Article

First report of *Nodulosporium* (Xylariaceae) in *Theobroma cacao* L. in Chiapas, Mexico and pathogenicity tests

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Abstract

Nodulosporium is a widely distributed fungus found in tropical areas and has been reported as a phytopathogen causing descending death, rot and resinosis, it is also considered as a potential agent of biological control of other fungi and oomycetes. The teleomorphic state is solitary and in clusters, and has been reported as an endophyte, saprophyte or weak phytopathogen. The creole cacao variety *Theobroma cacao* is of great ecological, economic and cultural relevance in the state of Chiapas; however, it presents high susceptibility to diseases unlike other important varieties, affecting quality and production. In Villa de Comaltitlan Chiapas, cacao fruits with typical characteristics of rot symptoms were observed. The objective was to identify the fungi associated with sick cocoa fruits with symptoms of rot. The fungi *Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp., *Trichoderma* sp., and *Nodulosporium* sp. were identified morphologically. The presence of *Nodulosporium* sp. is reported for the first time, in cacao fruits with rot symptoms of Villa de Comaltitlan, Chiapas, Mexico. The morphological and morphometric characteristics of *Nodulosporium* sp. and *Hypoxylon* sp., and its molecular identification. The pathogenicity of *Nodulosporium* sp. was confirmed in foliage of the crop causing chlorosis and dehydration of the leaf, and it was recovered in the form of pycnidia and mycelium with conidia, corresponding to its teleomorph, identified as *Hypoxylon* morphologically and molecularly with the oligonucleotides ITS4-ITS5.

Keywords: *Hypoxylon*, *Nodulosporium*, Chiapas, cocoa, phytopathogenic fungi.

Reception date: March 2019
Acceptance date: June 2019

Introduction

Cocoa *Theobroma cacao* L. (Malvales: Malvaceae) is a crop of great economic, social and environmental importance, as well as being a primordial species in the peasant agroforestry system (Jaimes and Aránzazu, 2010).

In Mexico the harvested area of the cocoa crop for 2017 corresponded to 59 837.8 ha, with a production of 27 287.25 t, and a value of the national production of $1 074 303.38. Cocoa production is concentrated in the states of Tabasco, Chiapas and Guerrero with 17 430.21, 9 611.63 and 245.41 t, respectively (SAGARPA-SIAP, 2018). In the state of Chiapas, cocoa is grown in four agronomic regions: Soconusco, North, Center and jungle Palenque, occupying the sixth place in cultivated area, behind corn, coffee, beans, oil palm and mango. Of the Mexican varieties of cocoa, in the Soconusco region the creole is cultivated, for its pleasant flavor and aroma, although with greater susceptibility to diseases with respect to other important varieties such as the trinitarian and the foreigner that have a genetic improvement resulting from the cross-linking with the creole (González and Amaya, 2005).

Several factors, such as the scarce technology for crop management, the use of materials of low agronomic quality and the frequency of diseases caused by fungi, insects, birds and rodents, have affected the quality and production of the crop, generating a shortage crisis, that forces the country to import seed (González and Amaya 2005; Hernández *et al*., 2015). Of all these factors, diseases are those that cause considerable losses of 40-100% of production (Jaimes and Aránzazu, 2010; Hernández *et al*., 2015).

In the cocoa agroecosystem, the disease with the greatest distribution is the black spot caused by *Phytophthora palmivora* Butl. /*Phytophthora capsici* Leonian (Peronosporales: Pythiaceae), witch's broom *Moniliophthora perniciosa* (Sthael) and moniliasis of cocoon *Moniliophthora roreri* (Cif.) HC Evans, Stalpers, Samson and Benny (Agaricales: Marasmiaceae), these last two being the most destructive of this crop, causing losses that range from 50 to 80% of production (Aime and Phillips-Mora, 2005). In Villa de Comaltitlán, Chiapas, cocoa fruits with typical rot characteristics were observed, with sporulation in the form of white powder on the tissue, brown/chocolate spots, oily/shiny and with the shape of a hump. The objective was to identify fungi associated with cocoa fruits and plant tissue with symptoms of rot.

Materials and methods

Collection of cocoa fruits

100 fruits (ears) of 15-20 cm of length of the cacao crop, with typical signs of rot, were collected in five points of a non-commercial plot in the municipality of Villa de Comaltitlan, Chiapas (15° 13’ 00’’ latitude north 92° 34’ 00’’ west longitude). The biological material was placed in sterile transparent polyethylene bags with hermetic closure and placed at room temperature (25 °C) in expanded polystyrene coolers and transferred to the Phytopathology laboratory of the Department of Parasitology of the Autonomous Agrarian University Antonio Narro (UAAAN) Saltillo, Coahuila.

Isolation and purification of the fungus

The isolation of the fungus to obtain pure colonies was carried out in two ways, the first being to take spores directly from the damaged ear tissue, using a sterile dissection needle and seeded in potato dextrose agar culture medium (PDA) (Bioxon®) and the second route consisted of cutting diseased plant tissue with a scalpel, which was disinfected with a triple wash with 3% hypochlorite, sterile distilled water (dH2O), 70% alcohol and dH2O and seeded in medium of cultivation PDA. In both isolation routes, they were incubated at 27 ±2 °C in a growth chamber (Binder®).

Morphological, morphometric and molecular identification

The morphological identification was carried out by means of mounts of mycelium structures and their respective conidia and spores in porta and coverslips, with a solution of lactophenol and cotton blue (to stain hyaline structures). With the help of a composite microscope, the structures at 40 and 100x were observed and they were identified with the taxonomic keys for imperfect fungal genera of Barnett and Hunter (1998).

The morphometric evaluation was carried out from mounts in porta and coverslips of the structures of the mycelium (conidia, hyphae, spores, septae and branches) in digital microscope with integrated camera (AM4023x) and with support of the measurement software DinoCapture 2 for obtaining of the dimensions of the structures evaluated.

The molecular identification was made for the fungus *Nodulosporium* sp., due to the lack of information on important crops and it was carried out by extracting and evaluating the DNA of the fungus, following the CTAB method (Almeyda *et al*., 2001) and subsequently the method PCR, using the universal initiators ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') (White *et al*., 1990). The amplification and visualization of the PCR product was carried out based on the protocol of Ahrens and Seemüller (1992), with modifications in the PCR reactions and whose volume was 25 µL [sterile ultrapure water: 11.9 µL, 10X Buffer (2.5 µL) dNTPs at 10 mM (0.4 µL), initiators 1TS4 and ITS5 at 5 mM (3 µL each), DNA polymerase (Green Taq DNA Polymerase, GenScript®) at 1U (0.2 µL) and shows DNA problem at 96.7 ng (4 µL)].

The PCR reaction conditions were: 1 initial denaturation cycle at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 10 s, 35 cycles of alignment at 57 °C for 30 s, 35 extension cycles at 72 °C for 45 s and 1 cycle of final extension at 72 °C for 5 min. The amplified product was visualized on a 1% agarose gel by electrophoresis. The PCR product was purified with the *In vitro* gene band kit (QuickClean II Gel Extraction Kit [100rxns], GenScript®) and the purified product was sent for sequencing in two directions (5' to 3' and 3' to 5') to the National Laboratory of Agricultural, Medical and Environmental Biotechnology (Lanbama) of the Potosino Institute of Scientific and Technological Research AC (Ipicyt), the result obtained was compared with the sequences in the database of the gene bank of the National Center for Biotechnology Information (NCBI, 2018).

Spore suspension

The source of inoculum was obtained from spores of the PDA medium, obtained with a sterile glass rod and placed in a 10 mL test tube diluted in sterile distilled water. The count was made in Newbauer chamber to determine the concentration of spores in the suspension ($1x10^8$ spores mL^{-1}).

Pathogenicity tests

Healthy 15-20 cm fruits were harvested from plants without the presence of any type of damage. The inoculation in the fruits was done by puncture, with dissection needle colonized with spores of the fungus *Nodulosporium* grown in PDA medium, placing the inoculum in the distal part (tip) of the fruit. In this test 10 fruits were used, and four replicas were made, placed on sterile brown paper during the study.

The foliar inoculation was performed with the detached leaf technique (Pettitt *et al*., 2011) on a plastic tray of 25 cm, with a suspension of $1x10^8$ spores mL^{-1}, placing this suspension on the adaxial surface (beam) in a total of 10 leaf, replicated three times. In both tests an absolute control was considered in which only sterile distilled water was applied following the same methodology. The tests were carried out under controlled conditions at a temperature of 25 ±2 °C, relative humidity of 70 ±5% and 12:12 light:dark. In addition, the foliage test was hydrated with a small cotton swab saturated with sterile water and replenishing this moisture when necessary. When symptoms appeared in the inoculated tissue, the pathogen was re-isolated in PDA culture medium and identified again.

Results and discussion

Morphological, morphometric and molecular identification

In the PDA culture medium, several spore fungi were isolated from the damaged tissue of the cob and diseased plant tissue with rot symptoms in cocoa. Each fungus was purified and identified, finding *Aspergillus* sp. Micheli (Trichocomaceae), *Penicillium* sp. Link (Trichocomaceae), *Rhizopus* sp. Ehrenb (Mucoraceae), *Trichoderma* sp., Persoon (Hypocreaceae) with an incidence of 85% and *Nodulosporium* sp. Preuss (Xylariaceae) with 15% incidence in isolates. The absence of the fungus *M. roreri* is highlighted, which is considered the causal agent of ear rot or moniliasis in several cocoa producing regions in Mexico (Phillips-Mora *et al*., 2006; Phillips-Mora *et al*., 2007).

In this study, the presence of the fungus *Nodulosporium* (= *Nodulisporium*) was registered for the first time in fruits of the cocoa crop in Chiapas, Mexico. In Mexico, the genus *Nodulosporium* has been found in mesquite (*Prosopis laevigata* L.) (Fabaceae) in the state of Puebla (De la Torre-Almaraz *et al*., 2009), in the flower chalice of Jamaica *Hibiscus sabdariffa* L. (Malvaceae) in the state of Guerrero (Ruiz-Ramírez *et al*., 2015) and in cacahuananche *Gliricidia sepium* (Jacq.) Kunth ex. Walp. (Fabaceae) in the state of Morelos (Sánchez- Fernández, 2016). In the cultivation of cocoa, the genus *Nodulosporium* has been mentioned by Márquez-Dávila *et al*. (2013), isolated from leaves and stems of native cocoa in upper Amazon basins of Peru.

Nodulosporium sp., presented a filling of the Petri dish (9 cm diameter) in 7 days, developing at first a mycelial colony of whitish color and of cottony appearance, 5 days after sowing, the maturation of conidia appeared, becoming beige color (similar to what was observed in diseased tissue), later on the seventh day, mature colonies of conidia became dark brown (Figure 1).

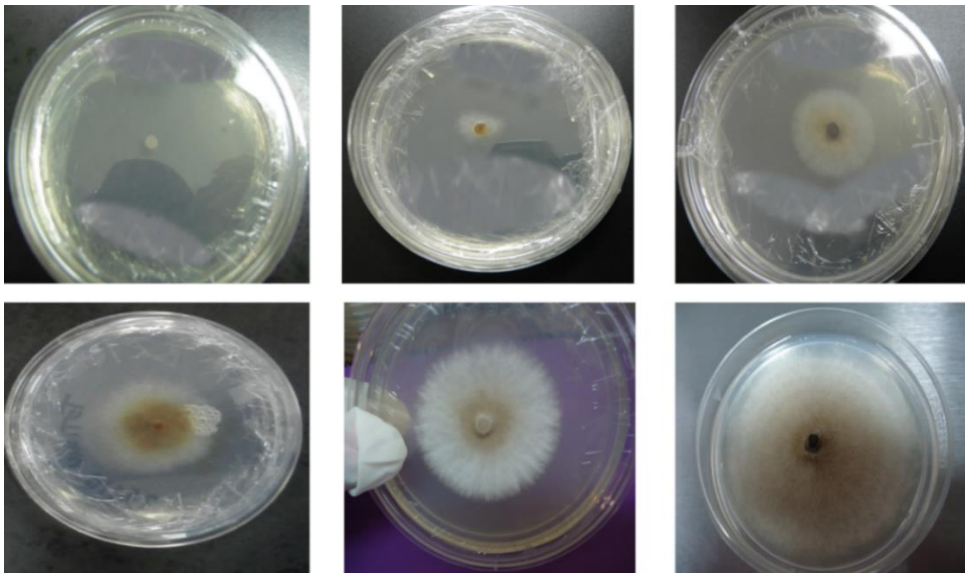

Figure. 1. Radial growth of *Nodulosporium* sp., in a Petri dish at 7 days.

Nodulosporium sp., presents slightly septate hyphae of light brown color, which are stained light blue in the center (effect of lactophenol and cotton blue), conidioespores in the sarcinated conidiophore, almond-shaped hyalines, which took on a light green color (effect of lactophenol and cotton blue). The wall or membrane of the spore slightly yellow (Figure 2). In addition to presenting a characteristic smell of moss.

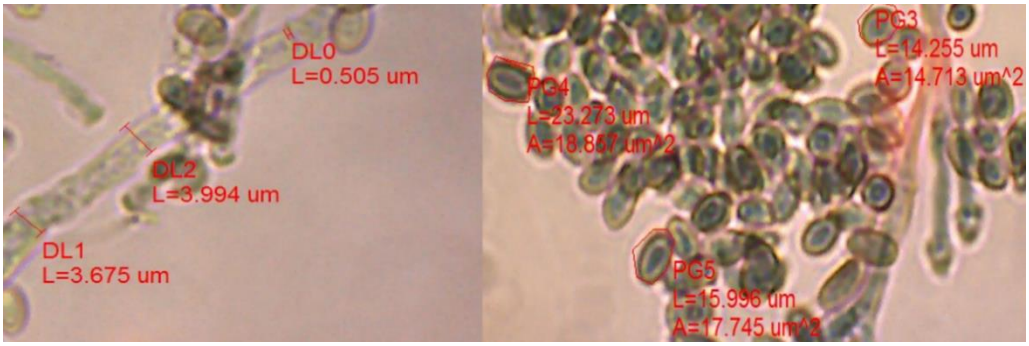

Figure. 2. Detail of hyphae and spores of *Nodulosporium* sp., at 40x.

The observation of the digital microscope allowed observing the structures of the mycelium, with a size (L= length) of conidia of 117.189-117.589 µm, thickness of hyphae of 3.675-3.994 µm, septa thickness of 0.505 µm, branch length of 34.797- 75.848 µm, branching thickness of 3.194-3.761 µm, spores of 13.415-23.273 µm in polygonal shape (the periphery) and an area of 12.816-21.666 μm^2 (A= area) and a spore membrane thickness of 1.197- 1.249 µm (Figures 2 and 3).

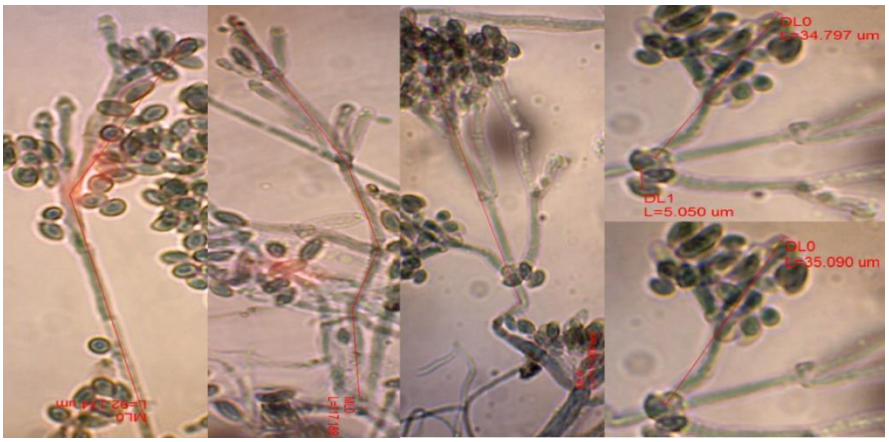

Figure. 3. Detail conidia and branches of *Nodulosporium* sp., at 40x.

From the pathogenicity tests on foliage, the fungus was recovered, which was isolated from the tissue and placed in PDA culture medium. Only mycelium and conglomerates of hyphae (pycnidia) were obtained with a colored gelatinous substance (mucus) of light orange color on and on the periphery of the conglomerate (Figure 4).

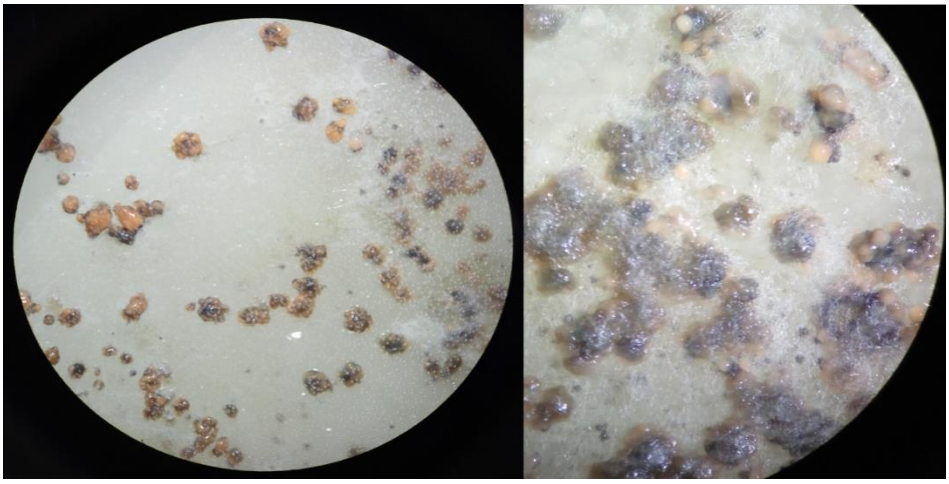

Figure 4. Observation of pycnidia of *Hypoxylon* sp., in PDA media.

These structures were mounted in porta and coverslips with lactophenol solution and cotton blue, where the formation of stroma was observed with spores, which are hyaline in color (they never turned dark, even in the dark for a period of 24 days), unicellular, ellipsoid-inequilateral, with slightly more rounded ends, with elongated germinal opening (11.5 µm) and presence of dehiscent perispore (length: 13.188-14.526 µm, width 4.342-5.427 µm) hyaline (Figure 5a, 5b), structures characteristics that based on the keys of Barnett and Hunter (1998), is identified as the genus *Hypoxylon* Bull. (Xylariaceae) teleomorph of *Nodulosporium*, for which both are related; in this sense Ju and Rogers (1996), recognize *Nodulosporium* as anamorph or sexual phase of several species of the genus *Hypoxylon*.

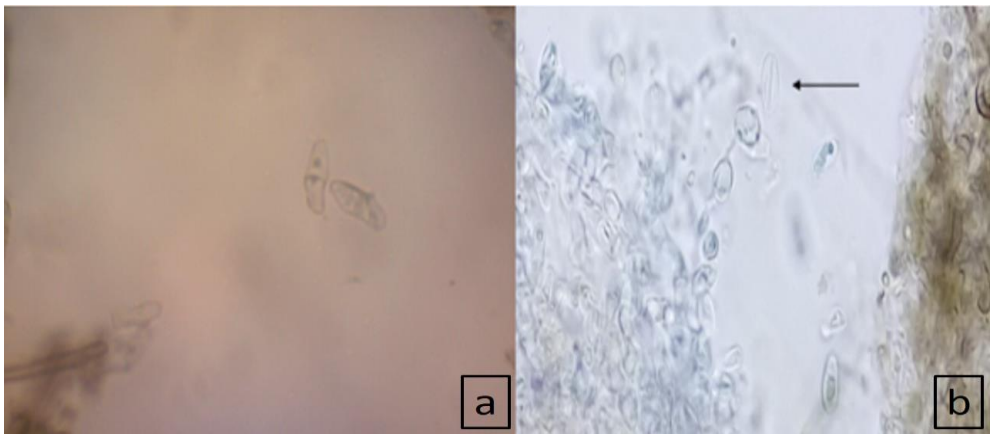

Figure 5. a) stroma of *Hypoxylon* sp., differentiating; b) hyaline spore without maturing.

The PCR product was obtained from the DNA of the mycelium of *Nodulosporium* and of pycnidia of *Hypoxylon*; both with a 600 bp product with oligonucleotides ITS4-ITS5. Two sequences were obtained for *Nodulosporium* and 3 for *Hypoxylon*, for comparison in the database of the gene bank of the BLAST algorithm (NCBI, 2018). The identity of the isolated fungus of cocoa fruits with rot and that recovered in the foliage pathogenicity test obtained from the municipality of Villa de Comaltitlán in the state of Chiapas was confirmed, revealing *Hypoxylon* in the 5 homologous sequences in two isolates in the GenBank., from the United States of America (accession number: KF496192.1) with a 100% identity (Table 1).

Table 1. Comparison of sequences in the NCBI gene bank.

Morphological identification	Primer's of sequence	Total score	Valor E	Identity (%)	Access no.	Molecular identification
Hypoxylon sp.	ITS4- ITS5	501	4e-138	100	KF496192.1	*Hypoxylon* sp.
Nodulosporium sp.	ITS4- ITS5	501	4e-138	100	KF496192.1	*Hypoxylon* sp.
Nodulosporium sp.	ITS4- ITS5	501	4e-138	100	KF496192.1	*Hypoxylon* sp.
Hypoxylon sp.	ITS4- ITS5	501	4e-138	100	KF496192.1	*Hypoxylon* sp.
Hypoxylon sp.	ITS4- ITS5	501	4e-138	100	KF496192.1	*Hypoxylon* sp.

Pathogenicity tests

The pathogenicity test with cocoa fruits during a period of 15 days of evaluation showed slight symptoms of growth of the inoculated fungus; however, the fruits of this test coming from field, were naturally sick with rotting, this condition was even observed in the fruits that were used as a control inoculated only with sterile distilled water. The natural infection under laboratory conditions showed rapid growth, which indicates that the interior of the fruit was already invaded before the inoculation. From the fourth day of inoculation, the first asymmetric dark brown and bright spots were observed, after the eighth day the fruit was completely covered by the spot with the presence of mixture of whitish, orange, green and brown mycelium, observing rottenness in high severity.

The pathogenicity test with foliage showed sensitivity 24 h after inoculation, with chlorosis observed on the leaf which exhibited a dehydration process (drying without losing the green color completely) from the inoculation point and after 48 h presented growth of whitish mycelium, remaining in this way until the end of the trial (7 days in total) (Figure 6). This lesion was observed only in 30% of the inoculated samples. In the control inoculated with sterile distilled water, no symptoms or lesions of the presence of the pathogen were observed. In the symptomatic samples the fungus was re-isolated, finding only the fungus inoculated in the form of pycnidia and mycelium with conidia, corresponding to the teleomorph of *Nodulosporium*, identified as *Hypoxylon*. This result indicates that Nodulosporium is slightly pathogenic in cocoa foliage, causing chlorosis and dehydration of the leaf.

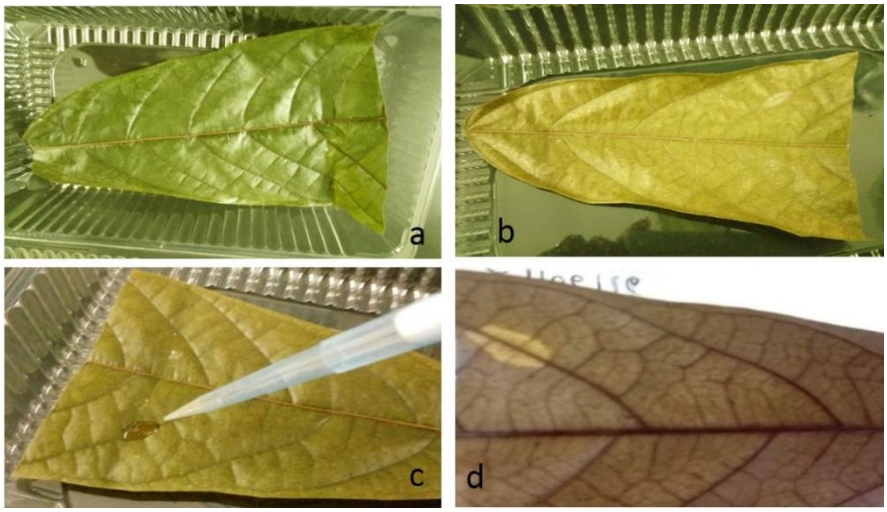

Figure 6. a and b) cocoa leaf without presence of damage by *Nodulosporium* sp. (control); c and d) presence of damage by *Nodulosporium* at 48 h in cocoa foliage.

The genera *Nodulosporium* and *Hypoxylon* have been reported as phytopathogens, causing descending death, black trunk rot and resinous mesquite branches *P. laevigata* (De la Torre-Almaráz *et al*., 2003; De la Torre-Almaráz *et al*., 2009). *Nodulosporium* has also been found to cause basal rot of the stipe in African oil palm *Elaeis guineensis* Jacq. (Arecaceae) (Mestizo *et al*., 2012) and basal rot in the chalice of Jamaica flower *H. sabdariffa* (Ruiz-Ramírez *et al*., 2015).

The genus *Nodulosporium* has been associated as an endophyte to various plants; Collado *et al*. (2001) report it in trunk bark, leaves and healthy and dry branches of *Quercus ilex* L. (Fagaceae). Salgado and Cepero (2005) found it in leaves of *Rosa hybrida* L. (Rosaceae), Márquez-Dávila *et al*. (2013) isolated from cacao leaves and stems *T. cacao*, Lizarazo-Medina *et al*. (2014) in leaves of two species of orchids *Cattleya percivaliana* and *Cattleya trianaei* (Orchidaceae) and was also isolated from cacahuananche (*G. sepium*) with potential as an antagonist against fungi and oomycetes (Sánchez-Fernández, 2016).

Species of the genus *Hypoxylon* have been reported as endophytes in tissues of woody or herbaceous plants (Petrini and Petrini, 1985), as well as weak saprophytes or phytopathogens in wood from temperate or tropical trees (Miller, 1961). For Mexico, this genus is the second with the

highest number of species in the Family Xylariaceae (San Martín, 1992) with 41 identified species, 14 registered for the state of Chiapas in seven municipalities, found on wood, mainly in high evergreen tropical forest, medium tropical subdeciduous forest, tropical low deciduous forest, pine forests and mesophilic mountain forest (San Martín, 1999).

Recognizing the diversity of pathogens associated with the cultivation of cocoa is of utmost importance, because they can pose a threat to the crop in the future.

Conclusion

The fungus *Nodulosporium* sp., isolated from diseased plant tissue with symptoms of rot in *Theobroma* cacao pod for the state of Chiapas, is identified and its teleomorph identified as *Hypoxylon* is confirmed. Also, its pathogenicity on cocoa foliage causes chlorosis and dehydration of the leaf.

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