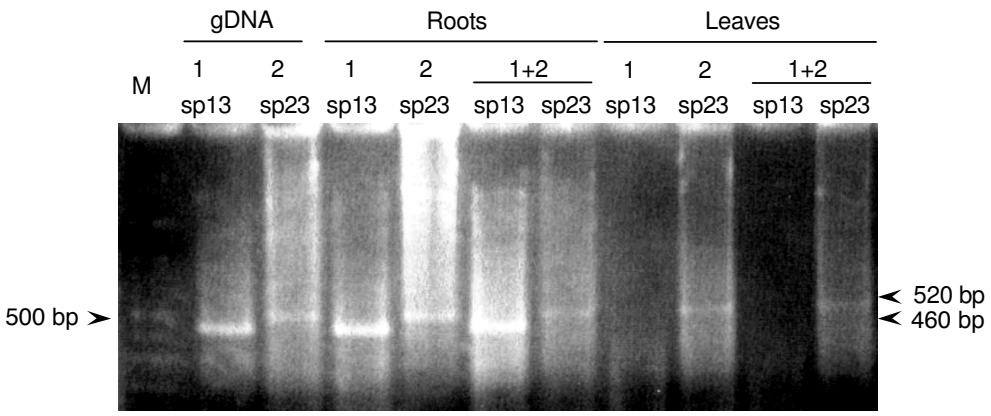

Fig. 8. Detection of *Fusarium oxysporum* f. sp. lycopersici race 1 and race 2 from tomato cv. Momotaro tissues 24 days after inoculation. Lanes: M, 100-bp DNA marker; gDNA, fungal genomic DNA extracted from *Fusarium oxysporum* f. sp. *lycopersici* race 1, and race 2 was used as a template; roots, leaves: Total DNA extracted from the roots and the leaves of the tomato cv. Momotaro inoculated singly with race 1, and with race 2, and jointly with races 1+2 was used as a template; sp13, the primer set sp13 amplifies a ca. 460-bp fragment specifically from race 1; sp23, the primer set sp23 amplifies a ca. 520-bp fragment specifically from race 2.

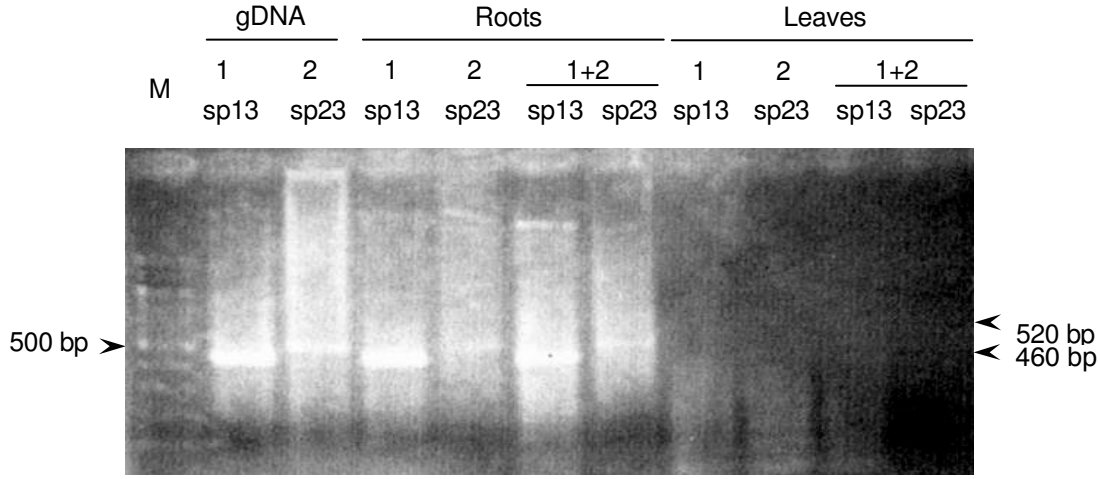

Fig. 9. Detection of *Fusarium oxysporum* f. sp. *lycopersici* race 1 and race 2 from tomato cv. Ponderosa tissues 24 days after inoculation. Lanes: M, 100-bp DNA marker; gDNA, fungal genomic DNA extracted from *Fusarium oxysporum* f. sp. *lycopersici* race 1 and race 2 was used as a template; roots, leaves: total DNA extracted from the roots and the leaves of tomato cv. Ponderosa inoculated singly with race 1, and with race 2, and jointly with races 1+ 2 was used as a template; sp13, the primer set sp13 amplifies a ca. 460-bp fragment specifically from race 1; sp23, the primer set sp23 amplifies a ca. 520-bp fragment specifically from race 2.

line with Yoshida *et al.* (personal communication) who found that in cabbage the critical point to determine the successful establishment of *F. oxysporum* f. sp. *conglutinans*, the cabbage yellows fungus was reached when the fungus penetrated from the root cortex into the vessels through the endodermis.

The different amplifications of race 1 and race 2 in the pre-symptom, race 1+2 inoculated leaf samples of the two tomato cultivars, and the later symptom patterns, indicated that there was a measure of synergism between the two races in race 1+2 inoculated plants. However, significant synergism occurred between the fungal races in the tomato pathosystem only when the host cultivar was sensitive to both races. As far as we know this is the first such report on synergistic interaction involving fungal races in tomato, although such synergism has also been reported for the interaction between potato virus X (PVX, genus *Potexvirus*) and tomato mosaic Virus (ToMV, genus *Tobamovirus*) in tomato hosts (Balogun *et al.,* 2005).

The PCR using the IGS region primers and the specific primer sets sp13 and sp23 at the pre-symptom stage of disease development, showed that random sampling of the lower leaves of tomato plants was sufficient to detect fusarial DNA, in apparently healthy plants. This method therefore enables symptom development to be predicted, especially in fields that have a history of Fusarium attacks. An added advantage of leaf sampling is that it is much less disruptive to the plant than root sampling, which may often damage or kill the plant.

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