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Standard methods for inoculations of *F. oxysporum* and *F. solani* in Passiflora

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Soil fungi, *Fusarium oxysporum* FO and *F. solani* FS (teleomorph: *Nectria hematococca*), are pathogens of economic importance passion fruit crops. The present work was developed in order to standardize the methodology of inoculation, as an initial step to confirm the etiology of diseases associated with Fusarium wilt and collar rot. Strains of FO for A14, A16, A22, A27, A29, A32, A34, A48, A54, A64 and FS A11, A23, A62, A63 were used; they were obtained from symptomatic crops of *P. edulis*. Inoculations were carried with and without wounds, on seedlings of two and four months of *P. edulis*. To assess incidence and severity, a scale designed for symptoms and growth variables was used. An incubation period of 14 to 19 days for FO, and was found highly virulent strains (A54, A64, A34). The symptoms are characterized by vascular wilt corresponded to a pattern of descending necrosis. Cross sections showed discoloration in vascular vessels and roots showed necrotic processes that lead to delayed development of seedlings. FS causes disease but the evolution in most strains is very low and exceeds 100 days. Wounds are further evidence for the fungus required in the plant tissue. Symptoms are manifested in the collar area with redness, mild canker associated with cracking and dry appearance on the injury.

Key words: Pathogenicity, collar rot, Fusarium wilt, passion flower, *Passiflora edulis*, Koch´s postulates.

INTRODUCTION

Fusarium Link 1809 is a genus that includes important plant pathogens, and some species are mycotoxin producers associated with human and animal health hazards. The fungi can attach to human, animal and plant tissues (Oechsler et al., 2013; Eldridge et al., 2014; Salter et al., 2012; Sarmiento-Ramírez et al., 2014; Kirkpatrick et al., 2013). Exhaustive *Fusarium* studies have been conducted in many fields, such as molecular biology, ecology, phytopathology, medical mycology, toxicology, and others (Torching and Mitchell, 2004;

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Watanabe et al., 2011; Zhang et al., 2006). The genus, *Fusarium*, also known by its teleomorphs, *Nectria* and *Gibberella*, comprises plant pathogenic fungi with a wide variety of hosts and infection strategies (Michielse and Rep, 2009).

Fusarium sp., a plant pathogen of Passifloraceae, *F. oxysporum* f. sp. *passiflorae,* is the agent of *Fusarium* wilt in *Passiflora edulis* (McKnight, 1951), *P. mollissima* (Gardner, 1989), *Passiflora edulis flavicarpa* X *P.edulis* (Ploetz 1991, Ploetz, 2003), *Passiflora* spp (Fischer and Rezende, 2008). Meanwhile *F. solani* is reported as the causal agent of collar rot in *P. edulis* f. *edulis* Sims (Cole et al., 1992), *P. edulis* f. *flavicarpa* (Ponte, 1993; Fischer et al, 2005), *P. ligularis* and *Passiflora* spp. (Ploetz, 2006; Fischer and Rezende, 2008). One of the difficulties in studying interactions of plant-*Fusarium* is that the taxonomy does not determine its pathogenicity; so there is need to conduct pathogenicity tests or Koch's postulates. A common example is *Fusarium oxsyporum* that differs in symptomatology, epidemiology and susceptibility of cultivars and can be distinguished by pathogenicity tests with suitable hosts (Vakalounakis and Fragkiadakis, 1999).

In addition to confirm the etiology of disease, pathogenicity tests also determine the pathogenic variability of a causal agent and assess potential sources of resistance. In *Fusarium* plant pathogens it is possible carry out Koch´s postulates; four steps are adapted to plant pathology in microorganisms that can grow in axenic media: i) The microorganism must be found in large numbers in all diseased plants, but not in healthy ones. ii) The organism must be isolated from a diseased plant and grown outside the body in a pure culture. iii) When the isolated microorganism is “injected” into other healthy plants, it must produce the same disease. iv) The suspected microorganism must be recovered from the experimental hosts v), isolated, compared to the first microorganism, and found to be identical (Kaufmann and Schaible, 2005).

Considering the third step of these principles, the "injection" of the pathogen refers to the way of inoculating the microorganism on its potential host; the route of entry determines the subsequent results, and that is why it is necessary to revise the technique of inoculation into the host tissue. Correct diagnosis of diseases can be reached through determination of specific factor that predominates other causal factors (Wallace, 1978). The ability of a factor to produce disease may depend on the earlier influence of another determinant which itself makes little direct contribution to disease, and inoculation is one of those. Inoculation must be as similar as possible to what occurs in natural inoculations.

With Koch's postulates it is possible to define the infective cycle of a pathogen, through the incubation period defined as time between infection and disease symptom expression in host and latency is the period between infection of host and production of inoculum (De Wolf and Isard, 2007). Fungus in *Fusarium* genus, produces three types of asexual spores: macroconidia produce sporodochia on the surface of infected plants parts; microconidia occur on aerial mycelium. Both macroconidia and microconidia may also be formed in the xylem vessel elements of infected hosts plants, but microconidia are usually the predominant type in infected plant tissue (Nelson, 1981). Those spores can be produced simultaneously to symptom expression in passion fruit plants (Ortiz et al., 2014), so those periods can lead to outlining of the relevance of control measures, and epidemiological tools (Kranz, 2012). Third spore are chlamydospores, formed in axenic culture and dead host plant tissue, in the final stages of wild-disease development. These spores survive for an extended time in plant debris in soil in the absence of a suitable host plant, and chlamydospores are the primary soil borne propagule of *F. oxysporum* (Bennett and Davis, 2013).

This research aims to standardize tests of pathogenicity of *F. oxysporum,* causal agent of *Fusarium* wilt and *F. solani* agent of collar rot on *Passiflora edulis*, which will allow experiments in physiology of host-pathogen interactions, resistant materials testing, pathogen suppression methods, among others.

MATERIALS AND METHODS

Pathogenicity tests on *P. edulis*

We used commercial seedlings of *P. edulis*, analyzed to exclude plant pathogens. Pathogenicity tests were carried out under greenhouse conditions with average temperature of 25 ° C and average relative humidity of 70%. In order to produce inoculum to use in these tests, isolates previously identified as *F. oxysporum* corresponded to A14, A16, A22, A27, A29, A32, A34, A48, A54 and A64 and *F. solani* A11, A23, A62, A63. For all tests a completely randomized design was applied with 10 replicates per treatment, except for the pathogenicity tests on nine month old plants with 5 replicates per treatment. Statistical analyses were performed using Kruskal–Wallis one-way analysis of variance (nonparametric data) SAS software, version 6.1.

F. oxysporum causal agent of Fusarium wilt

The *F. oxysporum* isolates A27, A32 and A32 were grown in liquid medium malt extract, according to the formulation indicated by Pancreac, 2003, with a modification consistent on agar remotion. A 250 mL Erlenmeyer flask was inoculated with 3 discs with young mycelium (5 days), and then prepared a conidial suspension at a concentration of 1.10 6 UFC mL-1. The incubation conditions were temperature of 25 ° C with stirring in shaker at 125 rpm under absence of light.

To simulate natural inoculations were proven two ways to impregnate plant roots with pathogen:

Immersion of roots without wound: Forty five days old seedlings, with two true leaves, were immersed in a conidial suspension for two minutes (Gardner, 1989; Vakalouonakis, 1996). Inoculated volume by plant was 15 mL with the methodology described by Ortiz et al. (2012). Immediately after inoculation, were planted seedlings in sterile peat with nutrients, previously saturated with

Table 1. Ordinal scale used to assess the severity of Fusarium wilt in *P. edulis**.

Description	Class
No symptoms	0
Light to moderate wilting, chlorosis.	1
Severe wilt with stem discoloration, defoliation.	2
Seedling death	3

Modified from Vakalounakis et al. (2005).

Table 2. Treatments for testing pathogenicity of *F. solani/ N. hematococca* isolates in seedling of *P. edulis.*

Treatment	Stage	Strain	Method
T1	*F. solani*	A11	Root immersion in 1.10 6 UFC mL-1
T2	*F. solani*	A23	Root immersion in 1.10 6 UFC mL-1
T3	*F. solani*	A62	Root immersion in 1.10 6 UFC mL-1
T4	*F. solani*	A63	Root immersion in 1.10 6 UFC mL-1
T5	Does not apply	Does not apply	Root immersion of malt extract broth
T6	*N. hematococca*	A11	Direct contact with mycelium plug, in the collar.
T7	*N. hematococca*	A11	Direct contact, with mycelia and perithecia plug, in the collar.
T8	*N. hematococca*	A23	Direct contact with mycelium plug, in the collar.
T9	*N. hematococca*	A62	Direct contact with mycelium plug, in the collar.
T10	*N. hematococca*	A62	Direct contact with mycelia and perithecia plug in the collar.
T11	*N. hematococca*	A63	Direct contact with mycelium plug in the collar.
T12	*N. hematococca*	A63	Direct contact with mycelia and perithecia plug in the collar.
T13	Does not apply	Does not apply	Direct contact with a non-colonized plug in the collar.

water. As a negative control, an equivalent volume of medium malt extract in plants was spread.

***Inmersion of roots with wound*:** The technique is similar to the above; the only difference was that about 0.5 cm of the end portion of the root system was removed (Haglund, 1989, modified). After identifying the most appropriate inoculation methodology, we proceeded to confirm reproducibility through a screening test of more virulent isolates, which is described hereunder.

Screening of more virulent isolates

F. oxysporum isolates A14, A16, A22, A29, A34, A48, A54 and A64, were evaluated in two months old plants. The isolate A54 was used as positive control since it was the most virulent in the standardization of the methodology of inoculation. After inoculation, all plants were kept in a tunnel with plastic cover under greenhouse conditions, with environment temperature and humidity mentioned above. The assessed variables were: incubation period, incidence, number of leaves and plant height (weekly), one month follow-up, and severity, using the scale of Vakalounakis et al. (2005), modified (Table 1). The characterized symptoms and fourth Koch´s postulate were verified.

F. solani causal agent of collar rot

Inoculation without wound: To analyze if a wound is needed to have infection of *F. solani* through the root system and collar in *P. edulis,* two months old plants were evaluated with treatments shown in Table 2. Treatments T1 to T5 were performed by immersion of roots without wound, following the same protocol as described for *F. oxysporum*. Treatments T6 to T12 consisted in direct contact disc 0.7 mm in diameter with fungal growth, located over collar plant, without wound. For anamorphic stages (*F. solani*) 5 days mycelium grown on PDA was inoculated, and for teleomorphic stages (*Nectria haematococca*) mycelium with perithecia grown on agar V-8.

Inoculation with wound: Four months old plants grown in sterile soil were inoculated by direct contact of mycelial disks in the collar area using a modification of the methodology described by Ploetz (1991) and Fischer et al. (2005). Cultures of *F. solani* A11, A23, A62 and A63 grown in PDA medium, incubated for five days at 25 ° C, were cut into discs about 10 mm in diameter. These plugs were located over a small incision on the collar plant, to which previously added 1 mL of sterile water in order to facilitate adhesion. On controls were added clean PDA discs of plants.

In order to verify the reproducibility of the inoculation method with wounds, pathogenicity tests were conducted in four months old plants grown in sterile soil. All plants were kept in a tunnel with plastic cover under greenhouse conditions for 9 months, with environment temperature and humidity mentioned above.

RESULTS

F. oxysporum

Pathogenicity tests indicated an incubation period of 18

Table 3. Incidence and severity at 20 days posterior inoculation (dpi), of *P. edulis* seedlings inoculated with *F. oxysporum* under greenhouse conditions.

Treatment		Incidence (%)	Severity* (index)
1	A27 - immersion without wound in roots	40	0.4
2	A27- immersion + wound in roots	60	0.7
3	A32- immersion without wound in roots	60	0.7
4	A32- immersion + wound in roots	60	0.7
5	A54- immersion without wound in roots	80	0.8
6	A54- immersion + wound in roots	90	0.9
7	Absolute control	0	0
8	Relative Control (non-inoculated + wound in roots)	0	0
	Chi square	66.996	50.839
	Pr > Chi square	0.244	0.4057

Table 4. *P. edulis* seedling inoculated with *F. oxysporum* isolates: Incidence and severity.

Treatment		Incidence	(%)	Severity	Index
		Mean	Group	Mean	group
1	*F. oxysporum* A14	25.5	c	23.5	c
2	*F. oxysporum* A16	52.15	b	61	ab
3	*F. oxysporum* A22	25.5	c	23.5	c
4	*F. oxysporum* A29	56.9	ab	56.5	b
5	*F. oxysporum* A34*	64.05	ab	62.2	ab
6	*F. oxysporum* A48	25.5	c	23.5	c
7	*F. oxysporum* A54*	68.3	a	69.55	a
8	*F. oxysporum* A64*	66.1	a	66.25	ab
9	Control	25.5	c	23.5	c
	Chi square	54.567		62.781	
	p-value	5.36E-06		1.32E-07	

Incidence and severity accumulated during test analyzed by means of Kruskal-Wallis test; (*) the most virulent isolates, early symptoms.

to 19 days, symptoms of mild chlorosis associated with slight to moderate wilt. When comparing methods of inoculation no statistically significant differences were found at 20 dpi; however, the incidence and severity tended to be higher causing wound in the root (Table 3).

Symptoms in infected plants corresponded to *Fusarium* wilt, displaying progression in severity scale used (Figure 1), with an index of severity ranging from 0.1 to 1.0 from 19 to 21 dpi, 1.1 to 2.0 of 22 24 dpi and 2.1 to 3.0 of 25-30 dpi.

Incubation period for two months old plants was 14 days. Isolates of *F. oxysporum* A54, A64 and A34, showed statistically significant differences analyzed by means of Kruskal-Wallis test, with higher incidence values (50 to 80%) and severity (0.5 to 0.8) (Table 4). Additionally, for these isolates the collapse of seedlings was early, at 24 dpi. Meanwhile, the least virulent isolates showed at 14 dpi low incidence values (20-30%) and severity (0.2 to 0.3) and, beginning the collapse of the plant 28 to 30 dpi. At the end of the trial (28 dpi), the incidence was similar for *F. oxysporum* A16, A29, A34, A54 and A64 (90-100%) isolates; however, *F. oxysporum* A34, A54 and A64 isolates reveal an increased severity index (2.2 - 2.7) and lower height of plants (3.2 - 4.0 cm). From ten isolates tested, three were to be non-pathogenic (A14, A22 and A48), showing statistically similar to the control values in variables assessed (Table 4). Regarding number of leaves, analysis showed significant differences ($P<0.00324$) but control was included in two groups formed by Tukey test; therefore, it shows variability in plant species, *P. edulis*, but is not effect of pathogens. Symptoms characterized vascular wilt corresponded to a pattern of descending necrosis, cross sections showed discoloration in vascular vessels and roots showed necrotic processes that led to delayed development of seedlings. From these lesions was obtained *F. oxysporum*, a 60-80% frequency confirmed the fourth Koch´s postulate. In transversal section of

Figure 1. Progression of symptoms caused by *F. oxysporum* in *P. edulis* seedlings, according to the scale of Vakalounakis *et al.,* 2005 modified. A. no presence of symptoms (0-18 dpi), B. mild to moderate wilting and chlorosis (19-21 dpi), C. severe wilt with stem discoloration and defoliation (22-24 dpi), D. death of seedlings (25-30 dpi).

stem, discoloration was observed in the vascular vessels. The results indicated that this pathogen does not require wounds to cause infection.

F. solani

This fungus is less aggressive than *F. oxysporum* in terms of incidence, during the time of evaluation. Two months old plants of *P. edulis* inoculated with *F. solani* strains without wound, showed an incubation period of 108 dpi for two plants: 1 for the treatment 2 (*F. solani* A23) and the other for treatment 12 (*N. hematococca* A63). The symptoms manifested in the collar area were redness, mild canker associated with cracking and dry appearance on the injury. The progress of the lesion showed a non-uniform pattern across the collar with 1.2 cm long x 1.9 cm wide at 120 dpi and 2.2 cm long x 3 cm wide at 128 dpi, for treatments 2 and 12 respectively.

As for the aerial part of plants, severe chlorosis in the lower leaves appeared. At 180 dpi, two additional plants of treatment 12 (N. haematococca A63) showed in the collar zone a slight reddish canker of dry appearance associated to the presence of crazing. The cankers length range was 1.1 to 1.3 cm; plants showed slight chlorosis of lower leaves. In these treatments, at 245 dpi cross sections of the collar revealed chancre with progress towards the pith.

At 210 dpi 2 plants, from treatment 1 (*F. solani* A11) and 4 (*F. solani* A63), exhibit browning color in the collar area, this lesion presented a fast advancing, leading to rot in the collar and necrosis in the stem to 5-6 cm height up, at 240 dpi occur wilting and death of plants. Not teleomorph stages were observed in any treatment. Table 5 summarizes the results of incidence and mortality rate of the test.

Inoculations in fourth month old plants with wound showed symptoms in one plant inoculated with *F. solani* A62 with an incubation period of 47 dpi. Expressed changes included chlorosis primarily in lower leaves, stunted growth, general decay, posteriorly a reddish brown canker in the collar caused constriction and rot to +/- 2 cm of root. At 50 dpi, numerous reddish perithecia on the lesion could be observed (Figure 2) and at 54 dpi started a defoliation. At 90 dpi, cross section of the stem showed discoloration of vascular bundles after verifying the fourth Koch postulate, it was confirmed that *F. solani* is the agent of collar rot in *P. edulis*.

DISCUSSION

External symptoms of wilting consist of an incipient chlorosis of lower leaves, followed by a permanent wilting of these leaves; symptoms gradually move up the plant. Sometimes, they can occur on one side of the plant. Used scale is optimum to assess wilting evolution in *P. edulis* seedlings, because it has few levels, and clearly detailed. Besides in practice test is easy to follow and analyze.

The inoculation of *P. edulis* with *F. oxysporum* shows that the pathogen does not require wounds to cause disease although wounds, injuries or senescence are predisposing factor to *Fusarium* wilting. Some authors state that wounding enhanced *Fusarium* invasion and establishment (Rekah et al., 2000; Kang and Buchenauer, 2000; Sakamoto and Gordon, 2006; Szczechura et al., 2013).

Pathogenicity screening of *F. oxsyporum* allowed detection of the corresponding A54, A64 and A34, as virulent isolates. All strains evaluated showed similar incubation periods 14 days posterior inoculation, but the most virulent isolates showed during the tests higher values of incidence and severity. This suggests that these attributes are reliable and practical for the rapid detection of pathogenic isolates. Number of leaves was

Figure 2. Symptoms of collar rot caused by *F. solani* A 62 in *P. edulis* at 50 dpi. A. canker with perithecia formation on collar plant, B. collar control plant, C. general chlorosis and wilting in aerial organs, D. control plant.

no significant at the beginning of the experiment differences; however at the end of the tests there was noticeable reduction in the number of sheets, which explains defoliation by the process generated by the pathogen.

The occurrence of non-pathogenic isolates (A14, A22 and A48) shows that the presence of *F. oxysporum* does not necessarily imply pathogenicity thereof on the host plant. This behavior may be due to variability pathogenic mechanisms or lack of pathogenicity for the host in question. O'Donnell et al. (2009) mentioned that although there have been non-pathogenic strains, the null hypothesis that some isolates are nonpathogenic is virtually impossible given the large number of potential host plants and no plants as proved in *P. edulis*. Sáenz (2011)'s *personal communication* demonstrated that strains *F. oxysporum* A34 and A54 inoculated in peas (*Pisum sativum*) and beans (*Phaseolus vulgaris*) do not exhibit symptoms, although the fungus can survive and stay in these species without causing disease.

It was proved by indexing, suggesting that they are avirulent fungal hosts. *F. oxysporum* f. sp. *passiflorae* is not mentioned in this paper, since test has demonstrated that *F. oxysporum* A54 is not specific to Passiflora, attacking carnation *Dianthus cariophyllus* (Maldonado *et al*., 2015) and tomato (*Solanum sculentum*) (Rozero et al., 2015).

Pathogenicity tests with *F. solani* revealed that all isolates are pathogenic, causing symptoms ranging in severity depending on the type of inoculation and the age of the plants. But, death occurred in plants inoculated by direct contact of mycelium on injury induced collar (A62) or dipping roots without induced injury (A11, A62), suggesting that presence of wound, in collar tissues or the points of lateral root formation, plays an important role in the development of this disease. In case of *F. solani* A11 and A62, there were no wounds, but Cole et al. (1992) and Fischer et al. (2005) reported that plant transplantation inevitably leads to damage to roots and stem injuries, increased susceptibility to *Fusarium* in plants. Ploetz (1991), who in pathogenicity tests with *N. haematococca* on *P. edulis X P. edulis F. flavicarpa*, establish that only plants inoculated with wound collapsed, made similar observations to those found with *F. solani* A62.

Inoculated plants through direct contact on collar tissue, only display symptoms with A63 teleomorph stage *N. haematococca*, without causing death of the plant. This pathogen can cause infection without the presence of an induced wound; however, under these conditions the plant is able to generate defense mechanisms that counteract pathogen attack. Similar observations were made by Fischer et al. (2005), *P. edulis* f. *flavicarpa*, where plants survive inoculations of the pathogen.

The highest percentage of plants affected by the teleomorph *N. haematococca*, suggesting an important role of this on pathogenicity; nevertheless affected plants were also presented by the anamorphic state *F. solani*, which is in this work referred to as causal agent.

The low incidence observed in these tests can be explained by two factors: i) *N. haematococca* is not considered a particularly aggressive pathogen in passion fruit (Ploetz, 2003). *F. solani* strains compared with *F. oxysporum*, display long incubation periods ii) it could be that oscillations of environmental factors such as soil and weather can modulate the development of disease, which were stables under research conditions.

Finally, standardization of the methodology of inoculation of these pathogens is a tool to consider in future studies aimed at finding sources of resistance, likewise, severity scale developed allows the evaluation of these diseases in a more versatile manner.

Conflict of Interests

The authors have not declared any conflict of interests.

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