Net Journal of Agricultural Science
Vol. 2(3), pp. 107-112, September 2014
ISSN: 2315-9766
Full Length Research Paper

Diagnosis of *Fusarium oxysporum* in the cultivation of pineapple *Ananas comosus* (L) Merr

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Accepted 29 August, 2014

ABSTRACT

Since about 2004 there has been a symptom in pineapple (var MD-2) in the humid tropics of Costa Rica, characterized by dieback of plants and different forms of stem rot in both growing vegetative, how after forcing, in the latter case, the crop undergoes a more rapid deterioration. Though productivity due to small fruit can be reduced up to 3000 boxes ha^{-1} in first crop plantations, the fruit has no internal damage caused by the causal agent. This fitosanitary problem was listed by other authors as an emerging disease in the crop in Costa Rica, therefore, it is associated with a causal agent "unconventionality in the pineapple production in Costa Rica", exists the risk that the disease have different diagnostics by some specialists. It makes possible that the recommendations oriented to control and combat the disease, not exert any effect, on the contrary, the inoculum level tends to rise and hence the intensity of the disease is becoming increasingly critical. For these reasons, we applied protocols for morphological and molecular classification for this disease, we have called "pineapple cultivation dieback" and we have identified the causal agent as *Fusarium oxysporum*, a disease that is characterized by a drying of the leaves, noticeable loss of vigor of the plantation and severe injury to the vascular level, specifically in the stem.

Keywords: Molecular classification, *Fusarium oxysporum*, pineapple disease, dieback.

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INTRODUCTION

Summerell et al. (2003) indicate that the history of biology of *Fusarium* is full of publications and reports that are now difficult to interpret due either to the fact that the report is ambiguous or because it is completely wrong. They claim that such publications unfortunately continue to occur today, therefore, stressed the need to follow a logical and systematic identification through a series of steps that should lead to the molecular classification of organisms isolated. Moreover, Geiser et al. (2008) indicate that the species of filamentous fungi such as *Fusarium*, collectively represent the most important group of toxigenic plant pathogens. This researcher agrees with Summerell et al. (2003) that efforts to accurately convey what *Fusarium* species are responsible for plant diseases and toxin contamination of food and feed have been hampered because most species are rather difficult or indistinguishable by itself morphology. However, argued that to address this problem, they have constructed a web-accessible database called *Fusarium*-ID that identifies the individuals isolated of interest, with a partial DNA sequence of a gene called factor elongation. Once this sequence is generated from the isolate of interest, it is compared with the database of the *Fusarium*-ID, and rapid and accurate identification is obtained.

Obregon and Mata (2008) reported that in analyzing samples of pineapple plants that presented symptoms of "dieback of pineapple cultivation" in different farms, they isolated and identified by Koch's postulates that *Fusarium* sp. was the causal agent of the disease, however, until then, the kind of *Fusarium* liable for damages was not known.

This technical note presents part of a thesis of Instituto Tecnológico de Costa Rica, accomplished to characterize the species of *Fusarium* that causes the disease commonly known as "Pineapple cultivation dieback", description of symptoms the plant manifest, and

conditions to favor the disease, as well as management-oriented to control them, are part of this technote.

METHODOLOGY

Plants were collected from different parts of the growing area showing characteristic symptoms of the disease "pineapple cultivation dieback" in the initial state and intermediate, from farms in Venecia - San Carlos. The collected samples were conditioned for transport with purposes phytopathological analysis, and transported to Biocontrol Laboratory of the Instituto Tecnológico de Costa Rica, where the stalks were chosen with intermediate damage state of the disease, to this material we applied protocols isolation, to obtain the axenic multisporic isolated.

The morphological characterization was performed using samples prepared in sterile slides, mycelial mass was obtained of axenic multisport developed prior, it was observed under an optical microscope Nikon model 50i, as reference we take the morphological characteristics described by Booth (1977), Nelson et al. (1983), Nelson (1991), and Aoki et al. (2014). As for the molecular characterization, it was necessary to obtain a monosporic axenic culture, which is performed by selection of macroconidia obtained of decimal dilutions and incubated in petri dishes containing PDA, for periods of 24 h at 26 ± 2°C temperature and 80% relative humidity and then selected and transferred to petri dishes containing PDA and incubated for a period of 8 days at 26 ± 2°C temperature and 80% relative humidity. Once monosporic crops were obtained, liquid medium was prepared based on potato dextrose, fractionated monosporic crops and placed in this medium. They were sent to the laboratory of plant biotechnology (CIA), where DNA of the fungus was extracted, later at the Centre for Research in Cellular and Molecular Biology at the University of Costa Rica (CIBCM, UCR), confirmation of the specie was performed.

RESULTS

Cultural and morphological characteristics of the agent

In isolates from diseased plants, mycelial growth was observed initially as white and rose, as it advanced this became pale pink; regarding the medium, it was pigmented with tended to purple or lilac. Figure 1 is very similar to the characterization performed by Summerell et al. (2003). Furthermore, in the samples observed microscopically hyaline septate hyphae was determined, as well as specialized hyphae (Figure 2), on which according to Agrios (2005) are formed asexual structures, this also observed in the isolates obtained.

In micrographs taken to these isolated by scanning electron microscope (SEM) model S570 from the University of Costa Rica, we observed the presence of these structures, called macroconidia and microconidia. The macroconidia according to the species may consist of three to five cells, and gradually become thinner and curve towards the ends (Leslie and Summerell, 2006), whereas microconidia may have only one or two cells and the structures are that the fungus produces in greater abundance (Agrios, 2005). Aoki et al. (2014) indicates

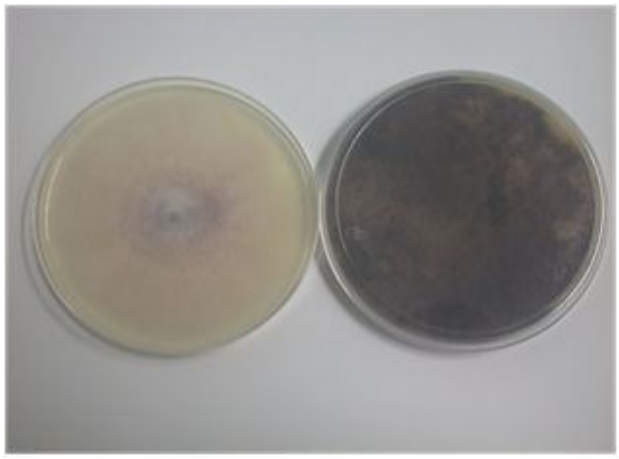

Figure 1. Isolated colony development in potato dextrose agar (PDA), isolated from the stem of pineapple, with plant dieback symptoms.

that macroconidas are the structures typical in *Fusarium* fungi.

We determined the presence of other types of structures known as chlamydospores, these are constituted by one or two cells are thick-walled and have a round shape maybe terminally or intercalarmente in the hyphae. Figure 2 shows structures for survival and can remain on the substrate or soil for long periods (Nelson et al., 1983; Nelson, 1991; Agrios, 2005; Lorens et al., 2006).

According to the structures obtained in the isolated and the description of the same, the results show that the agent that causes the disease known as dieback in the cultivation of pineapple is due to *Fusarium* fungi, which agrees with the results obtained by Obregón and Mata (2008), who also reported, among the pathogens that affect the cultivation of pineapple, *Fusarium* is the only causal agent invades the vascular system of the plant.

Molecular characterization of the causal agent

In the laboratory of plant biotechnology “CIA” (Centro de Investigaciones Agronómicas de la Universidad de Costa Rica), it was determined that the samples belonged to *Fusarium* spp. according to the methodology described by Geiser et al. (2008). By applying the technique of PCR-RFLP was identified the *oxysporum* species. Finally at the Center for Research in Cellular and Molecular Biology (CIBCM, UCR), the identity of the species was confirmed by sequencing the gene segment elongation (EF-1) (Figure 3).

This classification is consistent with findings in Venezuela of Páez et al. (2001), which reported the species *F. oxysporum* characterizing it as a disease manifests necrotic symptoms at the vascular bundles and red coloration in the leaves, but this has been in the

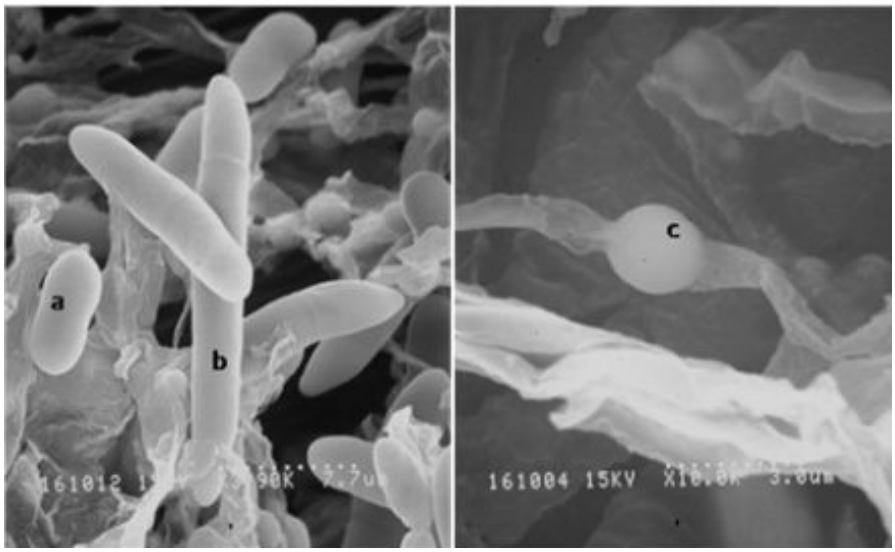

Figure 2. Resistance and reproductive structures of *Fusarium* fungi, isolated of stem of pineapple plant, with dieback symptoms, microconidia (a), macroconidia (b), chlamydospore (c).

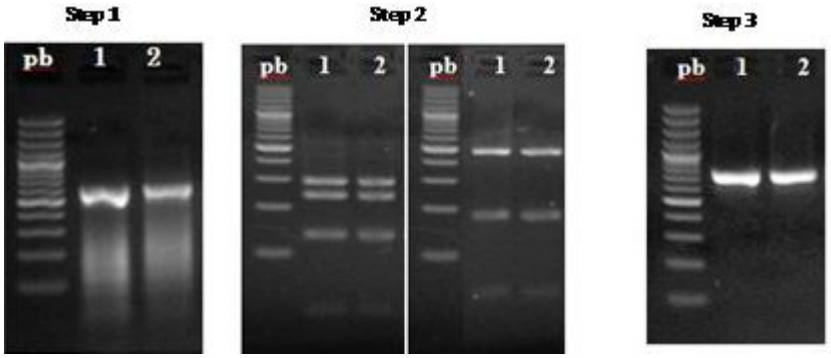

Figure 3. Molecular identification of *Fusarium* species isolated from pineapple plants symptomatic. Step 1 fragments obtained from PCR amplification to determine if the samples are from *Fusarium* sp. Step 2 fragments obtained from the PCR-RFLP technique which determines the presence of *F. oxysporum*. Step 3 fragments obtained from PCR amplification of the gene segment elongation factor (EF-1), confirming the *oxysporum* species (CIA - CIBCM, UCR), 2010.

Spanish and Smooth Cayenne variety.

DISCUSSION

Characterization of the disease symptoms and conditions favoring their development

In plantations suffering from this disease, shows symptomatic patches inside lots, until completely affected areas is characterized by dieback of infected plants, which have a drying area and yellow color in the leaves in the top (apex) towards the base.

When the disease is advanced, a total drying occurs in the first 10 to 15 inches of the blade, then the rest of the leaves take on yellowing symptoms like in the pineapple disease called "wilt virus" these symptoms may be evident from two months after sowing in heavily infested lots, presenting a retarded growth that leaves behind planting (Figure 4), showing a bearing not exceeding a plantation of four to five months even though it has even more than eight months of age after sowing, according to Obregon and Mata (2008), this is because the agent is in the vascular system of the plants, leading to blockage and/or translocation deficient water and nutrients to the upper portions of the plant.

Meanwhile Rivera (1999) and Agrios (2005) indicated that this agent can remain in the soil mainly as

Figure 4. Condition of a pineapple plantation affected by *F. oxysporum*, aged 5 months.

chlamydospores, and when the plants begin to develop these structures stimuli germinate by root exudates entering directly into the tips of the roots, through the area where the lateral root form or ultimately through wound. According to Agrios (2005), this pathogen is maintained in the xylem vessels, where it germinates and produces lots of microconidia that are carried to the top of the plant through the torrent of sap.

Besides the penetration of the agent can be facilitated by abiotic factors, such as excess moisture on the soil, that cause root rot due to deficient oxygenation, lead to toxic substances product of the metabolism of anaerobic microorganisms that are predisposed to the plant.

In this sense, Taiz and Zeiger (2006) mentioned that anaerobic microorganisms cause the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and nitrous oxide (N_2O) and molecular nitrogen (N_2) for energy and under more stringent conditions reducing Fe^{+3} to Fe^{+2} because of its higher solubility, the latter reaching concentrations that may be toxic to the roots when soils are under anaerobic conditions for many weeks. Also mentioned is that other anaerobic microorganisms can reduce (SO_4^{-2}) to hydrogen sulfide (H_2S), a poison that affects breathing.

According to Taiz and Zeiger (2006), where anaerobic microorganisms have abundant supply of organic substrate, bacterial metabolites such as acetic acid and butyric acid can be released from soil water and these acids, together with reduced sulfur compounds, leading to standing water with nasty smell. All these substances produced by the microorganisms under anaerobic conditions are toxic for the plants at high concentrations. These authors comment that respiratory rate and root metabolism are affected even before the dissolved O_2 is completely removed from the root environment.

In pineapple plantations that are without irrigation, it has been determined that *F. oxysporum* rapidly shows symptoms when the temperature reaches for extended periods of time (a month or more) of 28 to 35°C during the day, with relative humilities above 80%, and soil below field capacity; these conditions favor more the causal agent when the affected areas leave periods or periods contrary extreme conditions, this is, high rainfall, saturation conditions of soil, mainly in heavy soils (Ultisols and Inceptisols) or soils without structure with high percentages of limo (entisols of Huetar Atlantic Region of Costa Rica), poorly drained and compacted by rain even after being well prepared (loose soil) in summer, as the slimy texture and lack of structure allows rain in the winter, filtrate the thin materials of the soil texture and create a "profile compaction" in the first 30 cm or less, preventing good root development and favoring the latter's death from lack of oxygen.

This may explain the poor soil aeration (compaction, water logging) and drainage problems (high water table), low rise beds; promoting the development of the disease. In addition to this, several attacks arthropod, symphylans genera and species of insects as *Phyllophaga* spp and nematodes can also favor the incidence of the disease in primary inoculum of soil, this could pose major routes of entry of the pathogen into the vascular system, while in dry conditions may favor the infection when the man enters the plantation to perform manual labor such as weed control or the samplings plan.

Individually the plants show in the first 15 cm, top down necrosis of the intermediate leafs, loss of overall color and loss turgidity leaf (Figure 5).

In the transverse sections of stems, it is possible to see an injury that depending on the weather may be aqueous or dry (rots) reddish brown to black, with presence or absence of a compact "cork" in the center of the affected area is made (mainly in summer), odorless or very little odor. The few roots that are attached to the stem are only fibrous/corky remnants (Figure 6).

It was determined that the affected plants weight decrease rapidly due to death of the root system and the destruction of the vascular system in the stalk that does not allow the transpiration stream. Agrios (2005) indicated that this is in response to the combination of obstruction of the vessels and the pressure of the proliferation of adjacent parenchyma cells. Barahona and Sancho (2000) mentioned that in this condition the plant closes their stomata. Observations in the field suggest that the plant is dehydrated due to high temperatures and cannot be cooled by transpiration, these leaves lose their rigidity and become malleable to the touch, but do not break (Figure 7), as a healthy leaf of a suitable moisture content, turgid cells and causes the cell wall will provide rigidity to the whole leaf, this is the reason why affected plantations with *F. oxysporum* show loss of vigor, color, size and foliar coverage, still above 5 months age.

In blocks of less infested plantations but with the presence of this pathogen, symptoms can be delayed for up to 6-7 weeks after the induction of flowering, at this

Figure 5. Symptoms of *F. oxysporum* in pineapple plant. Fruit 100 days after flowering induction.

Figure 7. Typical of a sick plant by *F. oxysporum* foliar symptoms. Age 60 days after flower induction.

point in planting begins the manifestation of symptoms. Lots affected can reach the fruit crop with yield losses from 2000 to 4000 boxes ha^{-1}. Importantly, the fruit is not rejected by direct problems of the pathogen on the fruit quality (gummosis, injury or stains), but underweight because the plant did not manage the translocation of water and nutrients needed for the filled with fruit, which is different from the indicated yield losses in Brazil due to "Fusariosis" by Py et al. (1987), Rohrbach and Marchall (2003) and Ventura et al. (2008), among others, and that the causal agent *Fusarium* is clearly characterized as *Fusarium guttiforme* (Syn: *Fusarium subglutinans* f. sp.

Figure 6. Damage of *F. oxysporum*. on stem of pineapple plant. Plant stems 5 months old.

ananas), according to Ventura et al. (2008).

RECOMMENDATION

The leaves and stem are the main sump for the plant vegetative propagation of flower induction, appropriate cultural practices of land preparation and good nutrition management can significantly mitigate the manifestation of symptoms in this stage of vegetative development. However, if these lots have inoculum of *F. oxysporum*, they are at risk of developing symptoms after induction of flowering because this practice reduces the natural defenses of plants and the agent can win space. Because of this, microorganisms such as species of fungi of the genus *Trichoderma* can stimulate the defense mechanisms of the plant and indirectly prevent the action of the agent.

It should be noted that areas that come under suitable conditions of nutrition, weight and health at induction moment can achieve adequate productivity even when the attack of *F. oxysporum* comes after "forcing". This is because from the planting until forcing, leaf activity is remarkably important and is where the translocation of photo-assimilated for storage in the stem is more important (vegetative phase); however, use of these areas for ratoon crops or seed is not wise, because the population is greatly affected after fruit harvest.

In heavy soils (Ultisols and Inceptisols) or soils with high percentages of silt, use of plastic mulch mitigates the effects of rain as relates to compaction and providing a temperature, humidity and ventilation very compatible with growth and healing the root system. Use of this type of coverage coupled with suspensions of conidia over soil or seed with some species corresponding to fungi of the genus *Trichoderma* can minimize the chances of infection of *Fusarium oxysporum* on soils inoculated.

Figure 8. Symptom crystal spot on the stem of the pineapple plant. Age of 4 months post-planting.

Another important aspect to consider is the symptom of "crystal stain pineapple cultivation" (Figure 8). This is common in pineapple plantations mainly in the transition:winter to summer and summer to winter (although it can also occur by pests attack like symphylans snails or nematodes, etc), where drought in the first case and anaerobic conditions is given in the second death of a major lateral roots, translocation causing problems at the level of vascular bundles and leave stains aqueous inside the stem. This symptom does not necessarily imply that the lot, block or section is infected with *Fusarium oxysporum* but is an indicator that should be considered for extreme care and frequent monitoring of the plant pathogen diagnosis, as this indicates that the roots died are an important and latent path for the pathogen in the soil. It is also wise to consider the application of existing chemical alternatives in the market with usage record for pineapples, for example Carbendazim (0.5 mg ai L^{-1}).

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