Preinfection of *Botrytis cinerea* on poinsettia leaves (*Euphorbia pulcherrima*)

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ORIGINAL

Preinfección de *Botrytis cinerea* en hojas de nochebuena (*Euphorbia pulcherrima*)

Resumen. Se comparó el desarrollo de *Botrytis cinerea* sobre las hojas de dos cultivares ("Nutcracker White" y "Supjibi") de nochebuena (*Euphorbia pulcherrima*), con diferentes grados de susceptibilidad. Las hojas de ambos cultivares fueron inoculadas con conidios de *B. cinerea* suspendidos en agua destilada y en una solución de glucosa. La germinación de los conidios se inició a las 2 h formándose un tubo germinativo en uno de sus polos. Las diferencias en los porcentajes de germinación sobre las hojas de los dos cultivares fueron evidentes; a las 12 h el porcentaje de germinación en las hojas del cultivar "Supjibi" fue significativamente más bajo (α=0.05) con el inóculo suspendido en agua destilada. La longitud del tubo germinativo a las 12 h fue mayor sobre las hojas del cultivar "Nutcraker White" y hubo una diferencia significativa con el inóculo suspendido en la solución de glucosa. Se formaron más apresorios sobre las hojas de ambos cultivares cuando se añadió glucosa al inóculo.

Palabras clave: Flor de nochebuena, germinación conidial, apresorios.

Abstract. The development of *Botrytis cinerea* on leaves of poinsettia (*Euphorbia pulcherrima*) cultivars ("Nutcracker white" and "Supjibi") with different degrees of susceptibility is discussed. Leaves of both cultivars were inoculated with conidia suspended in water and glucose solution. Conidia germination began 2 h after inoculation, forming a germ tube at one pole. The difference in percentages of germination on the leaves of the two cultivars and with both types of inoculum was evident; at 12 h, the lowest percentage of germination in the leaves of the cultivar "Supjibi" was significantly lower (α= 0.05) with the inoculum suspended in distilled water. Germ tubes length at the 12 h was greater on the leaves of the cultivar "Nutcracker White", and there was a significant difference with the inoculum that contained glucose. More appressoria were formed on the leaves of both cultivars when 3% glucose was added to the inoculum.

Key words: Poinsettia, conidial germination, appresoria.

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Introduction

Botrytis cinerea Pers. is a geographically widely distributed pathogen which causes major diseases in many foods and ornamental crops [7] and has even been reported as a pathogen of conifer seedlings cultivated in nurseries [4]. It is the most important pathogen in greenhouse poinsettia plants (*Euphorbia pulcherrima* Willd. ex Klotzch) during their propagation, on foliage during the growth stage, or on bracts during the flowering period [21]. Under favorable moisture conditions, fungus infections begin mainly on the edges of leaves and extend to cover most of the surface. Damaged

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tissues necrose rapidly, and the symptoms reported by other authors appear [2, 21].

Although no information was found in the literature on the infective process of *B. cinerea* in poinsettia plants, differences in susceptibility to infection by *B. cinerea* have been reported to exist among poinsettia cultivars. For example, Witte & Miller [22] reported that in the cultivar "Reddy Light" development of lesions was not observed and that the cultivars "Annette Hegg Diva", "Annette Hegg Lady", "Scarlett Ribbons" and "Eckspoint Laurie Pink" were highly susceptible. Also, Manning *et al* [11] reported a high degree of resistance in leaves and bracts of the cultivars "Mollgard", "Eckespoint C-1" and "Imperial Red Rochford". For other hosts, differences in susceptibility to infection by *B. cinerea* have been detected among cultivars. For example the rose cultivars "Sonia", "Madelon" and "Melody" are highly susceptible, while "Carambola", "Gabriella", "Pasadena" and "Rubinette" are more resistant to the infection [15]. These authors conclude that the milder nature of the disease in the more resistant cultivars is due to inhibition of hyphae growth after penetration. However, among the cultivars "Supra" and the more resistant "Royalty" the difference in susceptibility was related to cuticle thickness, among other factors [7].

Plant surface structures and chemicals can strongly influence the susceptibility of a plant to disease by inducing tropic mistakes by the fungus which may result in a failure of pathogenesis [14]. Before penetration, the fungus responds to the plant surface at different development stages, including germination, germ tube growth and the formation of appressoria [23]. The prepenetration stages of infection have been studied extensively in a range of plant pathogens and, in some cases, have shown to play a role in host resistant [8].

Differences in susceptibility to *B. cinerea* exist between the cultivars "Nutcracker White" and "Supjibi", the former being more susceptible, according to the experience of poinsettia producers. This study was conducted to compare the development of *B. cinerea* on the leaves of the cultivars with different degrees of susceptibility. The particular objective was to determine whether, in resistant poinsettia cultivar, the fungus was less successful in completing the essential prepenetration stages of development.

Materials and methods

Inoculum. *B. cinerea* was isolated from leaves and stems of diseased poinsettia plants. The fungus was cultivated in PDA (potato dextrose agar) until sporulation. The monosporic colonies used in bioassays were12 to 15 days old. Two types of inoculum were prepared: 1) the conidia suspended only in sterilized distilled water and 2) the conidia were suspended in 30 g/L glucose. In both cases the concentration of inoculum was 1 x 10^5 conidia per mL.

Plant material. The poinsettia plants used were from two cultivars: "Nutcracker White" (A) and "Supjibi"(B), two months old, obtained from greenhouses located in municipality of Jiutepec, Morelos (Mexico).

Inoculation of detached leaves. To determine the germination and formation of the structures involved in *B. cinerea* infection, only the five upper leaves of plants were used, since these are more affected by greenhouses conditions. The leaves were separated from the plants and disinfected with 5% sodium hypochlorite and rinsed with sterile distilled water. The inoculum in water was applied to 30 leaves of each cultivar and the 30 g/L glucose inoculum was applied also to another 30 leaves. With an atomizer, approximately 5 mL of inoculum was sprayed over the leaf surface. The leaves were then placed in a humid chamber and incubated at 20-22 °C under continuous artificial light conditions.

Microscopic studies. Four 1 cm^2 samples were taken from each leaf, of each cultivars and inoculum, at intervals of 2 h up to 12 h after inoculation. Samples of leaf tissue were clarified in a Carnoy solution (60 mL ethylic alcohol, 30 mL chloroform, and 10 mL glacial acetic acid) for 24 h. Samples were rinsed with distilled water, stained with fuchsin acid to 0.05% in lactophenol, and mounted in glycerin for observation. Germination of conidia and formation of the structures of infection were determined using the 40X objective, through observation of 25 conidia from four leaf tissue samples. In five cross-sections of 12 h leaves, 20 conidia were observed to determine the length of the germ tubes and appressoria formation. Following the procedure indicated above, another five 0.5 cm^2 pieces of inoculated leaves from each cultivar were taken and studied with an electronic scanning microscopic. These samples were fixed in glutaraldehyde at 3% for 2 h and washed in phosphate buffer, pH 7.2, 0.1 M. Later, they were post-fixed in osmium tetraoxide for 1 h and washed in the phosphate buffer, pH 7.2, 0.1 M. These samples were dehydrated in gradual solutions of ethanol, dried to critical point with carbon dioxide (Samdri-780A. Mod. 20852. USA) and placed on adhesive tape on slides. The samples were covered with a layer of gold Å thick (Ion Sputter JFC-1100 Jeol, Tokio, Japan) and observed under an electronic scanning microscope JEOL JMS 35-C.

Results

Conidia germination. *B. cinerea* conidia germination initiated 2 h after leaf inoculation in both poinsettia cultivars. Generally, only one germ tube emerged from one of the conidia poles (Figure 1). Germination was greater after 2 h, when the inoculum suspension was enriched with glucose, 10.7% in B and 34.2% in A. During this time the conidia suspended in sterilized distilled water had a germination rate of 10.2% and 13.7%, respectively. The highest germination rate occurred between 10 and 12 h after inoculation on the leaf surface of both poinsettia studied (Table 1). The difference in germination percentage on the leaves of the two cultivars and with both types of inoculum was evident. At 12 h the lowest germination percentage on B leaves was significantly lower (α= 0.05) with the inoculum suspended in distilled water.

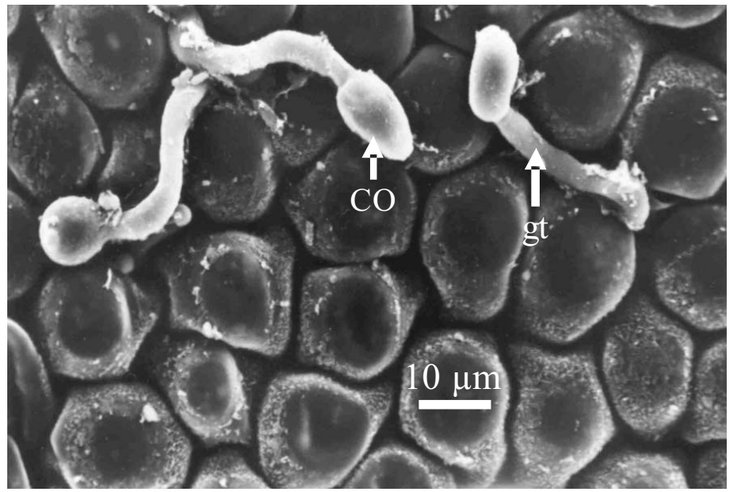

Figure 1. Conidia (co) germination of *Botrytis cinerea* 2 h after inoculation on the leaves of *Euphorbia pulcherrima* cultivar A. The conidia show a germ tube (g t).

Table 1. Germination of *B. cinerea* on poinsettia leaves of the cultivars A and B. Conidia were applied suspended in sterilized distilled water (W) or in a solution of 3% glucose (G)

	time					
Treatments	2h	4h	6h	8h	10h	12h
AW	13.7 b[1]	45 b	60.5 b	70.7 b	81.3 b	93 ab
AG	34.2 a	66.7 a	75.2 a	83.2 a	91.7 a	95.7 a
BW	10.2 bc	30.7 d	40.5 c	47.5 c	61 c	69.7 c
BG	10.7 c	39.7 c	62.7 b	74.7 b	84.7 b	90.7 b

[1]In each column, the figures with the same letter are not significantly different (Tukey, α= 0.05). Figures represent the average of four replications (25 conidia per replication).

Growth of germ tubes. During their growth, the germ tubes followed the epidermal cell contour until they lodged themselves in the anticlinal walls junctures, where they penetrated. At this point, the apex of the germ tubes thickened to form the appressoria. All along the germ tube, a mucilaginous substance was observed; this may have favored its adherence on the epidermal cells surface (Figure 2).

The length of germ tubes at 12 h was greater on the leaves of cultivar A, and there was a significant difference with the inoculum aditionated with glucose. The conidia germ tubes suspended in distilled water on cultivar B was significantly smaller (Table 2).

Appressoria formation. The appressoria formed at anticlinal wall junctures of the epidermis cells on leaves in both cultivars (Figure 2). Data on frequency of appressoria analyzed using a contingency table and the X^2(II) test [20] indicated significant differences, and there was greater formation when 3% glucose was added to the inoculum (Table 2).

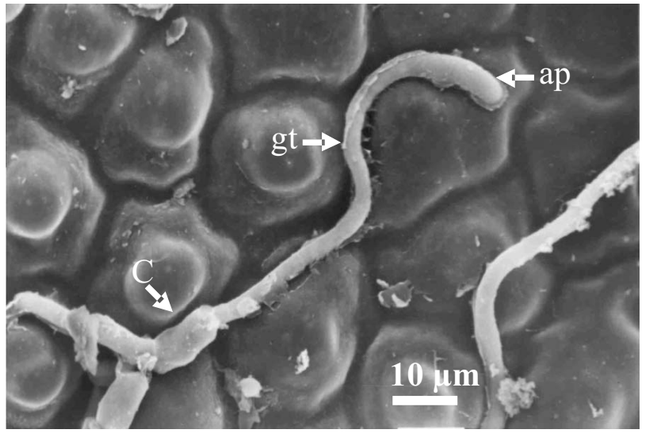

Figure 2. *Botrytis cinerea* on leaves of *Euphorbia pulcherrima* cultivar B with a conidium (c) with a germ tube (gt) and an appressorium (ap).

Table 2. Influence of glucose in the percentage of germ tubes with appressoria and mean length of the germ tubes of *B. cinerea* 12 h after inoculation on poinsettia leaves of the cultivars A and B

	Lengh of the germ tubes[1] (m)		Appressoria
Treatments	Mean	Range	12 h
A W	48.99 ab[2]	18.4 – 126.0	45 c
A G	54.36 a	18.4 – 126.5	82 a
B A	37.66 c	13.8 - 92	36 d
B G	43.34b c	16.1 – 115.1	69 b

[1]Average of 100 conidia.
[2]In each column the figures with same letter are not significantly different (Tukey,α = 0.05).

Discussion

The behavior of *B. cinerea* on the surface of poinsettia plants showed several features which are also found in many other fungal species [14]. The process of *B. cinerea* conidia germination began 2 h after inoculation on leaves of the two poinsettia cultivars. From 4 h on, it was evident that glucose had a favorable effect in inducing greater germination, mainly in the susceptible cultivar A (66.7%). *B. cinerea* germination on its hosts is variable, thus, it has been reported that on tomato fruits, germination of the conidia of this fungus began 3 h after inoculation [17] and at 4 h on gerbera petals [18, 19]. For other hosts, variable percentages of conidia germination have been reported: 21% on broad bean leaves at 3 h [3] and 99% at 4 h [12], 70% on nectarine and prune fruits at 3 h and 80% on flowers of these hosts at 3 h [6]. The percentage of germination of *Botrytis* spp. has been related to the age and concentration of conidia in the inoculum suspension [10], as well as to the effect of physical factors such as humidity and temperature [18]. The addition of glucose in the inoculum is a determinant factor for stimulating the *B. cinerea* germination on its hosts [3, 5] like was observed on the poinsettia leaves.

Botrytis cinerea germ tubes on the surface of poinsettia leaves at 12 h varied greatly in length, from 14 to 115 µm on B and 18 to 126 µm on A, with the inoculum with glucose or water (Table 2). Previous reports have indicated that *B. cinerea* germ tubes are short, 2-4 µm in length, in some cases exhibiting an apex thickening similar to an appressorium on tomato fruits [17]. McKeen [12] also reported that *B. cinerea* on broad bean leaves forms germ tubes 10-20 µm long, while others are 40-100 µm long, forming an appressorium on occasions. Pie & De Leew [16] reported germ tubes of up to 30 µm on rose petals, while on the same tissues, Hammer & Evensen [7] observed short germ tubes of 1-2 µm, typically ending in an appressorium. Heuvel van den & Waterrus [9] reported than on common bean leaves (*Phaseolus vulgaris*), *B. cinerea* formed protuberances at the tip of germ tubes, appressoria or cushion structure; growth of one structure or another was determined by conidial concentration. Previous reports describe the *B. cinerea* development on the leaves of several hosts forming different types of appressoria; however, this pathogen growing on the poinsettia leaves only formed the typical appressorium in both kinds of inocula.

The addition of glucose to the inoculum used in the present study increased the percentage of *B. cinerea* conidia germination on leaves of the two poinsettia cultivars. It was also evident that sugar had the effect of incrementing the length of germ tubes and the formation of appressoria, especially in the more susceptible A. In this sense, Clark & Lorbeer [1] mentioned that nutrients in the inoculum provide the energy necessary for appressoria formation and penetration. However, Heuvel van den & Waterreus [9] stated that the inoculum with glucose is important for germination and growth of the germ tubes, but it does not influence penetration.

It was observed that during their growth the *B. cinerea* germ tubes followed the contour of the epidermal cells on the leaves of the two poinsettia cultivars. The

presence of mucilage on the germ tubes during their progress among the epidermal cells was also detected at the sites of appressoria adhesion. Secretion of mucilage on the surface of *B. cinerea* germ tubes has already been reported [12, 16]. This mucilage in some fungal species contains several enzymes that are important to the infection process [13]. During the development of *B. cinerea* infection structures on the poinsettia leaves, the appressoria were formed on the anticlinal wall cell junctures like was observed in the tomatoes fruits [17] and in the onion leaves [1]; however, the appressoria were formed near of the stomata in the plum and nectarine fruits [6] and in the western larch needles [4].

Several differences in the development of *B. cinerea* on the surface of the susceptible and resistant poinsettia plants were found. Both the light microscopy and SEM studies showed that conidia germination, the average germ tube length and the number of appressoria were higher on the leaves of susceptible cultivar A. Apart from the disponibility of nutrients, other factors involved in host recognition, such as the morphology and chemistry of the epicuticular wax layer, or the presence of antifungal toxins could play a role in the different behavior patterns of *B. cinerea* observed on the two poinsettia cultivars.

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