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Conidial germination of *Botryosphaeria dothidea* Mough.: Fr (Ces. & De Not.) and histological alterations on stems of pitahaya (*Hylocereus undatus* H.) (Haworth) Britton & Rose

Germinación de conidios de *Botryosphaeria dothidea* Mough.: Fr (Ces. & De Not.) y alteraciones histológicas en tallos de pitahaya (*Hylocereus undatus*) (Haworth) Britton & Rose

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ABSTRACT

Conidial germination of *Botryosphaeria dothidea* (anamorph*: Fusicoccum*) in sterile distilled water and 1% sterile dextrose solution was evaluated at 4, 6, 12, 24 and 36 h after incubation. Also, it was described the anatomical changes on pitahaya stems induced by this fungus, collected in the field and artificially inoculated in the laboratory. Conidial germination was less than 30% in water and it was improved when 1% dextrose was added to the water. In 1% dextrose solution the germination was 90% after 4h of incubation and 100% at 6 h. Pathogen germ tubes had entered through wounds and sometimes through stomata and hyphae colonized intra and intercellularly in the parenchyma-chlorenchyma tissues. On naturally and artificially diseased stems the main alterations were: destruction of cuticle, hyperplasia of epidermal and collenchymatous

RESUMEN

Se evaluó la germinación de conidios de *Botryosphaeria dothidea* (anamorfo: *Fusicoccum*) en agua destilada estéril y en una solución de dextrosa 1% a las 4, 6, 12, 24 y 36 h de incubación. También, se describieron los cambios anatómicos en tallos de pitahaya inducidos por este hongo, tanto aquellos naturalmente infectados en campo como inoculados artificialmente en laboratorio. La germinación de conidios en agua estéril solo alcanzó el 30%, mientras que la adición de dextrosa al 1% mejoró la germinación. En una solución de dextrosa al 1% la germinación a las 4 h fue de 90% y del 100% a las 6 h. Los tubos germinativos del hongo penetraron a través de las heridas y algunas veces a través de los estomas y se multiplicaron inter e intracelularmente en los tejidos del parénquima-clorénquima. En tallos enfermos natural y artificialmente, las

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hypodermal cells and conform the advance of the pathogen a layer of lignified periderm was formed surrounding the damaged tissues; however, it couldn't stop the advance of the pathogen and the cells that surrounded the lesion suffered necrosis.

principales alteraciones fueron: destrucción de la cutícula, hiperplasia de las células epidermales e hipodermales colenquimatosas. Conforme el avance del patógeno se formó una capa de peridermis lignificada que rodeó el tejido dañado; sin embargo, no se detuvo el avance del patógeno y las células que rodeaban la lesión se necrosaron.

Keywords

Hylocereus undatus • dragon fruit • histopathology • stem spot

Palabras clave

Hylocereus undatus • fruta del dragón • histopatología • mancha del tallo

INTRODUCTION

Pitahaya or dragon fruit, *Hylocereus undatus* (Haworth) Britton & Rose (Cactaceae), is a semi-epiphyte plant of anthropocentric importance for its fruits and stems at national and international level (27). In addition, some parts of the plant have uses in herbal medicine and also in agro industry (20, 21). This cactus species is cultivated in at least 10 different countries worldwide and presents a promising future production increase (7). Mexico ranks third in acreage and volume of production (145 hectares and 1450 tons). The second place is occupied by Colombia (250 hectares) with a volume of production of 2500 tons, and the first place is occupied by Nicaragua with 560 hectares and 5600 tons (24, 26). In those countries, the plant sanitary measures are limited with higher risks for incidence of the diseases.

Stem spot caused by *Botryosphaeria dothidea* (anamorph: *Fusicoccum*) is an important disease of pitahaya (figure 1) that has been spreading in intensive pitahaya productions areas. This disease can reduce the yield up to 44% (26). The disease was first reported in Mexico in 2003 (25).

Figure 1. Symptoms of stem spots caused by *Botryospaheria dothidea* (anamorph: *Fusicoccum*) on *Hylocereus undatus* (Haworth) Britton & Rose. Barr = 1 cm.

Figura 1. Síntomas de manchas en tallo causado por *Botryosphaeria dothidea* (anamorfo: *Fusicoccum*) en *Hylocereus undatus* (Haworth) Britton & Rose. Barra = 1 cm.

Since then, the disease has been observed in main areas of pitahaya production in Mexico. No extensive research has been carried out on this disease with special reference to the histological changes induced on pitahaya tissues.

Additional reports has been conducted to confirm the causal agent of this disease, pathogenicity of this fungus and determine better conditions for *in vitro* cultivation a strain of *B. dothidea* (27).

The fungus *B. dothidea* is the main plant pathogen that can affect the productivity on this crop. Other pathogens and its correspondent diseases on stems are *Pectobacterium carotovorum* (soft rot), *Enterobacter cloacae* (11), *Cactus virus* X (Pitahaya Mosaic Virus) (8), *Collectotrichum gloesporioides* P. (antracnose) (14). Recently, *Alternaria* sp. was found causing similar spots on stem of pitahaya. However, pathogenicity of *Alternaria* has not been confirmed. On fruits, *Botrytis cinerea* can cause rots.

B. dothidea is a pathogen that affects wooden trees penetrating by wounds, lenticels or stomata (1, 13, 16); although the way of penetrating into the plant tissues is variable depending on the host (2).

Stress conditions may be a factor for entering to the plant tissues (28). Just only one report on *Cistus ladanifer* was found on this fungus acting as a primary pathogen (17). In the family Cactaceae, *B. dothidea* was found causing disease on the genera *Selenicereus* and *Rhipsalis* (18, 26).

A particular insect, *Leptoglossus* sp. (Hemiptera:Reduviidae) has been associated as vector transporting spores and disseminating the disease among the fields. Spots will be observed just on the site where the insect feed (26).

On the anatomy of the health *Hylocereus* species not extensive research has been made. Initially, cuticle and three general tissues (epidermal and hypodermal and chlorenchymal tissues) were mentioned (3, 5).

The most recently description on the stem anatomy of *Hylocereus* was made by García *et al.* (4). They differentiate structural components of the primary tissue of three species of *Hylocereus* (*e. g.*, cuticle, epidermis, hypodermis, chlorenchyma and reserving parenchyma) and secondary tissue (*e. g.*, vessels elements with simple perforation plates, scalariform and pseudoscalariform intervascular pits, librifrom fibers, scanty paratracheal parenchyma, and heterogeneous rays) but no changes of these tissues has been recorded to plant pathogens affecting stems of pitahaya.

The purpose of the present study was to evaluate germination of conidia in water and 1% dextrose solution and to determine histological changes in artificially and naturally infected pitahaya stems tissues.

MATERIALS AND METHODS

Fungus isolation and inoculum production

The isolate of *B. dothidea*, obtained consistently from a diseased commercial pitahaya orchard in 2001 from Huitziltepec and Xochitlán Todos Santos, Puebla State in Mexico, was used throughout this study.

To isolate the pathogen, small pieces (approximately 5 mm in diameter) from infected pitahaya stems with brown lesions were surface sterilized with 1% sodium hypochlorite for 2 min. After rinsing with sterile distilled water, tissues were blotted on sterilized paper towels, plated on fresh potato- dextrose-agar (PDA), and incubated at 22-25°C. Fungi growing from the plated tissues were individually transferred again onto PDA (Bioxon, USA), and incubated at 22-25°C under continuous fluorescent light for two weeks. The stock culture of *B. dothidea* was stored in distilled sterile water vials in the refrigerator at 4°C for long term. To prepare spore suspensions, Petri dishes were flooded with sterile water, and the resulting spore suspension was then filtered through two layers of sterile cheesecloth. To study the infection process spore concentration was adjusted with a Neubauer counting chamber to a 50000 spores per milliliter.

Conidial germination

In Petri dishes, five milliliter of sterile distilled water and 1% sterile dextrose solution (three replications) were inoculated with 50000 spores/milliliter and incubated at 22 ± 3°C for 4, 6, 12, 24 and 36 h.

After 24 h of incubation 1% of dextrose was added to the water to determine the effect of the dextrose on the germination of spores. Aliquots from each suspension were taken and the percentage of those that produced hyphal growth was counted (100 spores per replicate) microscopically.

Artificially inoculated pitahaya stems for histology studies

The fungal suspension of *B. dothidea* was adjusted to a concentration of $5x10^4$ spores per milliliter using a Neubauer counting chamber. The conidial suspensions were deposited (20 µL) onto the 15 cm wounded stems using Pasteur pipette.

The inoculated stems were put into plastic boxes and incubated at 22 ± 3°C until the time of collection of samples (36 h and 95% of relative humidity). Control stems (three) were wounded and inoculated with sterile distilled water and incubated using the same methods employed for inoculated plants. Stem samples (5 mm) were collected at 2, 4, 6 and 36 hours after inoculation (hai). Stem samples from control plants were also collected and processed for histology.

Pitahaya stem naturally infected with stem spots for histology of studies

Pitahaya stems with typical symptoms of naturally occurring spots were collected from plantings in Huitziltepec and Xochitlán Todos Santos, State of Puebla, Mexico in 2000. Most samples were taken from red fleshy cultivars of pitahaya or dragon fruit (*H. undatus*).

Histology studies

For histology studies, 15 samples of naturally and artificially inoculated stems were examined. Tissues were also collected from the control plants to elucidate anatomy of the healthy stems of pitahaya. The stem tissues including the lesions were cut and fixed in Formalin-Aceto-Alcohol (FFA, No. 1 solution of 90 mL of 50% ethanol, 5 mL of formalin, and 5 mL of glacial acetic acid) for at least 1 day. The samples were soaked with 70% ethanol for a few minutes to soften the tissues, then rinsed in running tap water and dehydrated through ethanol series: 50, 70, 85, 95, and 100% (6).

They were embedded in Paraplast (Sherwood Medical Industries, St. Louis, MO) embedding medium mixed with *n*-buthyl alcohol two hours each. Serial sections (10 µm thick) were cut on a Spencer 820 rotation microtome (American Optical Company, Ramsey Minnesota, USA). The ribbons were mounted on clean glass slides with grenatine as an adhesive in a water bath at a temperature of 60°C (6). After that, sections were stained using differential fast safranine-green reaction (6).

The paraplast was removed from the tissue sections by means of three changes of xylene, and were dehydrated in a gradual series of ethylic alcohol 100, 96 and 50% during three minutes in each one. Then, the tissues were stained with 1% safranine in 50% ethylic alcohol during 30 min; later they were washed in ascending series of ethylic alcohol at 50%, 70% and 96% (3 min each one).

After, two to three drops of rapid green were applied at 1% in ethylic alcohol at 96% for 45 seconds, the supernatant was decanted and washed with ethylic alcohol at 96% and 100% and three changes of xylene (3 min each one). Finally, tissues were mounted with synthetic resin between slides for their observation. Micrographs were taken in a light microscope Carl Zeiss (Jena, Germany) integrated with a digital camera.

RESULTS AND DISCUSSION

Conidial germination

In distilled water, less than 30% of conidia of *B. dothidea* germinated until 24 h, although germination was increased as 1% dextrose were added to the water. In 1% dextrose solution, germination of conidia reached 90% at 4h, emitting germ tube (gt) in average of 24.3 µm (long) (16.8-26.5 µm) and 0.24 µm (width). In the same solution, at 6 h all conidia were reported as germinated measuring its gt of 51.99 µm (long) (45.6- 54.6 µm) and 0.24 µm (width).

As reported by Wrona & Grabowski (29) sugar present in the substrate that fungi colonize are their primary source of nutrients and may help to germinate the spores. Sometimes conidia were observed with one or two septa when germination started or previous to the germination. This fact has been recorded as a particular characteristic of this fungus by Mass (10). At 36 h after inoculation conidia were observed with long germ tubes frequently with many ramifications (figure 2a, 2b: pág. 298).

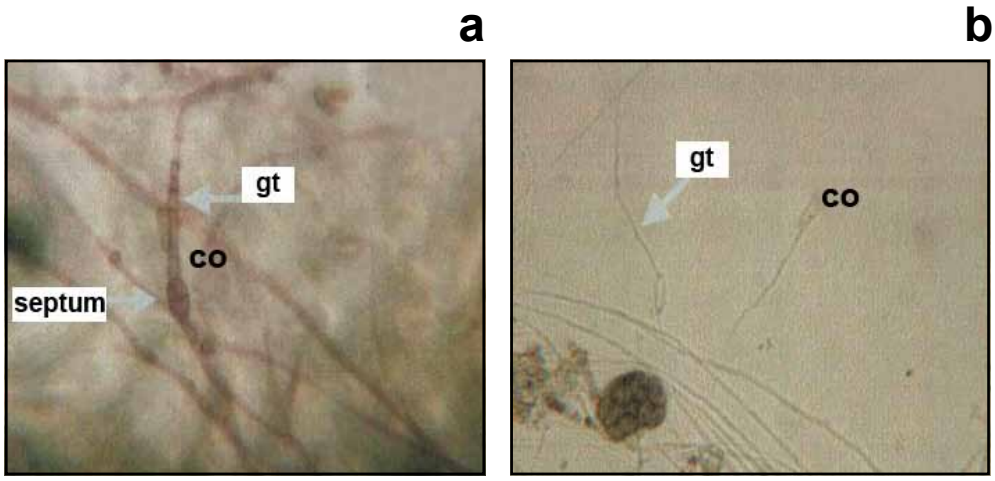

Figure 2. a), b). Conidial germination of anamorph *Fusicoccum* state of *Botryospaheria dothidea* in 1% dextrose suspension at 36 h after incubation. gt = germ tube, co = conidium. Magnifications 40X. Barr = 13.59 µm.

Figura 2. a), b). Germinación de conidios de *Fusicoccum* estado anamorfo de *Botryosphaeria dothidea* en suspensión de dextrosa 1% a las 36 h después de incubación. gt = tubo germinativo, co = conidio. Aumentos 40X. Barra = 13,59 µm.

Anatomy of the healthy stem of *Hylocereus undatus*

In non-inoculated control stems, a smooth cuticle was firstly observed, after it was found a layer of epidermis with cells in rectangular form containing prismatic calcium oxalate crystals (figure 3a, 3b: pág. 299) as reported by species of *Cephalocereus* and *Neobuxbamia* (Cactoideae subfamily) (23).

Subsequently, a collenchymatous hypodermis was observed (figure 3a, page 299) as found in concordance with the results of the study of García *et al*. (4) for three species of *Hylocereus* and others individuals of the subfamily Cactoideae (9). Below the hypodermis, chlorenchyma and reserving parenchyma regions were observed.

Generally, chlorenchyma region is characterized by 3 to 5 of palisade parenchyma cells and reserving area is constituted by parenchyma cells and circular idioblasts containing mucilage (figure 3c, page 299) as well lenticular and sandstone crystals deposited in the lumen of some parenchyma cells (4).

Vascular tissues constitute the secondary tissue and it consists on a unidirectional periderm (4, 22) and secondary xylem presenting simple perforation plates (figure 3d, page 299), alternative scalariform and pseudoscalariform intervessel pits, rare paratracheal parenchyma and heterogeneous radios that characterizes the subfamily Cactoideae (4, 12, 23).

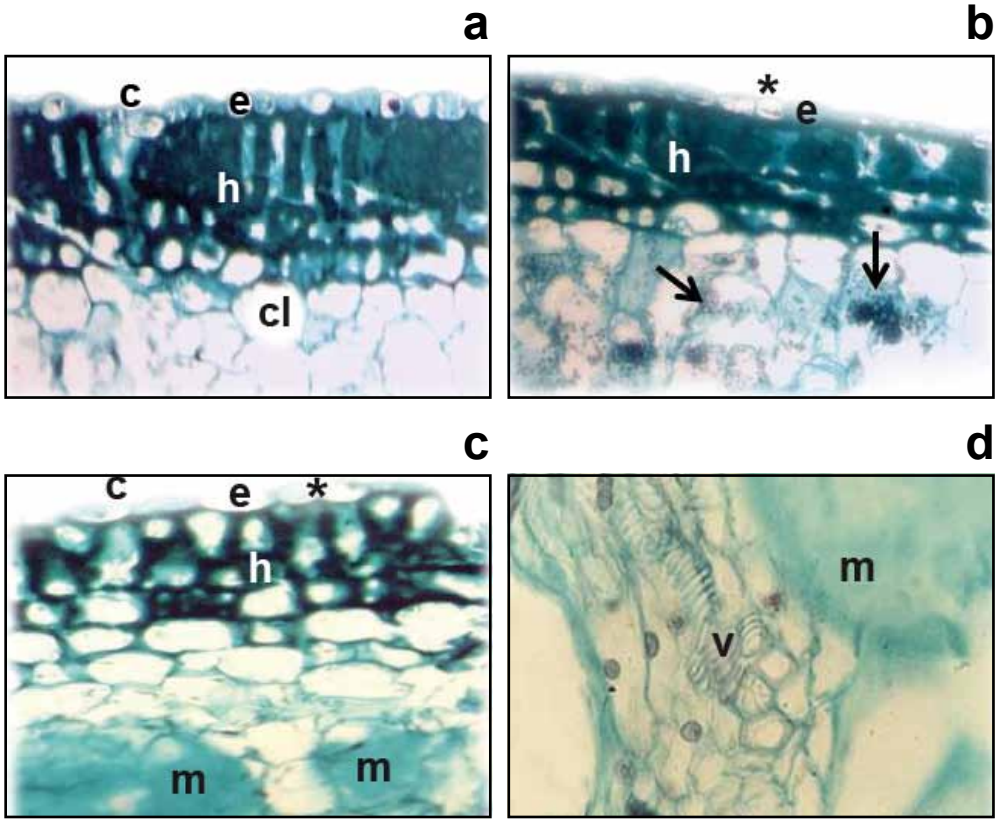

Figure 3. Healthy anatomy of pitahaya (*Hylocereus undatus*) stem. Transversal views. **a)** and **b)** Smooth cuticle, Collenchymatous hypodermis, lenticular crystals; **c)** Reserving parenchyma with mucilage cells, prismatic crystals; **d)** Secondary xylem with scalariform and pseudoscalariform vessels. c = cuticle; cl = chlorenchyma; e = epidermis; h = hypodermis; m = mucilage; v = vessel pits; * = prismatic crystals; arrows = lenticular crystals. Magnifications 40X.

Figura 3. Anatomía de tallo sano de pitahaya (*Hylocereus undatus*). Vistas transversales. **a)** y **b)** Cutícula suave, hipodermis colenquimatosa, cristales lenticulares; **c)** Parénquima de reserva con células de mucílago, cristales prismáticos; **d)** Xilema secundario con vasos escaleriformes y pseudoescaleriformes. c = cutícula; cl = clorénquima; e = epidermis; h = hipodermis; m = mucílago; v = punteaduras de vaso; * = cristales prismáticos; flechas = cristales lenticulares. Aumentos 40X.

Histological changes in natural and diseased stems

Conidia deposited on the surface of stems germinated four hour after inoculation. Wounds on stems favored entrance for penetration and colonization of *B. dothidea*. Once into the tissue fungus hyphae was found colonizing intra and intercellularly the parenchyma and chlorenchyma regions.

Similar changes were observed for natural and artificially diseased stems. The first alteration observed was a destruction of the cuticle and hyperplasia of epidermal cells, and granular deposits of polyphenols within the cells (figure 4a, 4b), as well as development of intertwined hyphae of the fungus in the epidermis and into the hyperplasic cells. Later, the fungus continued toward the collenchymatous hypodermis and some suffered hyperplasia (figure 4c).

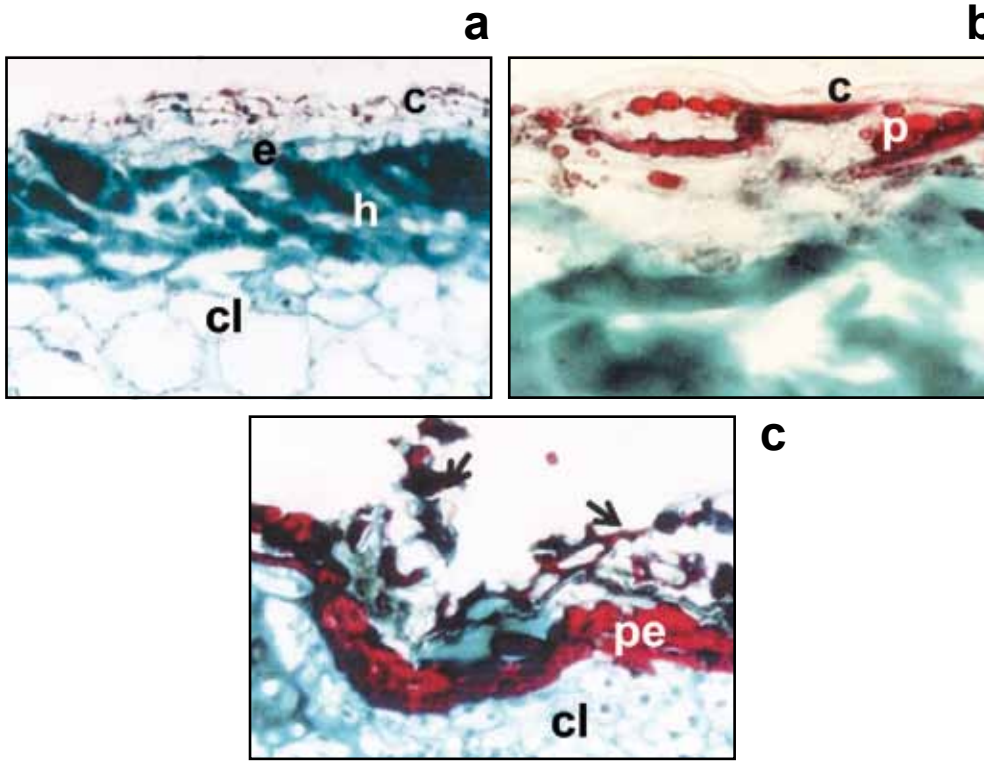

Figure 4. Photomicrographies of the transversal sections of anatomical changes of artificially and naturally infected stems. **a)** Collapsed cuticle and hyperplasia of epidermal and hypodermal cells. **b)** Polyphenols and collapse of cuticle. **c)** Lignified periderm, hyperplasia of the chlorenchymal cells and necrosis of the cuticle, epidermis and hypodermis in conjunction with the hyphae of the *B. dothidea*. c = cuticle; cl = chlorenchyma; e = epidermis; h = hypodermis; p = polyphenols; pe = periderm; arrows = necrotic tissues. Magnifications 40X.

Figura 4. Fotomicrografías de secciones transversales de cambios anatómicos de tallos naturalmente y artificialmente infectados. **a)** Cutícula colapsada e hiperplasia de células epidermales e hipodermales. **b)** Polifenoles y colapso de la cutícula. **c)** Peridermis lignificada, hiperplasia de células del clorénquima y necrosis de la cutícula, epidermis e hipodermis en conjunto con la hifa de *B. dothidea*. c = cutícula; cl =clorénquima; e = epidermis; h = hipodermis; p = polifenoles; pe = peridermis; flechas = tejidos necróticos. Aumentos 40X.

The final stage of the infection was characterized by a necrosis of the cells of the chlorenchyma region affecting palisade parenchyma cells and reserving area. As a response of the plant to the progress of the infection a lignified layer of the periderm and necrotic lesions were formed to prevent the advance of the fungus toward the interior of the tissues. Similar responses were observed in other studies with ophiostomatoid fungi (15, 19). Sometimes all necrotic tissue can detach and holes were observed in the stems. Lignified cells of periderm were founded in healthy stem of *Wilcoxia* species. In our study we found no periderm in healthy tissue, so we suggest that lignification of the periderm cells is a response of the plant cells to the progress of the infection.

CONCLUSIONS

In conclusion, conidial germination *in vitro* was favored by 1% dextrose. The main anatomical changes were: collapse of the cuticle, hyperplasia of epidermal cells and collenchymatous hypodermis, presence of polyphenols in the epidermis and formation of lignified periderm.

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