Factors involved in the early events of spore germination and host colonization by Botrytis cinerea.

Salem Nassr[1] and Radwan Barakat [2]

[1, 2] Department of Plant Production and Protection, Faculty of Agriculture, Hebron University, Hebron, P.O.Box 40, Palestine

Introduction

Botrytis cinerea Pers. Fr. (teleomorph: *Botryotinia fuckeliana*) the causal agent of gray mold disease contributes to substantial fruit rots worldwide and in Palestine. *Botrytis* exhibits great morphological variability in mycelial growth rate, conidial germination, pathogenicity, sporulation, production of sclerotia, and resistance to fungicides (Lorbeer, 1980; Kerssies *et al.,* 1997). The early events of infection by *B. cinerea* were intensively studied lately by many researchers (Doehlemann *et al.,* 2006; Schumacher *et al.,* 2008) whilst the regulatory role of external factors on the germination mechanisms have rarely been investigated. Conidial germination of *B. cinerea* is induced by different physical and chemical signals, including the presence of nutrients and in particular sugars and surface hydrophobicity (Osherove and May, 2000). A deeper understanding of the host-pathogen interaction plus the microenvironment in which the fungus operates is essential.

Material and methods

Conidia of *B. cinerea* isolates B05.10, PBC1 and PBC3 were harvested from 10 days old sporulating cultures grown previously on (PDA+10% bean leaves) medium with sterile distilled water. Spore concentration was set to 2.5×10^4 conidia/ml. Sarstedt microtitre plates (24 wells) were used to monitor the germination development. A completely randomized design was used with 3 replicates for each treatment. Germination counts were done after 2, 5, 8, 10 and 24 hours. To monitor the influence of temperature, fructose was prepared into five concentrations: 1µM, 10µM, 100µM, 1mM, and 10mM. (Doehlemann, 2006). Fructose solution (475 µl of each concentration) was suspended carefully into the middle of a 25 µl conidia in each of the 4 wells. Conidial germination was then determined after 8 hours of incubation under the temperatures of 5, 10, 15, 20, 25 and 30 ^{o}C. Each treatment consisted of 4 replicates (wells) and the number of germinated conidia out of 100 randomly selected conidia was determined under an inverted microscope. Disease severity was measured 4 days after inoculation with mycelium discs (5mm) as mean lesion diameter. The experiment was done on detached bean leaves (Cul. Celina) under 5, 10, 15, 20, 25, and 30 ^{o}C and continuous light. For monitoring conidial germination in the absence of exogenously applied nutrients, five different surfaces were tested; The Sarstedt plate surface, the Spherical glass coverslip surface (15mm Roth, Karlsruhe. Germany), the Polypropylene film surface, the Polypropylene film surface coated with grease and the surface of the glass slides (76×26mm, Knittel Glaser-Germany) coated with the same grease. All surfaces were placed on moist filter papers inside closed sterile Petri dishes. Conidial suspension was prepared from the wild type isolate B05.10 and fixed at a concentration of 1×10^5 conidia/ml. The surfaces were then inoculated with 4 separate droplets of conidial suspension (10 µl each) and then placed in an incubator under dark light conditions. Germination counts were done after 19 hours of incubation by counting germinated spores out of 100 randomly selected spores in each droplet. The role of additional factors such as age and concentration of conidia, pH, amino acids, sugars, salts and inorganic nitrogen forms was evaluated and still under investigation. The data were analyzed statistically using analysis of variance (one way ANOVA) and fisher least significant difference (LSD) test with the aid of SigmaStat 2.0.

Results

Conidial germination was significantly affected by the incubation temperature. The highest germination rate (93%) was obtained at 20 ºC; germination was almost zero at 5 and 30 ºC. Similarly, the effect of temperature (5-30 ºC) on pathogenesis of *B. cinerea* mycelium expressed as gray mold lesion diameter was significantly affected by temperatures but to a lesser extent compared to conidial germination. The highest disease severity was obtained at 20-25 ºC. However, disease severity was almost zero at the lowest temperature (5 ºC) and the highest temperature tested (30 ºC). No significant variations were found between the isolates.

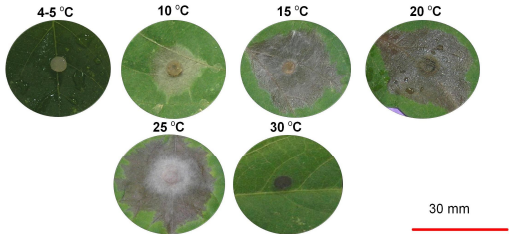

Figure 1. Imaging infection of Beans (Cultivar: Celina) with *B. cinerea* under different incubation temperatures.

Surface hardness is known to affect fungal spore germination. In this experiment, the influence of different surfaces on conidial germination of *B. cinerea* was evaluated. In the absence of externally added nutrients, surface modification with the grease induced almost complete germination of B05.10 conidia on polypropylene (94%) and on glass (96%) after 18 hours. Statistical differences between treatments were minor. The lowest germination rate however, was recorded on the polypropylene surface.

Table 1. Influence of surface hardness and hydrophobicity on germination of *B. cinerea*.

Surface	% Germinated conidia
Sarstedt surface	88 cd
Glass coverslips	91 bc
Polypropylene film	85 d
Polypropylene film+Grease	94 ab
Glass slides + Grease	96 a

- (LSD= 4.049, n=4).

In addition, germination was impaired by extreme pH values and strongly induced (>90% after 24hours) in the presence of sugars at concentrations above 100mM. However, the cations (Ca^{2+}, Mg^{2+}, K^{+}, and Fe^{2+}) had no influence on conidial germination at a wide range of concentrations (up to 1mM). In the presence of inorganic nitrogen forms, conidial germination responded similarly with no particular influence on germination, whilst germ tube growth and elongation responded positively with increasing concentrations of $NH4^{+}$ and $NO3^{-}$.

Discussion

The work in this study describes various factors that influence conidial germination of *B. cinerea*. Germination starts with spore swelling, localized outgrowth of the germ tube and subsequent polarized growth of the new hyphae. (Fig. 2)

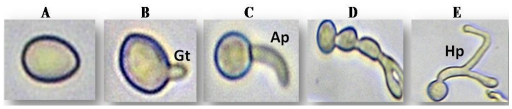

Figure 2. Germination of *B. cinerea* at 200X. (A): Non-germinated conidium; (B): Germination starts after 2 hours; (C): Germinated conidium after 5 hours, notice the variation in germ tubes lengths (D), (E): Germinated conidia after 24 hours. Gt: Germ tube; Ap. Appressorium, Hp: hyphae.

Similar results on the influence of temperature on Ascomycetes were found by Tomioka *et al.,* 1999 using 3 different isolates of *Botrytis cinerea*. The temperature requirements for germination are usually in the same range as for growth, but the differences in the optimum for germination and growth maybe existing between or within fungal species (Griffin, 1994). Elad and Younis (1993), however, found that germination and the infection process in *Botrytis cinerea* occur at a wide range of temperatures up to 25°C. Doehlemann *et al.,* (2006) using the same hydrophobicity procedure reported high germination rates (91-99 %) on onion epidermis and polypropylene hydrophobic surfaces. In the same direction, Kim *et al.,* 1998 reported that in the fungus *Colletotrichum gloeosporioides,* hard surface contact was required to prime conidia for the perception of plant signals that induce germination and appressorium formation. Furthermore, several researchers have reported similar findings for other plant pathogenic fungi such as *Colletotrichum graminicola* (Chaky *et al.,* 2001) and *Phyllosticta ampelicida* (Kuo and Hoch, 1996). The results of the present study have definitely shed some light on some of the factors that influence the early event of germination and infection by *B. cinerea*; other factors such as substrate pH, nitrogen forms, amino acids and others, are still under investigation and will be reported soon. However, further investigations are needed at the molecular level to reveal more information about the signalling cascades that are involved in the early events of pathogenesis of this important plant pathogen.

References

Chaky, J., Anderson, K., Moss, M., and Vaillancourt, L. 2001. Surface hydrophobicity and surface rigidity induce conidia germination in Colletotrichum graminicola. Phytopathology 91: 558–564.

Doehlemann, G., Berndt, P. and Hahn, M. 2006. Different signalling pathways involving a Gα-protein, cAMP and a MAP kinase control germination of B. cinerea conidia. Molecular Microbiology 59: 821-835.

Elad, Y. and Younis, H. 1993. Effect of microclimate and nutrients on development of cucumber gray mold (Botrytis cinerea). Phytoparasitica 21: 257-268.

Griffin D.H. 1994. Spore dormancy and germination, Fungal Physiology, 2nd ed. pp: 375-398. John Wiley and Sons. New York. US

Kerssies, A., Zessen, A.I., Wagemakers, C.M. and Van Kan, J. 1997. Variation in Pathogenicity and DNA polymorphism among B. cinerea isolates sampled inside and outside a glasshouse. Plant Disease 81: 781-786.

Kim, Y. K., Li, D., and Kolattukudy, P. 1998. Induction of Ca2+ calmodulin signalling by hard-surface contact primes Colletotrichum gloeosporioides conidia to germinate and form appressoria. Journal of Bacteriology 180: 514-515

Kuo, K.C., and Hoch, H.C. 1996. Germination of Phyllosticta ampelicida pycnidiospores: prerequisite of adhesion to the substratum and the relationship of substratum wettability. Fungal Genetics and Biology 20: 18-29.

Lorbeer, J.W. 1980. Variation in Botrytis and Botryotinia. In: Coley-Smith, J.R., Verhoeff, K. and Jarvis, W.R. (Eds.). The biology of Botrytis. Academic Press Inc. (London) LTD. pp 19-40.

Osherov, N. and May, G. 2000. Conidial germination in Aspergillus nidulans requires RAS signaling and protein synthesis. Genetics: 155, 647-656.

Schumacher, J., Kokkelink, L., Huesmann, C., Jimenez-Teja, D., Collado, I., Barakat, R., Tudzynski, P., and Tudzynski, B. 2008. The cAMP-dependent signaling pathway and its role in conidial germination, growth and virulence of the grey mould B. cinerea . Molecular Plant-Microbe Interaction 21: 1443-1459.

Tomioka, K., Mori, M., and Sato T. 1999. Identification and pathogenicity of causal agent of Gray Mold of Buttercup (Ranunculus asiaticus L.). Annals of Phytopathological Society of Japan. 65: 620-623.