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RESEARCH ARTICLE

Identification of *Fusarium oxysporum* f. sp. *lactucae* race 1 as the causal agent of lettuce wilt in Brazil

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ABSTRACT

The objective of this work was to assess the virulence to lettuce cultivars and host specificity and to identify races of *Fusarium oxysporum* f. sp. *lactucae* (FOLac) isolates associated with lettuce wilt in Brazil. Thirty-one isolates identified based on morphology were inoculated in a set of lettuce cultivars for race determination. Plantlets of 'Elisa', 'Vera', and 'Red Salad Bowl' cultivars were inoculated with all isolates using a root-dipping method. Isolates were also inoculated in species of Asteraceae and other botanical families. 'Elisa' and 'Vera' were susceptible to 27 isolates while 'Red Salad Bowl' was resistant to all isolates. Plants other than lettuce were not infected by any of the isolates, suggesting their specificity to lettuce. All pathogenic isolates were assigned to race 1 and used to evaluate a PCR protocol with primers targeting race 1. Only amplicons associated with race 1 pattern were observed for all pathogenic isolates but not for one non-pathogenic isolate, thus leading us to conclude that FOLac race 1 is so far the sole causal agent of lettuce wilt in Brazil.

Key words: *Lactuca sativa*, Fusarium wilt, virulence, pathotypes.

INTRODUCTION

Fusarium wilt caused by *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *lactucae* Matuo & Motohashi is one of the most economically important diseases of lettuce (*Lactuca sativa* L. - Asteraceae) around the world. Yield losses may vary from 20 to 70% depending on the cultivar, management practices and environmental conditions (Pasquali et al., 2005; 2007). First symptoms of the disease are a poor development and an overall chlorosis. As the disease progresses, older leaves may collapse leading to plant death. An intense brownish color can be seen in the vascular vessels of diseased plants after transversal stem sectioning as a result of the host reaction to the colonization and toxin production by *F. oxysporum* f. sp. *lactucae* (FOLac) (Fujinaga et al., 2001; Garibaldi et al., 2004b; Scott et al., 2010).

Fusarium wilt of lettuce has a wide geographic distribution being reported in Japan (Matuo & Motohashi, 1967), United States (Hubbard & Gerik, 1993), Taiwan (Huang & Lo, 1998), Iran (Millani et al., 1999), Italy (Garibaldi et al., 2002), and Portugal (Pasquali et al., 2007). Three FOLac races have been identified (Fujinaga et al., 2003) and Japan is the only country where all three races are present (Fujinaga et al., 2001; 2003; Yamauchi et al., 2004). Race 1 is the most widespread around the world with reports in Italy, Portugal, Taiwan, Iran, and the United States (Pasquali et al., 2005) and race 3 has been reported in Taiwan (Chang et al., 2011).

The most commonly used strategy for FOLac race determination is based upon the ability of the isolates to induce (or not) wilt symptoms in a set of differential lettuce cultivars (Ogiso et al., 2002; Fujinaga et al., 2003), which is a cumbersome and time-consuming approach. The development of PCR-based molecular markers for FOLac race determination (Pasquali et al., 2007; Mbofung & Pryor, 2010) is useful for increasing the diagnostic power and conducting large scale survey of races. However, the usefulness of FOLac race-specific PCR primers is yet to be demonstrated for distinct groups of fungal isolates from Neotropical areas of the world.

In Brazil, lettuce vascular wilt caused by *F. oxysporum* isolates was first reported in Espírito Santo State (Ventura & Costa, 2008), and later in other lettuce production areas of the country. However, a thorough understanding of the causal agent of the disease in Brazil is lacking, especially the potential presence of distinct FOLac races associated with this disease.

In this context, the objectives of this study were to 1) assess the virulence profile and host specificity of *F. oxysporum* isolates associated with lettuce vascular wilt, 2) identify FOLac races based on a set of differential lettuce cultivars and 3) assess a PCR protocol for detection and identification of FOLac race 1 isolates.

MATERIAL AND METHODS

Collection of isolates

Thirty-one *Fusarium* isolates associated with lettuce plants displaying wilt symptoms and intense brownish color of the vascular vessels were collected in seven Brazilian States (Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Rio de Janeiro, Minas Gerais, and Espírito Santo) and in the Federal District between the years of 2008 to 2012 (Table 1). The isolates were obtained from symptomatic stem segments under aseptic conditions. Affected tissues were washed with tap water for two minutes, disinfested with 70% alcohol for one minute, washed again with sterile distilled water for 30 seconds, and air-dried on the laboratory bench. Five stem segments were allocated into equidistant points within Petri dishes containing Potato Dextrose Agar medium supplemented with 20 mg of tetracycline (PDA-t). The Petri dishes were maintained in a growth chamber for 12 h dark/light cycle at 25°C for three days. Thereafter, the fungus was transferred to a distinct Petri dish containing PDA-t medium aiming to obtain pure cultures. The isolates were assigned to species based on morphological description in the literature (Nelson et al., 1983; Leslie and Summerell, 2006). The isolates were maintained in Petri dishes containing PDA and in glass vials filled with sterile-distilled water (Castellani, 1963) and deposited in the culture collection of plant pathogenic fungi "Professora Maria Menezes" – CMM (Table 1).

Inoculation procedures

Three mycelial discs (5 mm in diameter each) were obtained from five-day old cultures grown in PDA-t and placed in Erlenmeyer flasks containing 100 mL of Potato + Dextrose liquid medium. These flasks were shaken for ten days (25°C and 12h dark/light cycle) to induce microconidia production. After this treatment, the suspension was passed

TABLE 1 - Information and attributes of 31 *Fusarium oxysporum* isolates associated with lettuce (*Lactuca sativa* L.) wilt in locations across seven States and in the Federal District, Brazil.

Isolate code	Location (municipality - state)	Year	Formae speciales[1]	Pathogenicity[2]	CMM code[3]
Fus-171	Alegre - ES	2008	*lactucae*	+	CMM-3569
Fus-172	Alegre - ES	2008	*lactucae*	+	CMM-3570
Fus-173	Antônio Carlos - SC	2008	*lactucae*	+	CMM-3573
Fus-174	Antônio Carlos - SC	2008	*lactucae*	+	CMM-3584
Fus-187	Caratinga - MG	2009	*lactucae*	+	CMM-3589
Fus-202	Muriaé - MG	2009	*lactucae*	+	CMM-3574
Fus-203	Marechal Floriano - ES	2009	*lactucae*	+	CMM-3592
Fus-205	Santa Cruz do Rio Pardo - SP	2009	*lactucae*	+	CMM-3575
Fus-206	Paulínia - SP	2009	*lactucae*	+	CMM-3593
Fus-207	Campinas - SP	2010	*lactucae*	+	CMM-3576
Fus-208	Nova Friburgo - RJ	2009	*lactucae*	+	CMM-3694
Fus-209	Nova Friburgo - RJ	2009	*lactucae*	+	CMM-3577
Fus-210	Sumidouro - RJ	2009	*lactucae*	+	CMM-3595
Fus-219	Colombo - PR	2010	*lactucae*	+	CMM-3580
Fus-220	Colombo - PR	2010	*lactucae*	+	CMM-3581
Fus-221	Colombo - PR	2010	Non-pathogenic	-	_
Fus-222	Itajaí - SC	2010	*lactucae*	+	CMM-3583
Fus-223	São José dos Pinhais - PR	2010	*lactucae*	+	CMM-3596
Fus-224	São José dos Pinhais - PR	2010	Non-pathogenic	-	_
Fus-225	São José dos Pinhais - PR	2010	Non-pathogenic	-	_
Fus-227	Itajaí - SC	2010	*lactucae*	+	CMM-3597
Fus-237	Brasília - DF	2011	Non-pathogenic	-	_
Fus-240	Pelotas - RS	2011	*lactucae*	+	CMM-3600
Fus-241	Santa Cruz do Rio Pardo - SP	2011	*lactucae*	+	CMM-3601
Fus-242	Santa Cruz do Rio Pardo - SP	2011	*lactucae*	+	CMM-3602
Fus-243	Santa Cruz do Rio Pardo - SP	2011	*lactucae*	+	CMM-3603
Fus-244	Marechal Floriano - ES	2011	*lactucae*	+	CMM-3605
Fus-252	Santa Cruz do Rio Pardo - SP	2011	*lactucae*	+	CMM-3606
Fus-253	Marechal Floriano - ES	2011	*lactucae*	+	CMM-3655
Fus-254	Três Pontas - MG	2012	*lactucae*	+	CMM-3656
Fus-255	Três Pontas - MG	2012	*lactucae*	+	CMM-3657

[1]isolates were assigned to a *formae speciales* based on pathogenicity and molecular assays.
[2]Pathogenicity assays were conducted using three lettuce cultivars. (+) = isolate able to induce disease; (-) = no-pathogenic isolate
[3]Code of the pathogenic isolates deposited in the culture collection of plant pathogenic fungi "Professora Maria Menezes" – CMM.

through a double-layer gauze filter in order to remove mycelium fragments. Spore concentration was determined using an hemocytometer and adjusted to a concentration of 2 *x* 10^6 microconidia/mL. All 31 isolates associated with lettuce wilt were employed in the pathogenicity tests using three commonly grown lettuce cultivars ('Elisa', 'Vera' and 'Red Salad Bowl'). The seedlings were produced in polystyrene trays (68 cm *x* 34 cm) with 128 cells filled with commercial solid substrate (Bioplant®; Nova Ponte, MG). The trays were kept in a greenhouse free of pest and pathogen infestation with daily irrigation until transplanting and without pesticide applications. Inoculation was performed in 25-day old seedlings by dipping the apical region of their roots (cut approximately 2 cm from the top by sterile scissors) in 50 mL of a conidial suspension for three minutes. Immediately after inoculation, the seedlings were transplanted individually to 1L plastic pots containing sterilized soil. After that, 3 mL of the conidial suspension were added to the collar area of each seedling. The pots were irrigated two hours prior to inoculation until soil saturation (Santos, 1999).

Host range experiment

The isolates were inoculated in a group of six species from the Asteraceae family including lettuce (cv. Elisa), *Cichorium endivia* L., *Cichorium intybus* L., *Sonchus oleraceus* L., *Emilia sonchifolia* L., *Bidens pilosa* L. and *Tagetes erecta* L. as well as several species from other botanic families such as Solanaceae (*Solanum lycopersicum* L., *Capsicum annuum* L., *Nicotiana tabacum* L.), Malvaceae (*Gossypium hirsutum* L.), Fabaceae (*Phaseolus vulgaris* L.), and Lamiaceae (*Ocimum basilicum* L.). Inoculation was made using the root dipping method of the wounded root system essentially as described. Mock-inoculated plants of each species were kept in the same greenhouse conditions to serve as controls. Evaluation was done at 20 to 25 days after inoculation, initially by visual analysis of leaf chlorosis, overall wilt, and vascular browning.

Race determination based on a differential set of lettuce cultivars

The isolates were inoculated in a set of cultivars comprising: 'Patriot' (susceptible to all races), 'Costa Rica No. 4' (resistance specific to race 1) and 'Banchu Red Fire' (resistance specific to race 2). The seedlings were produced in polystyrene trays (68 cm *x* 34 cm) with 128 cells filled with commercial solid substrate (Bioplant®; Nova Ponte, MG). The seedlings were removed from the substrate at 25 days after sowing. Inoculation production and inoculation procedures were essentially as described previously. Conidial suspension was adjusted to 2 *x* 10^6 microconidia. mL^{-1}. The evaluation was done 20 days after inoculation and the plant reaction was assessed using a disease severity ordinal and descriptive scale ranging from 1 to 5 (Santos, 1999). The isolates were classified as pathogenic/virulent to a given cultivar when the average disease severity grade was above score 2 of the scale (plants without vascular wilt and chlorosis symptoms but with vascular browning; score 1: plant without symptoms). Isolates unable to induce disease in all three cultivars were classified as non-pathogenic or avirulent. Race 1 isolates are pathogenic/virulent only to the cultivars 'Patriot' and 'Banchu Red Fire', whereas race 2 isolates are pathogenic only to 'Patriot' and 'Costa Rica No. 4'. Isolates able to induce disease in all three accessions would represent race 3, which has been reported only in Japan and Taiwan (Chang et al. 2011).

Molecular FOLac race identification

Twenty-seven isolates characterized as being FOLac race 1 based on the inoculation assays in the set of differentials were tested in additional molecular race phenotyping assays. One isolate of *F. oxysporum* f. sp. *lycopersici* (FOL) race 3 (named Fus-191) and one non-pathogenic *F. oxysporum* isolate (Fus-237) were also included. Fungal genomic DNA was extracted using the CTAB protocol with minor modifications (Boiteux et al., 1999). PCR reaction was carried out using the primer par Hani3' (5'CCC-TCC-AAC-ATT-CAA-CAA-CTG3') and Hanilatt3rev (5'ATT-CAC-TGT-ACA-CCA-ACC-TTT-T3'), which were designed for specific discrimination of FOLac race 1 isolates (Paquali et al, 2007). PCR reaction was composed of 3 µL of fungal genomic DNA (20 ng/mL), 2 µL 10X buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 0.48 µL $MgCl_2$ (50 mM), 2 µL dNTPs (2.5M each), 0.2 µL *Taq* DNA polymerase (5 units/µL), 3 µL of each primer and 6.32 µL Milli-Q water, with a final reaction volume of 20 µL. Samples were amplified in one My Cycler™ (BIO RAD) thermocycler. The amplification program employed was carried out as follows: initial cycle (94ºC /1min), ten denaturation cycles (94ºC for 15 seconds), annealing at 66ºC (30 s). The annealing temperature was reduced by 0.5ºC after each cycle, and then the program was shifted to 30 denaturation cycles (94ºC for 15 seconds), 61ºC annealing temperature (30 seconds) and extension of 72ºC (30 seconds). A final extension cycle was carried out at 72ºC for 2 minutes. The introduction of these final 30 cycles was a modification in relation to the original protocol described by Pasquali et al. (2007). Amplicons were analyzed in agarose gel (1% p/v) eletrophoresis, stained with ethidium bromide and visualized under UV light.

RESULTS AND DISCUSSION

The thirty-one purified *Fusarium* isolates showed the typical morphological features of the species *F. oxysporum* (Leslie & Summerell, 2006). Twenty-seven isolates were able to infect 'Elisa' and 'Vera' cultivars and were re-isolated from the diseased plants, thus fulfilling Koch's postulates. Four isolates were non-pathogenic to this subset of lettuce cultivars, including the one from the Federal District (Fus-237). The 'Red Salad Bowl' cultivar exhibited a resistant reaction against all isolates. Taken together, these results

allowed us to assign all pathogenic isolates to *F. oxysporum* f. sp. *lactucae*. The inoculation-based assay is so far the only available method for distinguishing saprophytic (non-pathogenic) isolates from pathogenic *F. oxysporum* isolates, since they are quite similar in terms of cultural and morphological traits (Booth, 1971; Burgess et al., 1994).

Additionally, the 27 pathogenic isolates were not able to induce wilt symptoms in the set of Asteraceae species as well in host species of other botanical families tested in our study, thus suggesting the specificity of these isolates to lettuce. Previously, Ventura & Costa (2008) described the causal agent of lettuce wilt disease in Brazil only to the species level (*F. oxysporum*).

The reaction of the race differentials to the set of pathogenic isolates was quite similar. 'Patriot' and 'Banchu Red Fire' were highly susceptible to all isolates (but not to the four nonpathogenic isolates), whereas 'Costa Rica No. 4' was found to be resistant, indicating so far the exclusive presence of FOLac race 1 in Brazil, which has been also found in the United States, Japan, Italy, Portugal, Taiwan and Iran (Fujinaga et al., 2001; McCreight et al., 2003; 2005; Garibaldi et al., 2004b; Pasquali et al., 2007).

Amplicons corresponding to the region between the clusters of class II transposons (Hua-Van et al., 2000) were amplified via PCR assays for the 27 pathogenic isolates. These amplicons were not present in the control FOL race 3 (isolate Fus. 191) and in one non-pathogenic *F. oxysporum* (Fus. 237) isolate (Figure 1). The size of the amplicon was approximately 200 bp. This same primer pair was employed by Pasquali et al. (2007) for specific detection of FOLac race 1 using a collection of race 1 isolates from Italy, United States (States of Arizona and California), Portugal, Taiwan, and Japan. In fact, these primers have been previously used as powerful diagnostic tools for FOLac race 1 isolates, but they were not evaluated with isolates from Neotropical areas of the world. All the results combined suggest that all 27 pathogenic isolates belong to FOLac race 1.

Molecular techniques are important tools to discriminate fungal species with intimate genetic relationships where few morphological diagnostic traits are available (Wuff et al., 2010) as well as to differentiate isolates within the same species (Chandra-Nayaka et al., 2010). In *Fusarium*, the identification to species based exclusively upon morphological information is challenging. This genus is especially difficult to fungal taxonomists because either the lack of informative traits or the phenotypic instability of several traits, which expression might be affected by *in vitro* and *in vivo* conditions (Fravel et al., 2003). Our study showed that the PCR primer pair Hani and Hanilatt3rev (Hua-Van et al., 2000) might reliably be used to discriminate FOLac race 1 isolates from Brazil. Robust PCR assays as this one able to differentiate isolates at their *formae speciales* and race levels are useful for large-scale surveys of fungal isolates.

It is well known from the literature that distinct *formae speciales* of *F. oxysporum* are very efficiently seed-transmitted. In fact, the spread of FOLac race 1 isolates into several countries of distinct continents in a short period of time suggests its spread via infested seed, although this has not been conclusively documented (Garibaldi et al., 2004a; Mbofung & Pryor, 2010). Likewise, the presence of FOLac race 1 in several and distant lettuce production regions of Brazil suggests that this pathogen may have been introduced into the country via infected seeds. Further molecular phylogenetic analysis of the Brazilian isolates might allow assessing whether these isolates are either endemic or introduced into the country from abroad.

The control of *Fusarium* wilt of lettuce via fungicide and soil desinfestation treatments is usually not economically and ecologically feasible. An effective

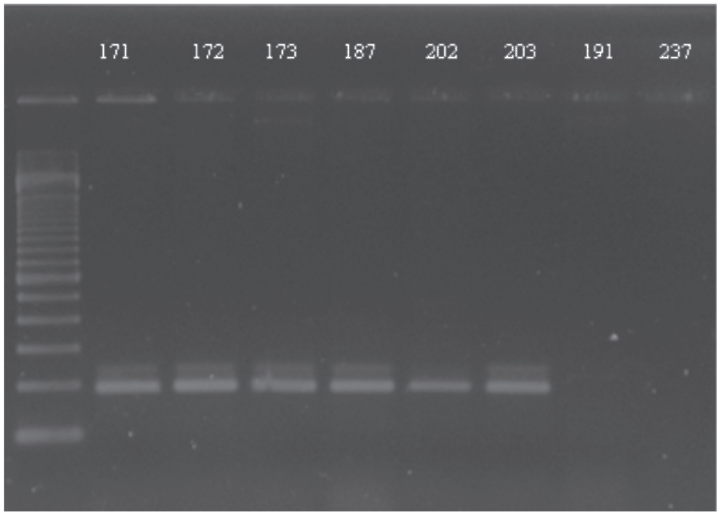

FIGURE 1 - PCR amplicons of around 200 bp obtained with the *Fusarium oxysporum* f. sp. *lactucae* (FOLac) race 1-specific prime pair Hani (5' GAA-CCC-TCC-AAC-ATT-CAA-CA 3') and Hanilatt3rev (5' CCT-CCA-ACA-TTC-AAC-AAC-AAT-G 3'). The lanes 171, 172, 173, 187, 202, and 203 are PCR the amplicons obtained with genomic DNA extracted from six distinct isolates that were pathogenic to lettuce. The lane 191 corresponds to the results obtained with the same prime pair using genomic DNA extracted from one isolate of *F. oxysporum* f. sp. *lycopersici* (FOL) race 3 and the lane 237 corresponds to the results obtained with one *F. oxysporum* isolate non-pathogenic to lettuce. M = Size marker 1 Kb Plus DNA Ladder® (Invitrogen).

disease management has been achieved using pathogen-free propagative (seed) material, crop rotation and resistant cultivars (McCreight et al., 2003; 2005). Information about the genetic variability of the pathogen is important when breeding for disease resistance and the identification of a single race of the FOLac present in Brazil provides useful information for lettuce breeding programs in the country. This will help to develop resistant cultivars using either 'Red Salad Bowl' or 'Costa Rica No. 4', as well as other already selected commercial cultivars (Cabral & Reis, 2013), as sources of resistance alleles. The information is also important to growers and extension service, guiding them to select resistant cultivars in at least seven Brazilian States. The ability of the fungus to transmit through infected seeds and the high yield losses imposed by Fusarium wilt also indicates the need for preemptive breeding programs aiming to pyramid distinct race-specific genetic factors into a single cultivar even for races not yet present in Brazil.

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