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Morphological and biochemical indicators of *Fusarium oxysporum* f sp. *fragariae* in strawberry crops (*Fragaria* × *ananassa* Duch) in the province of Pichincha, Ecuador

S. Salazar[1], F. Bayas-Morejón[1,*], Z. Niño[2], J. Guevara[1], M. Ruilova[1], O. Merino[1], W. Donato[2] and J. Guamán[1]

[1]Universidad Estatal de Bolívar, Facultad de Ciencias Agropecuarias Recursos Naturales y del Ambiente, Departamento de Investigación. Centro de Investigación y Desarrollo Biotecnológico, EC020150 Guaranda, Ecuador
[2]Universidad Estatal de Bolívar, Facultad de Ciencias Agropecuarias Recursos Naturales y del Ambiente, Departamento de Investigación. Centro de Investigación del Ambiente, EC020150 Guaranda, Ecuador
[*]Correspondence: isba@doctor.upv.es; fbayas@ueb.edu.ec

Abstract. *Fusarium oxysporum* f. sp. *fragariae* is a fungal pathogen, transmitted by soil in crops of strawberry (*Fragaria* × *ananassa* Duch.), which causes wilt disease that, kill the strawberry cultivars. The disease caused by *Fusarium oxysporum* f. sp., *fragariae* is hard to detect as the of the symptoms are similar with other soil-borne diseases. In spite of detection methods targeted *F. oxysporum* using molecular criteria have been developed, they have not been shown to successfully identity the unique identity of *F. oxysporum* strain that causes this disease. In Ecuador, the cultivation of strawberry has acquired great importance for the consumption, promoting the increase of its production. However, the process of importing plant material from producing countries for the purpose of improving production has contributed to the spread of the fungus. The objective of this study was to identify the presence of the *Fusarium oxysporum* f. sp *fragariae* by means of morphometric identification and the application of biochemical methods (BIOLOG) in the province of Pichincha. Fifty-two diseased strawberry plants and 52 asymptomatic plants were analyzed. Of these, 13 isolates were identified by morphometry as *F. oxysporum*. However, through BIOLOG four strains were identified as *F. oxysporum*, 5 as *Fusarium* sp., 2 *F. lateritium*, 1 *F. udum* and 1 strain as *F. sacchari*. The results obtained through the identification and evaluation confirmed the presence of *F. oxysporum* f. sp., *fragariae* in evaluated strawberry cultivars, thus determining the high risk to exist if the pathogen spreads in new plantations in Ecuador.

Key words: *Fusarium oxysporum*, BIOLOG, Morphological Indicators, strawberry.

INTRODUCTION

The big strawberry fruit (*Fragaria* × *ananassa* Duch.) is being cultivated since the year 1800, constituting the starting point of the modern varieties that dominate the world production (López-Aranda et al., 2011).

Its consumption has great acceptance worldwide, as its cultivation has spread to U.S.A, Canada, Mexico, Colombia, Chile, Argentina, Guatemala, Costa Rica, Ecuador and almost entire Europe, generating a growing expansion of consumers, favored by the aerial transport (Fernández, 2005).

Diseases such as Botrytis, grey mould (González et al., 2013) and Fusarium wilt (Koike et al 2009; Henry et al., 2017) infected strawberry. The latter is a very serious disease caused by Fusarium oxysporum f. sp. fragariae. According to Koike et al. (2009), the symptoms consist of foliage wilting, drying and wilting of older leaves, delayed plant growth and reduced fruit production, causing the plants to finally collapse and die. Studies in California by Koike et al. (2009) reported that since 2006, this disease has increased in incidence and severity. The initial problems in 2006 consisted of multiple small patches of infected plants, and by 2009, in some fields, the disease affected large sections. Henry et al. (2017), found infected strawberry population in California of a considerable diversity of F. oxysporun f. sp. fragariae, an indication that horizontal gene transfer might have occurred. This fungus was first reported in Australia in 1965 by Winks & Williams (1965), then in Japan (Takahashi et al., 2003) and Korea (Kim et al., 1982). This pathogen has caused serious losses in strawberry fruit productions fields in Chile (González et al., 2005), Spain (Arroyo et al., 2009), USA (Gordon et al., 2016), Australia (Fang et al., 2012) and in Mexico (Dávalos-González et al., 2016), attributing that its worldwide dissemination is due to its difficult diagnosis (van Diepeningen et al., 2015).

According to Koike & Gordon (2015), the field diagnosis of Fusarium wilt is complicated since other soil-borne diseases also showed very similar symptoms. Methods for detecting F. oxysporum based on molecular criteria have been developed but have not yet been shown to uniquely identify F. oxysporum strain causing strawberry Fusarium wilt. In Ecuador, intensive strawberry cultivation began in 1983, mainly in the province of Pichincha, (Bejarano, 1993). According to the Ministry of Agriculture, Livestock, Aquaculture and Fisheries of Ecuador (MAGAP), this crop constitutes an important socioeconomic alternative for this Province (MAGAP-DPP, 2015). This research is the continuation of the molecular characterization of *Fusarium oxysporum* f. sp. *fragariae* in Pichincha-Ecuador to be able to establish or discard their presence in the country, with the following objectives: to identify the morphology and morphometry of the phytopathogen and to evaluate its biochemistry through the use of the BIOLOG identification system.

MATERIALS AND METHODS

The research work was carried out in the Molecular biology laboratory of the State University of Bolivar, Ecuador. Strawberry plants (*Fragaria* × *ananassa*) were analyzed with 13 inoculums of the pathogenic fungus (*Fusarium* sp.), the treatments are shown in the Table 1.

The microscopic characteristics of the fungus were observed and identified through the use of morphometric keys (Garibaldi et al., 2015), and it was determined with descriptive statistics considering the average lengths of 20 macroconidia (average of maximum and minimum lengths).

Sampling was carried out in strawberry producing areas located in the province of Pichincha, of which 52 plants with symptoms and 52 asymptomatic plants were obtained; these plants were in phases of vegetative development and fruiting stages (Table 2).

Table 1. Treatments used in this work

Treatment	Description
T0:	Strawberry plant without inoculum
T1	Strawberry plant + strain 1
T2	Strawberry plant + strain 2
T3	Strawberry plant + strain 3
T4	Strawberry plant + strain 4
T5	Strawberry plant + strain 5
T6	Strawberry plant + strain 6
T7	Strawberry plant + strain 7
T8	Strawberry plant + strain 8
T9	Strawberry plant + strain 9
T10	Strawberry plant + strain 10
T11	Strawberry plant + strain 11
T12	Strawberry plant + strain 12
T13	Strawberry plant + strain 13

Table 2. Information of the analyzed samples

Cod. Health surveillance	Place of the province	N° of Sample	Sample	Detected pathogens
17-01-85-401	Yaruquí	1	root	*Fusarium* sp.
17-0185-410	El Quinche	1	root	*Fusarium* sp
1/-1	yaruquí	1	Soil / plant	*Rizoctonia* sp
17-2	Yaruquí	1	Soil / plant	*Fusarium* sp
17-3	Yaruquí	1	leaves	*Mycosphaerella* sp
17-4	Yaruquí	1	leaves	*Mycosphaerella* sp
17-7	San Carlos	3	Soil / plant	*Fusarium* sp
17-8	Yaruquí	1	Soil / plant	*Pestalotía* sp
17-9	Yaruquí	2	Soil / plant	*Pestalotía* sp
17-10	Checa	3	Soil / plant	*Fusarium* sp
17-11	Checa	1	leaves	*Mycosphaerella* sp
17-201	Pifo	10	Soil / plant	*Fusarium* sp
17-202	Pifo	3	Soil / plant	*Pestalotía* sp
17-203	Pifo	10	Soil / plant	*Fusarium* sp
17-205	El Quinche	2	Soil / plant	*Pestalotía* sp
17-206	El Quinche	2	Soil / plant	*Pestalotía* sp
17-207	El Quinche	4	Soil / plant	*Pestalotía* sp
17-209	Puembo	1	leaves	*Mycosphaerella* sp
17-210	Puembo	4	Soil / plant	*Fusarium* sp
	TOTAL	52		

The identification method was by isolation on PDA, then it was identified by microscopic observation.

Sowing of plant material and media preparation

Longitudinal cuts of the crown were made and 1–2 cm segments containing areas with or without vascular wilt lesion were removed. After this process, four segments were cultured in Potato dextrose Agar (PDA medium) in triplicate, then incubated at 24 °C for 7 days. The isolates that presented asexual structures of *Fusarium* sp., (conidia, hyphal formation) were selected. On the other hand, 10 g of soil was added in flasks with

90 mL of distilled water, of which, 100 µL of each sample, was cultured in duplicate on PDA agar and incubated for 7 days at 24 °C.

For purification and obtaining monosporic cultures, the PDA cultures were transferred on Malt Extract Agar (MEA), from these pure cultures a suspension of the inoculum was extracted. The suspension was loaded in a Neubauer chamber by counting propagules with the aid of an optical microscope. From this suspension, continuous dilutions were made up to a concentration of 20 propagules / mL of distilled water (macroconidia or microconidia). Finally, the suspension was reseeded on MEA and incubated at 24 °C for 7 days.

Storage of field samples

The samples collected were stored in paper bags with a silica gel envelope and stored in a drying chamber.

Massification of mycelium in liquid medium

In order to obtain a sufficient amount of biomass, two agar blocks of monosporic culture medium were introduced into one flask in duplicate for each isolate. The media was allowed to stand for 5 days. After this period, the solution was filtered using a vacuum pump, the mycelium obtained was rinsed, transferred to eppendorf tubes for storage at -20 °C.

Biochemical evaluation by the BIOLOGY identification system

Pure and monosporic strains were cultured in MEA medium and incubated at 24 °C for 7 days. From this culture, with the help of sterile handle, the mycelium was extracted from the fungus and a suspension of fungi was made. The readings were made by spectrophotometry until a turbidity of 75% ± 2 was obtained for each isolate. Subsequently, the fungus was inoculated into microplates (FF MicroPlate TM), dispensing 100 µL in each of the 96 wells. Microplates were incubated at 24 °C for 7 days, at which time the respective readings were performed, the intervals are shown in the Table 3.

Table 3. Intervals of readings by the BIOLOG system

1° Reading	2° Reading	3° Reading	4° Reading	5° Reading
24 hours	48 hours	72 hours	96 hours	168 hours
8:00	8:00	8:00	8:00	8:00
	13:30	13:30	13:30	
	12:00	12:00	12:00	
	15:00	15:00	15:00	
	17:00	17:00	17:00	

RESULTS AND DISCUSSION

From the total of samples, analyzed after isolation and confirmed by microscopic characteristics, was evidenced in 13 isolates the presence of asexual structures of Fusarium sp., representing 25% of the total samples; In a study carried out by Juber at al. (2014), the incidence of *Fusarium* sp., found in strawberry plants was of 40–60%, these differences are due especially to the environmental characteristics in which the crops are found.

Characteristics of the colony morphology of the 13 isolates planted in PDA

The treatment T1-T4 present pink color; however, the T1 presented a withish-pinkish color in its aerial part and the treatment T2, T3 and T4 a rosy aerial mycelium. The treatments T5, T6, T11-T13 presented a violet color, with a whitish-violet aerial mycelium. The treatments T8 and T9 presented a purple color and in their aerial part whitish mycelium. Whereas, only the T7 treatment presented a yellowish coloration andaerial mycelium different from the others. As far as its growth, a concentric growth was visualized in treatments T4,T5, T6, T7, T10 and T11, and in the rest was a diffuse radial growth (Fig. 1). Similar results were reported by Domsch et al. (2007), of which, the isolates were cultured on PDA and presented different characteristics in color and form of the mycelium.

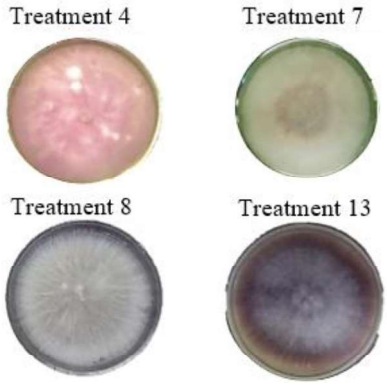

Figure 1. *Fusarium* colonies showing various colony colors on PDA medium.

According to a study by Quilambaqui, (2005), colonies identified as *Fusarium oxysporum* developed violet or purple mycelium with macroconidia, and another group of Fusarium sp., developed white mycelia that turned to a yellow coloration.

The asexual structures of Fusarium sp. isolated on PDA media are shown in the Fig. 2.

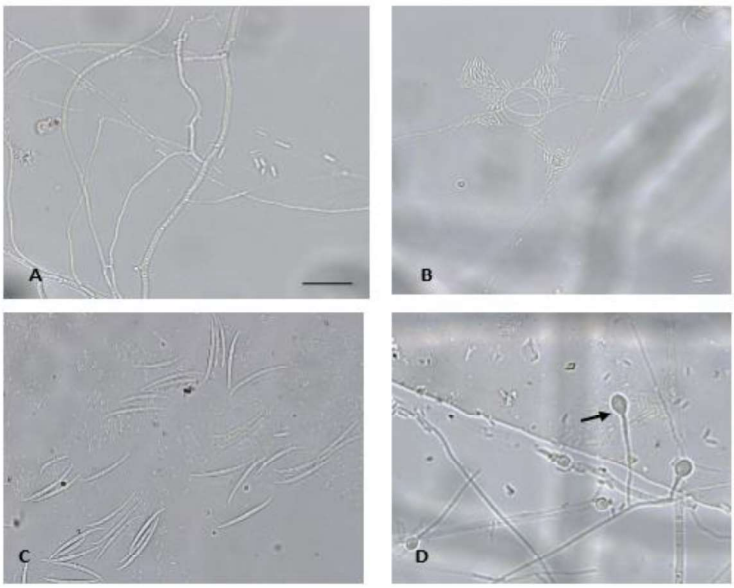

Figure 2. Asexual structures of *Fusarium* sp. isolated on PDA (40X). A: Mycelium; B: Microconidia; C: Macroconidia; D: Chlamydospore (40 μm).

Biochemical evaluation using the BIOLOG identification system

The reading of the color densities in the wells of the sensitivity tests were positive in the treatments T_2, T_5, T_6 and T_{11} for *Fusarium oxysporum*, evidencing a slight difference in the readings between treatments T_5, T_6 and T_{11} due to the absorbance difference. In treatments T_1, T_7, T_8, T_{12} and T_{13}, the limit of detection only indicates at genus level Treatment T_3 and T_9 had 97% identity for *Fusarium lateritium*. The percent results indicated turbidity and colorimetry readings during biochemical reactions between the substrate and the pathogen. On the other hand, a percentage of 99% identity was obtained for *Fusarium sacchari* and 94% for *Fusarium udum* in the treatment T_4 (Table 4).

Table 4. Microplate readings corresponding to the 13 isolates for *Fusarium* sp...

Treatment	Morphometric Identification	Biological Identification	Probability (%)
T1	*Fusarium oxysporum*	*Fusarium* sp.	*
T_2	*Fusarium oxysporum*	*Fusarium oxysporum*	0.99
T_3	*Fusarium oxysporum*	*Fusarium lateritium*	0.97
T_4	*Fusarium oxysporum*	*Fusarium udum*	0.94
T_5	*Fusarium oxysporum*	*Fusarium oxysporum*	0.77
T_6	*Fusarium oxysporum*	*Fusarium oxysporum*	0.78
T_7	*Fusarium oxysporum*	*Fusarium* sp.	*
T_8	*Fusarium oxysporum*	*Fusarium* sp.	*
T_9	*Fusarium oxysporum*	*Fusarium lateritium*	0.97
T_{10}	*Fusarium oxysporum*	*Fusarium sacchari*	0.99
T_{11}	*Fusarium oxysporum*	*Fusarium oxysporum*	0.79
T_{12}	*Fusarium oxysporum*	*Fusarium oxysporum*	*
T_{13}	*Fusarium oxysporum*	*Fusarium* sp.	*

*: No absorbance.

In a study carried out in Sweden by Khalil & Alsanius (2009), they detected five pathogens of vegetable roots, including *Fusarium oxysporum* f.sp. *radicis-lycopersici* and *Fusarium solani,* with a high percentage of identity. Frac et al. (2016), isolated strains of *Fusarium* sp., and identified them by the method of dilutions in filamentous fungi (FF) microplates BIOLOG TM2, but failed to discriminate at the classification level and through this technique also analyzed levels of resistance to fungicides. On the other hand, Gizaw et al. (2017) isolated *Fusarium* species present in the cultivated soil (Rizosfera) and were identified by the BIOLOG technique, being constituted 75% by filamentous fungi of which 16% corresponded to the genus *Fusarium* with the species *F. melanochlorum, F. juruanum, F. avenaceum,* with 62.5% similarity. These results demonstrate in part that the species of *F. oxysporum* are more commonly found in plant materials than in crop soil and that the diversity of *Fusarium* species changes relative to the geographical area.

The BIOLOG identification system method allows diagnose of the possible metabolic patterns of the biochemical profile and the phylogenetic relationship between different isolates, while the pathogen oxidizes the different carbon sources. This diagnosis is related to the manipulation and nature of microorganisms.

On the other hand, no significant statistical differences between the treatments for the distribution were detected, of which there are no differences between the variables. The coefficient of variation was 19.46% tolerable, indicate that it is acceptable for field

evaluation. This analysis establishes that the inoculation in the plants was effective both by the concentration of the inoculum and the method performed. The incidence of the disease was determined based on visual symptomatology. In this way, those plants that showed, wilt, necrosis and death of leaves, were considered infected by the pathogen.

CONCLUSIONS

By means of the pathogenesis evaluation, the presence of *Fusarium oxysporum* f sp., *fragariae* that causes the disease in the cultivation of the strawberry in the 13 treatments showing symptomatology of vascular wilt, which determines the presence of this disease in Ecuador. This study represents a first advance study in the recognition of the microbiont associated with the cultivation of strawberry in Ecuador, this determines the importance of the application of biochemical techniques namely using BIOLOG for the detection of the pathogen.

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