# CHANGES IN MYCELIAL STRUCTURE OF *BOTRYTIS CINEREA* INDUCED BY REMOVAL OF THE GLUCAN MATRIX

Nurit BAR-NUN[1], Annie L'HYVERNAY[2], B. DONÈCHE[2]* and A. M. MAYER[1]

1: Department of Plant Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
2: UMR 1219 INRA-ISVV/Faculté d'œnologie, University of Victor Segalen Bordeaux 2, 351 cours de la Libération, Talence 33405, France

## Abstract

**Aims**: β-1,3-glucanase is one of the main pathogenesis related proteins of plants, involved in plant-pathogen interactions. Its effect on fungal pathogens is not entirely known. The hyphae of *Botrytis cinerea* are covered by an extra cellular matrix, mainly composed of a β-1,3-D-glucan. This matrix also contains a variety of enzymes, lipids and melanin which may play a role in fungal virulence.

**Methods and results**: Cultures of *Botrytis cinerea* are made in presence of β-1,3-glucanase. The structure of the mycelium of *Botrytis cinerea* after exposure to β-1,3-glucanase during growth was examined by staining with Schiff's reagent and using the electron microscope. Without glucanase, hyphae have a normal diameter and were surrounded by a glucan matrix. Cytoplasm is dense and contains little vacuoles. The glucanase treatment removed most of the glucan sheath, but did not kill the fungus. The structure of the hyphae was changed by the treatment and their diameter increased. Membrane structure showed marked changes, the cytoplasm of the cells was less dense, but more inclusions were observed, including an increase in what appeared to be lipids.

**Conclusion**: The appearance of the mycelium, whose glucan sheath has been removed, was that of cells under stress. The possible implications of the function of the glucan sheath during the interaction of *Botrytis cinerea* with its host during pathogenesis are discussed.

**Significance and impact of study**: These changes following glucanase treatment would lead to a fungal mycelium which will be more sensitive to antifungal agents and might suggest ways of combating *Botrytis* infections by preventing the formation of the extra-cellular matrix.

**Key words**: *Botrytis cinerea*, mycelium, extracellular matrix, glucan, glucanase, ultrastructure, pathogenesis

## Résumé

**Objectif** : La β-1,3-glucanase est une des principales protéines liées à la pathogénèse chez les plantes, impliquée dans les interactions plante-pathogène. Ses effets sur les champignons pathogènes ne sont pas encore complètement connus. Les hyphes de *Botrytis cinerea* sont recouverts d'une matrice extracellulaire composée principalement de β-1,3-D-glucane. Cette matrice contient également diverses enzymes, des lipides et de la mélanine pouvant jouer un rôle dans la virulence du champignon.

**Méthodes and résultats** :Des cultures de *Botrytis cinerea* ont été réalisées en présence de β-1,3-glucanase et les hyphes obtenus ont été observés après coloration et en microscopie électronique. En absence de glucanase, les hyphes développés ont un diamètre normal et sont entourés d'une matrice de glucane. Le cytoplasme est dense et il contient peu de vacuoles. Le traitement à la glucanase entraîne de nombreux changements de structure. Il supprime totalement la matrice de glucane, mais ne provoque pas la mort du champignon. La structure des hyphes est modifiée et leur diamètre est augmenté. Le système membranaire apparaît sous forme de vagues. Le cytoplasme est moins dense et présente de nombreuses inclusions dont la nature semble lipidique.

**Conclusion** : Les hyphes traités par la glucanase présentent les caractéristiques de cellules ayant subi un stress. Les conséquences sur les modifications éventuelles du processus de pathogénèse sont discutées.

**Signification et impact de l'étude** : Le traitement à la glucanase conduit à un mycélium fongique qui sera plus sensible aux agents antifongiques. Cela peut permettre de développer de nouvelles voies de lutte anti-*Botrytis* en empêchant la formation de la matrice extracellulaire.

**Mots clés** : *Botrytis cinerea*, mycélium, extracellular matrix, glucane, glucanase, ultrastructure, pathogénèse

*manuscript received : 30th April 2007 - revised manuscript received: 27th August 2007*

*Corresponding author: ;bernard.doneche@oenologie.u-bordeaux2.fr



*J. Int. Sci. Vigne Vin*, 2007, **41**, n°3, 149-153
©Vigne et Vin Publications Internationales (Bordeaux, France)

## INTRODUCTION

The presence of an extra cellular matrix on the hyphae of *Botrytis cinerea* is well known. This matrix is composed of a β-1,3-D-glucan(Dubourdieu *et al.*, 1980; Cole *et al.*, 1998), which contains a variety of enzymes,lipids and melanin (Gil-ad *et al.*, 2001; Doss, 1999; Cooper *et al.*, 2000; Doss *et al.*, 2003). This matrix appears to have several functions, including possible regulation of the distribution of extra-cellular enzyme activity, adhesion of conidia to host surfaces, and melanin which may play a role in fungal virulence. During experiments on the removal of this matrix, using Glucanex® (β-1,3-glucanase) (Dubourdieu *et al.*, 1981), we observed changes in the appearance of the mycelium of *Botrytis cinerea*. These prompted us to look at the structure of the mycelium, both by simple staining and using electron microscopy.

Relatively little work has been published on the detailed structure of *Botrytis cinerea*. Some attention has been paid to the ultrastructure of dormant and non-dormant conidia of the fungus, to the conidial surface and to the ultrastructure of sclerotia(Hawker and Hendy, 1963; Buckley *et al.* 1966; Doss *et al.*, 1995; Nair and Martin, 1987). The vacuoles and spherosomes of the hyphae have also been studied (Weber *et al.*, 1999). The most detailed report on the ultrastructure of *Botrytis cinerea* is that of Tenberge (2004), dealing especially with the early stages of host infection and penetration into host tissue.

In order to obtain a better insight into the possibly importance of the glucan matrix we studied the effect of glucan treatment on the ultrastructure of the hyphae of the fungus.

## MATERIALS AND METHODS

*Botrytis cinerea*, INRA strain, spores were collected from 10 day old cultures grown on potato dextrose agar. The spores were sown into sterile Nunc 12 well cell culture plates containing 2 % malt extract in 10 mM phosphate citrate buffer pH 3.5, made up in tap water. The concentration of spores was adjusted to $2\ 10^6\ ml^{-1}$. The cultures were maintained at 20 °C either on a reciprocal shaker or under static conditions. As required, 0.2 mg/ml Glucanex®, a gift from Novo Nordisk Ferments Ltd. Switzerland, were added to the wells. For staining the mycelium was collected after seven days of culture. The mycelium was treated for 30' with 1 % periodic acid in 3 % acetic acid and then rinsed 4 times for 10 minutes and then for two hours with pure water. Next they were treated with Schiff's reagent (Sigma) in the dark for 60', rinsed three times for 10' with 0.5 % sodium metabisulphite and rinsed with distilled water to remove excess dye and examined under a light microscope, and compared with unstained mycelium. For electron transmission microscopy, the mycelium was treated with glutaraldehyde 2.5 % in 0.1 M cacodylate buffer, pH 7.2 at 4 °C, rinsed for 10' with cacodylate buffer, followed by fixation with 1 % osmic acid in 0.1 M cacodylate buffer andthe mycelium was stained with 1 % uranyl acetate for 1 hr at room temperature, then washed with water. The samples were dehydrated using graduated concentrations of ethyl alcohol, in 50 % for 10', in 70 % for 10', twice in

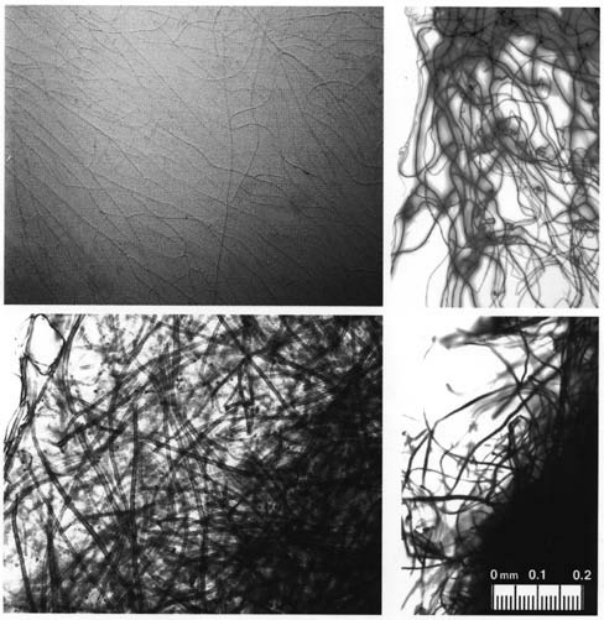


**Figure 1 - Top left: untreated mycelium, unstained. Top right: stained with Schiff's reagent. Bottom left: treated mycelium, unstained. Bottom right stained with Schiff's reagent. The cultures were grown on in malt extract medium for seven days.**


©Vigne et Vin Publications Internationales (Bordeaux, France)

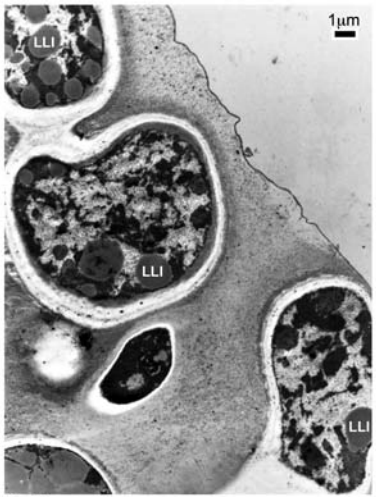


**Figure 2 - Cross section of a normally growing, seven day old, hypha of *Botrytis cinerea* .**

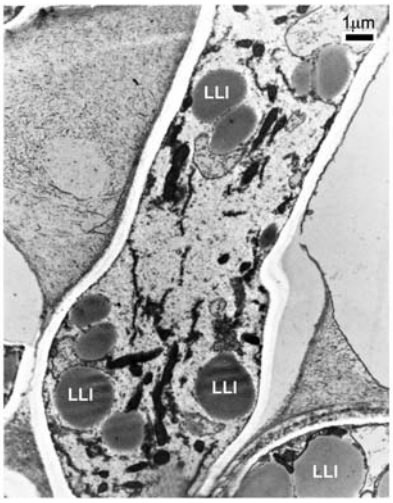


**Figure 3 - Section through a normally growing, seven day old, hypha of *Botrytis cinerea*.**

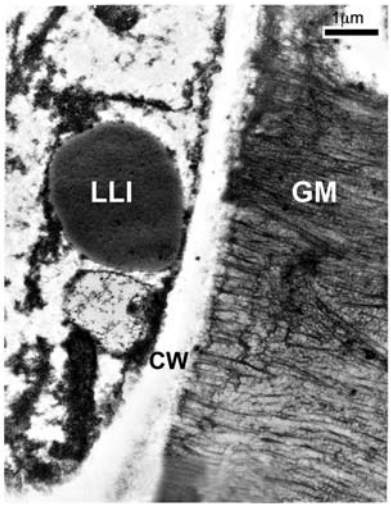


**Figure 4 - Extra-cellular matrix of a hypha of *Botrytis cinerea*, grown for seven days. Note the cell wall (CW) and the presence of lipid-like inclusion (LLI) in the cell.**

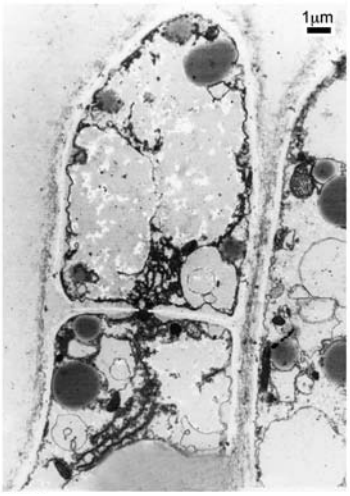


**Figure 5 - Section through a seven day old hypha of *Botrytis cinerea* grown in the presence of 0.2 mg/mlGlucanex®. Note absence of the matrix.**

90 % for 10', and in 100 %, twice for 15', then treated for 15' with ethyl alcohol/propylene oxide (1/1) and twice for 15' with propylene oxide. The samples were treated with 1/1 propylene oxide/EPON resin for 90' and then overnight at room temperature with pure EPON resin. Then the samples were incubated at 60 °C for 24 h for polymerization. Ultrathin sections were prepared and placed on metal grids (200 mesh) for observation.

## RESULTS AND DISCUSSION

Casual visual observation of the mycelium of *Botrytis* treated with Glucanex® showed the treated material to be fluffier and not to form aggregates. Preparations of the material grown in the presence of Glucanex® and parallel controls were stained with Schiff's reagent. A comparison of the preparations clearly indicates that the typical extra-cellular matrix is absent in the mycelium of *Botrytis* grown for seven days in the presence of 0.2 mg/ml Glucanex®. In addition the diameter of the hyphae of the treated material was clearly significantly greater than in the controls (figure 1). Average diameter size of mycelium is normally around 6-8 µm; it increases until 12-15 µm in the presence of Glucanex®.

In order to study this effect on mycelium morphology and ultrastructure, in the absence of the glucan matrix, we used electron microscopy.

Both normally grown mycelium of *Botrytis* and mycelium grown in the presence of Glucanex® were


©Vigne et Vin Publications Internationales (Bordeaux, France)

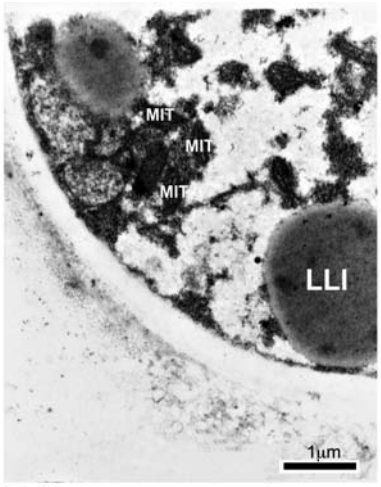


**Figure 6 - Section through a seven day old hypha of *Botrytis cinerea* grown in the presence of 0.2 mg/mlGlucanex®, note mitochondria (MIT) and numerous lipid like inclusions (LLI).**

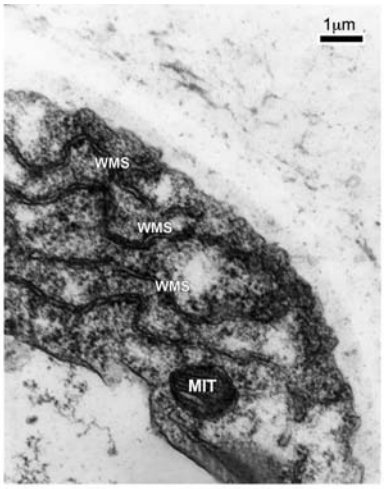


**Figure 7 - Section through a seven day old hypha of *Botrytis cinerea* grown in the presence of 0.2 mg/ml Glucanex®, showing a wavy membrane system (WMS).**

removed from the culture medium after seven days, fixed and prepared for observation under the electron microscope. The results are shown in the figures 2 and 7.

In figure 2, it can be clearly seen that the mycelium of the control sample is covered by an extra-cellular matrix. The hyphae contain dense cytoplasm and numerous lipid inclusions. Figure 3 shows the presence of these lipid inclusions within the cell and there is little vacuolization. Figure 4 shows the structure of the extra-cellular matrix at higher magnification.

These observations can be compared with those of the treated material. In figure 5, marked vacuolization can be observed, while in figure 6, the presence of numerous well defined mitochondria can be seen. Although in figure 5 close contact between hyphae can be observed, there is practically no extra-cellular matrix present, indicating that Glucanex® treatment was effective in preventing the formation of the β-1,3-D-glucan. Overall the cytoplasm of the treated hyphae is less dense than in the controls, more vacuolated and contains more inclusions, whose nature appears to be lipid. These differences clearly appear when figure 3 is compared to figure 5. The cell walls of the treated and control hyphae appear to be the same, although perhaps those of the treated cells may be a slightly thicker. Figure 7 shows the presence of a wavy system of membranes. This structure, never cited in previous works, is not explained.

These results tend to indicate that the culturing of *Botrytis* in the presence of Glucanex® results in a mycelium which is suffering from stress, as indicated by an increased number of mitochondria, increased vacuolisation, changes in membrane structure and changes in the inclusions in the cells. According to the suppression of the extracellular matrix of glucan, *Botrytis* accumulates compounds acting as osmoticum like glycerol and polyalcohols. The synthesis of these molecules needs an active energetic pathway and the presence of numerous mitochondria. These changes would lead to a fungal mycelium which will be more sensitive to antifungal agents and might suggest ways of combating *Botrytis* infections by preventing the formation of the extra-cellular matrix. In this case, some antifungal agents as chitosan will have greater efficacity on fungal membrane (Xu *et al.*, 2007). This matrix clearly has an important role in the growth and vigour of *Botrytis cinerea*. It is worth noting that β-1,3-glucanases are regarded as pathogenesis related proteins, the so called PR2 proteins, which are considered to afford some protection to plants against pathogens (Kaufmann *et al.*, 1987), although their role is not entirely clear cut (Van Loon *et al.*, 2006). It is possible therefore that although the cell wall of *Botrytis* does not seem to be damaged during its culture in the presence of the β-1,3-glucanase, the fungus is exposed to stress. Such a situation might be relevant to the interaction of host and pathogen when the formation of PR proteins is enhanced during pathogenesis.

**Acknowledgment**: We acknowledge with thanks the help of the SERCOMI department of the University of Bordeaux 2 in preparing samples and making electron microscopic observations. Our thanks are due to Dr. K. Tenberge, Dr. Y. Elad and Dr. D. M. Joel for helpful comments on the electron micrographs.


©Vigne et Vin Publications Internationales (Bordeaux, France)

## REFERENCES


BUCKLEY P. M., SJAHOLM V. E. SOMMER N. F., 1966. Electron microscopy of *Botrytis cinerea conidia*. *J. Bact*. **91**, 2037-2044.

COLE L. DEWEY F. M. HAWES C. R., 1998. Immunocytochemical studies of the infection mechanism of *Botrytis cinerea* (Part 1). *New Phytol*. **19**, 597-609.

COOPER L.L.D. OLIVER J.E., DE VILBISS E.D., DOSS R. P., 2000. Lipid composition of the extracellular matrix of *Botrytis cinerea* germlings. *Phytochemistry*, **53**, 293-298.

DOSS R.P. 1999. Composition and enzymatic activity of the extracellular matrix secreted by germlings of *Botrytis cinerea. Appl. Env. Microbiol*. 65, 404-408.

DOSS R.P. POTTER S.W., SOELDNER A.H., CHRISTIAN J. K., FUKUNAGA L.E., 1995. Adhesion of germlings of *Botrytis cinerea*. *Appl. Env. Microbiol*., **61**, 260-265.

DOSS R. P., DEISENHOFER J., KRUG VON NIDDA H-A, SOELDNer A. H., MCGUIRE R. P., 2003. Melanin in the extracellular matrix of germlings of *Botrytis cinerea*. *Phytochemistry*, **63**, 687-691.

DUBOURDIEU D., RIBÉREAU-GAYON P., FOURNET B., 1980. Structure of the extracellular β-D -glucan from *Botrytis cinerea*. *Carbohydrate Research*, **93**, 294-299.

DUBOURDIEU D. VILLETAZ J. C. DESPLANQUES C., RIBÉREAU-GAYON P., 1981. Dégradation enzymatique de glucane de *Botrytis cinerea*. Application à l'amélioration de la clarification des vins issus de raisins pourris. *J. Int. Sci. Vigne Vin*, **15**, 161-177.

GIL-AD N.L., BAR-NUN N. and MAYER A. M., 2001. The possible function of the glucan sheath of *Botrytis cinerea*: effects on the distribution of enzyme activities. *FEMS Microbiology Letters*, **199**, 109-113.

HAWKER E. AND HENDY R. J., 1963. An electron microscope study of germination of conidia of *Botrytis cinerea*. *J. Gen. Microbiol*., **33**, 43-46.

KAUFFMANN S., LEGRAND M., GEOFFROYP., FRITIG B., 1987. Biological function of pathogenesis-related proteins: four PR proteins of tobacco have 1,3-β-glucanase activity. *Embo J*., **6**, 3209-3212.

NAIR N.G. AND MARTIN A. B., 1987. Ultrastructure of sclerotia of *Botrytis cinerea* Pres. *In vitro*. *Phytopathologische Z*., **119**, 52-63.

TENBERGE K. B., 2004. The morphology and cellular organisation in *Botrytis* interactions with plants. *In: Botrytis: biology, pathology and control*, eds. Elad Y., Williamson B., Tudzynski P., Delen N. Kluwer Academic Press pp. 67-84.

VAN LOON L. C., REP M. and PIETERSE C.M. J., 2006. Significance of inducible defense-related proteins in infected plants. *Annual Review Phytopathology*, **44**, 135-162.

WEBER R.W.S. WAKLEY G.E. and PITT D., 1999. Histochemical and ultrastructural characterization of vacuoles and spherosomes as components of the lytic system of the fungus *Botrytis cinerea*. *Histochemical J*., **31**, 293-301.

XU W.T., HUANG K.L., GUO F., QU W., Yang J.J., LIANG Z.H., LUO Y.B., 2007. Postharvest grapefruit seed extract and chitosan treatments of table grape to control *Botrytis cinerea*. *Postharvest Biology and Technology*, **46**, 86-94.



©Vigne et Vin Publications Internationales (Bordeaux, France)