Structural changes of spores of tree fungal pathogens after treatment with the designed antimicrobial peptide D2A21

D. Rioux, V. Jacobi, M. Simard, and R.C. Hamelin

Abstract: In vitro effects of the antimicrobial synthetic D2A21 peptide on the structure of spores of four fungal pathogens causing important tree diseases were examined by microscopy in parallel with tests to measure inhibition of spore germination. With light microscopy, the use of SYTOX® green stain indicated that the peptide rapidly altered the plasma membrane of conidia of three Ascomycetes: *Gremmeniella abietina* (Lagerberg) Morelet var. *abietina* Petrini et al., *Ophiostoma ulmi* (Buism.) Nannf., and *Nectria galligena* Bres. With basidiospores of *Cronartium ribicola* J.C. Fisch., a difference between control and treated spores was also found, but it was less pronounced than with conidia of the Ascomycetes. In transmission electron microscopy, untreated conidia showed typical cytoplasmic contents with the regular presence of mitochondria, ribosomes, and nuclei, at times accompanied by vacuoles of various sizes. At concentrations of the peptide inhibitory to spore germination, plasma membranes, as well as nuclear and mitochondrial envelopes, were either generally difficult to discern or were distorted and swollen. At more advanced stages, the cytoplasm of treated spores contained numerous vesicles and was in places more electron-dense than in controls. Cytoplasm leakage was also regularly observed. Present observations strongly suggest that the primary site of action of this peptide is located at the plasma membrane level.

Key words: *Cronartium ribicola*, *Gremmeniella abietina*, *Nectria galligena*, *Ophiostoma ulmi*, D2A21, peptide.

Résumé : Les effets in vitro du peptide antimicrobien synthétique D2A21, sur la structure de spores de quatre champignons pathogènes responsables de maladies importantes des arbres, ont été examinés en microscopie conjointement avec des tests de germination. En microscopie photonique, l'emploi du réactif SYTOX® green a révélé que le peptide altère rapidement le plasmalemme des conidies des trois Ascomycètes : *Gremmeniella abietina* (Lagerberg) Morelet var. *abietina* Petrini et al., *Ophiostoma ulmi* (Buism.) Nannf., et *Nectria galligena* Bres. Une différence entre les basidiospores traitées et témoins des *Cronartium ribicola* J.C. Fisch. a aussi été notée bien que celle-ci ait été moins prononcée que chez les conidies des Ascomycètes. En microscopie électronique à transmission, les conidies témoins avaient un cytoplasme typique avec la présence régulière de mitochondries, de ribosomes et de noyaux, accompagnée parfois de vacuoles de différentes tailles. Aux concentrations du peptide inhibant la germination des spores, le plasmalemme ainsi que les enveloppes nucléaire et mitochondriale étaient soit difficiles à discerner, soit gonflées et difformes. À un stade plus avancé, le cytoplasme des spores traitées montrait plusieurs vésicules ainsi que des parties de cytoplasme plus opaques aux électrons. Des fuites de cytoplasme étaient également observées. Les observations de cette étude suggèrent fortement que le site d'action primaire de ce peptide se situe dans le plasmalemme.

Mots clés : *Cronartium ribicola*, *Gremmeniella abietina*, *Nectria galligena*, *Ophiostoma ulmi*, D2A21, peptide.

Introduction

Numerous mechanisms occur in plants and animals that confer protection against a variety of microorganisms. An important component of this defense system appears to be the production of small peptides displaying antimicrobial activities (Gabay 1994; Nicolas and Mor 1995; Rao 1995; Broekaert et al. 1997). These peptides, generally comprising no more than 50 amino acids, can be classified as follows: linear peptides, synthetic (designed) peptides, disulfide peptides, and lantibiotics (Rao 1995). Among the linear peptides, the best characterized are the cecropins identified in insects (Boman and Hultmark 1987) and the magainins isolated from amphibian species (Bevins and Zasloff 1990). The synthetic peptides produced so far are usually analogues of the linear peptides (Chen et al. 1988; Zasloff et al. 1988; Powell et al. 1995; Reed et al. 1997). Disulfide peptides have been isolated from many higher organisms and are the prominent type produced by plants against pathogens (Broekaert et al. 1995). Lantibiotics are found in bacteria and comprise those peptides that undergo modifications following their synthesis (Hansen 1993).

Besides the possibility of directly using these peptides as therapeutic treatments against many diseases, their antimicrobial activity, coupled with their relatively simple structure, makes them attractive candidates to improve plant disease resistance through genetic engineering. For instance, tobacco plants were transformed to produce an antimicrobial

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peptide upon infection with a foliar pathogen and were shown to better withstand the fungal aggression (Terras et al. 1995). Another reason for the increased interest in these peptides is that they represent a good alternative to the use of traditional chemical pesticides.

The strategy of using antimicrobial peptides might also be followed with great benefit in forestry where diseases that occur in natural stands, in plantations, and in urban environments often cause severe economic, ecological, and aesthetic losses. A recent in vitro study has assessed the potential of four peptides by measuring their antifungal activity against some fungal pathogens causing important tree diseases and by evaluating their phytotoxicity using a pollen germination test (Jacobi et al. 2000). This study revealed that the synthetic Peptidyl MIM™ D2A21 combined high antifungal activity with low pollen toxicity. A necessary parallel study, the object of the present paper, was to follow microscopically the effects of this peptide on the cellular structure of spores produced by four fungal pathogens: *Gremmeniella abietina* (Lagerberg) Morelet var. *abietina* Petrini et al., *Ophiostoma ulmi* (Buism.) Nannf., *Nectria galligena* Bres., and *Cronartium ribicola* J.C. Fisch. These fungi are responsible for scleroderris canker of conifers, Dutch elm disease, nectria canker, and white pine blister rust, respectively.

Materials and methods

Fungal strains and culture conditions

The isolate 94006 (North American race) of *G. abietina* var. *abietina* (provided by Dr. G. Laflamme, Laurentian Forestry Centre) was grown at 18°C in the dark on a medium containing 3% Bacto agar (BA) (Difco Laboratories, Detroit, Mich.) and 20% Campbell's V8 juice. To induce sporulation, 3-week-old cultures were exposed to light adjusted to 175–275 $\mu mol \cdot s^{-1} \cdot m^{-2}$. The isolate 205 of *Ophiostoma ulmi* (provided by Dr. P. DesRochers, Laurentian Forestry Centre) and the isolate Q943091 of *Nectria galligena* (provided by F. Plante, Université Laval, Faculté de foresterie et géomatique, Sainte-Foy, Que.) were grown at room temperature either on potato dextrose agar (BBL, Cockeysville, Md.) or malt agar (Difco) and spores were harvested directly from these cultures. Basidiospores of *Cronartium ribicola* were collected from naturally infected *Ribes* leaves.

Peptide bioassay

The antimicrobial peptide D2A21 was kindly provided by Demeter BioTechnologies (Durham, N.C.). This linear synthetic peptide (molecular weight = 3664) is composed of 23 amino acids having the following sequence FAKKFAKKFKKFAKKFAKFAFAF (A, alaline; F, phenylalaline; K, lysine). Treatments were made using the peptide at different concentrations after appropriate dilution with sterile deionized water and 0.05% Tween 20 (British BioCell International, Cardiff, U.K.), the latter preventing spore adhesion to test tube walls. The final concentrations of the peptide and the spore concentration are given below for each experiment. Prior to microscopy, 5 µL of each spore suspension was transferred onto 1.5% BA and incubated at room temperature to assess percent germination using either a Leitz Fluovert FS inverted microscope or a Wild M420 macroscope. Water-treated spores served as controls. All the plates were followed until the time when either there were no changes or when the numerous growing hyphae made it impossible to accurately evaluate percent germination.

Experiment 1

Gremmeniella abietina var. *abietina* was challenged for 2 h at room temperature with 50 or 250 µM of the peptide. The spore concentration was $7 \times 10^6 \cdot mL^{-1}$, and the percent germination was evaluated after 96 h.

Experiment 2

Gremmeniella abietina var. *abietina* and *O. ulmi* were challenged with 50 µM of the peptide at room temperature for 10, 30, 60, or 120 min. The spore concentration was $1 \times 10^6 \cdot mL^{-1}$ for *G. abietina* var. *abietina* and $4 \times 10^7 \cdot mL^{-1}$ for *O. ulmi*. Germination was assessed after 72 h for *G. abietina* var. *abietina* and 24 h for *O. ulmi*.

Experiment 3

Gremmeniella abietina var. *abietina* and *O. ulmi* were challenged with 6.25, 12.5, 25, 50, or 100 µM, and *N. galligena* was challenged with 50 and 100 µM of the peptide at room temperature for 2 h. The treatment of *N. galligena* conidia was repeated twice. The spore concentration was $5 \times 10^6 \cdot mL^{-1}$ for the three pathogens. Percent germination was examined after 68 h for *G. abietina* var. *abietina*, 40 h for *O. ulmi*, and 21 h for *N. galligena*.

Experiment 4

Telia of *C. ribicola* were carefully detached from *Ribes* leaves and were fixed with 2% BA to the lid of a Petri dish. Basidiospores produced were allowed to discharge underneath on a microscope slide covered with a thin layer of 2% BA. In an assay, ungerminated basidiospores collected after 18 h were counted and were treated with 100 µM of the peptide at room temperature for 2 h. In another test, basidiospores were let to discharge directly upon either 20, 50, or 100 µL of a 100 µM solution of the peptide. The SYTOX stain (see below) was applied after 2 h of treatment and the percent germination was evaluated after 48 h.

Epifluorescence microscopy

The SYTOX® green nucleic acid stain (Molecular Probes, Eugene, Oreg.) was developed to evaluate the integrity of the plasmalemma of microorganisms. Whereas it cannot cross the plasma membrane of live cells, it readily penetrates compromised plasma membranes before binding to nucleic acids where it induces an intense fluorescence emission when excited under blue light illumination. The SYTOX green stain was used in repeated experiments with control spores and with spores exposed to the D2A21 peptide. After the appropriate treatment, 10 µL of each fungal cell suspension was mixed with 10 µL of a SYTOX solution at 10 µM. After 5 min, the cells were examined with a Reichert Polyvar microscope under either differential interference contrast or blue light excitation using a BP 450–490 exciter filter combined with a DS 510 separator mirror and a LP 515 barrier filter. Except with *C. ribicola*, with which we observed at most 30–40 basidiospores per microscope slide, at least 100 spores per treatment were counted with the other pathogenic fungi.

Acridine orange, a stain that can penetrate unaltered plasma membranes, was also used with spores of *G. abietina* var. *abietina* at a final concentration of 0.1% to detect possible cytoplasm leakage. Under blue light illumination, acridine orange gives an orange colour to the cytoplasm, whereas the nucleus displays a yellow-green fluorescence.

Transmission electron microscopy

The conidia of *G. abietina* var. *abietina*, *O. ulmi*, and *N. galligena* were prepared for transmission electron microscopy (TEM). The basidiospores of *C. ribicola* were in too low a concentration to permit observation in TEM. Each conidia suspension was adjusted with glutaraldehyde to a final concentration of 2.5% of the fixative

Figs. 1 and 2. Differential interferential contrast (Figs. 1*a* and 2*a*) and blue epifluorescence after treatment with SYTOX stain (Figs. 1*b* and 2*b*). The fluorescence is almost undetectable with the control spores of *G. abietina* var. *abietina* (Fig. 1) but it is intense within a few minutes after treatment with 100 µM of the D2A21 peptide (Fig. 2). Scale bars = 25 µm (Fig. 1) and 30 µm (Fig. 2).
Figs. 3–6. Acridine orange staining of control spores (Fig. 3) and of spores of *G. abietina* var. *abietina* treated with 100 µM of the peptide for 10 min (Fig. 4), 25 min (Fig. 5, arrowhead), and 90 min (Fig. 6). The orange staining of the cytoplasm (Fig. 4, arrowheads), as well as the yellow-green fluorescence representing the nucleus, has become diffused in the minutes following the treatment, and later, some of it is seen outside the spores (Fig. 5, arrowheads). By 90 min, it was often difficult to detect any orange fluorescence, whereas the yellow-green fluorescence was less intense than in controls (Fig. 6, arrowheads). Scale bars = 12 µm (Figs. 3–5) and 30 µm (Fig. 6).

Table 1. Percent (%) of spore germination after exposure to D2A21 peptide.

	G. abietina var. *abietina*	*O. ulmi*	*N. galligena*	*C. ribicola*
Experiment 1				
Control	98	—	—	—
50 µM	0	—	—	—
250 µM	0	—	—	—
Experiment 2				
Control (120 min)	85	88	—	—
50 µM (10 min)	0	0	—	—
50 µM (30 min)	0	0	—	—
50 µM (60 min)	0	0	—	—
50 µM (120 min)	0	0	—	—
Experiment 3				
Control	92	95	>95[a]	—
6.25 µM	88	27	—	—
12.5 µM	52	7	—	—
25 µM	20	0	—	—
50 µM	<1	0	>90[a]	—
100 µM	0	0	89, 44[b]	—
			90, 52[b]	—
Experiment 4				
Test 1				
Control	—	—	—	93
100 µM	—	—	—	63
Test 2				
Control	—	—	—	97
20 µL	—	—	—	80
50 µL	—	—	—	61
100 µL	—	—	—	50

[a]For both macroconidia and microconidia (two repetitions).
[b]Proportions for macroconidia (89% and 90%) and microconidia (44% and 52%) respectively.

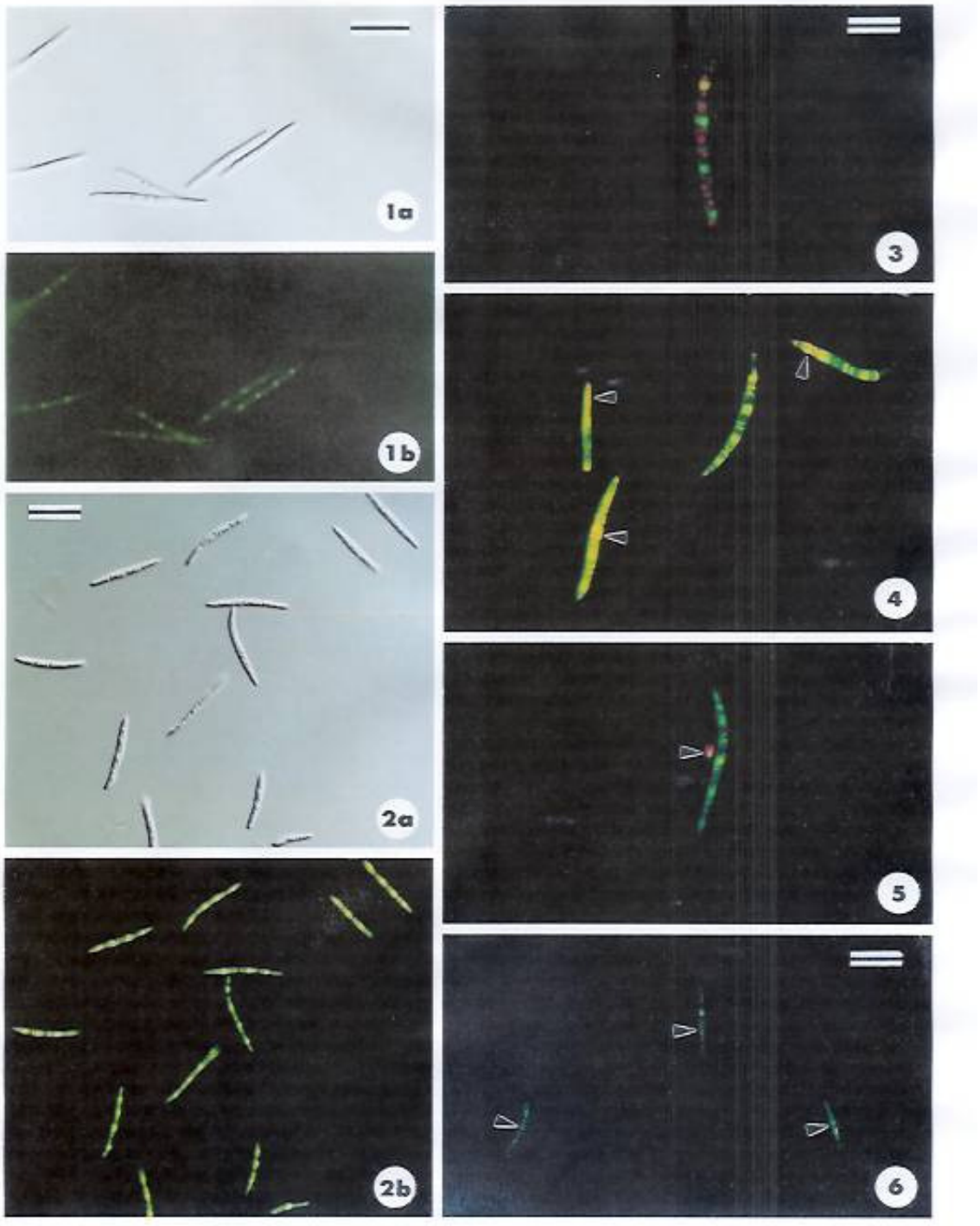
in 0.05 M sodium cacodylate buffer (SCB) (pH 7.2). Spores were fixed overnight at 4°C and thereafter centrifuged for 1 h at 2200 × *g* at room temperature. The supernatant was discarded and to the pellet was added an equal volume of a solution containing 10% bovine serum albumin and 5% glutaraldehyde. After the medium solidified, samples were rinsed three times with SCB and postfixed with 1% OsO_4 in SCB for 1 h. The spores were rinsed three times with SCB prior to being gradually dehydrated with ethanol and embedded in Epon 812.

Sections (0.5–1 µm) were preexamined with a Reichert Polyvar light microscope. In TEM, sections (90 nm) were contrasted with uranyl acetate and lead citrate and examined with a Philips 300 microscope operating at 80 kV.

Results

Data concerning the effect of the peptide on percent germination of fungal spores are presented in Table 1. At low concentrations (6.25–25 µM) of the peptide, inhibition of germination was more pronounced with *O. ulmi* than with *G. abietina* var. *abietina*. At 50 µM, spore germination was suppressed in both fungi. At that concentration, spores of *N. galligena* seemed less affected by the treatment but at 100 µM, about half of the microconidia did not germinate whereas most of the macroconidia germinated. With *C. ribicola*, the 100 µM concentration inhibited the germination by about 37–50%.

The proportion of spores that showed intense fluorescence in the presence of SYTOX stain corresponded with the reduction of germination obtained with the peptide. While almost all control spores displayed a fluorescence barely noticeable under blue light illumination (Figs. 1 and 7), in the minutes following treatment with 50 µM of the D2A21 peptide, spores of *G. abietina* var. *abietina* and *O. ulmi* emitted intense fluorescence likely as a result of the binding of the



Figs. 7–11. Differential interferential contrast (Figs. 7*a*, 8*a*, and 11*a*) and blue epifluorescence after treatment with SYTOX stain (Figs. 7*b*, 8*b*, 9, 10, and 11*b*). Fig. 7. Untreated microconidia and macroconidia of *N. galligena* showing the general absence of fluorescence except a weak one in some microconidia (Fig. 7*b*, arrowheads). Fig. 8. Intense fluorescence observed in conidia of *O. ulmi* after 2 h exposure to 50 µM of peptide. Figs. 9 and 10. *Nectria galligena* 2 h after treatment with 100 µM of the peptide. Fig. 9. About half the microconidia show intense fluorescence, whereas macroconidia do not (arrowheads). After germination of a conidium, the fluorescence is also noticeable in a developing hypha (arrow) and its mother cell. Fig. 10. Only the tip cell of this macroconidium displays fluorescence (arrowhead). Fig. 11. These two basidiospores have reacted positively with SYTOX and the right one shows two well-circumscribed nuclei. Scale bars = 25 µm (Figs. 7, 9–10) and 20 µm (Figs. 8 and 11).

SYTOX green stain to the nuclear DNA (Figs. 2 and 8). After treatment with 100 µM of the peptide, about half of the microconidia of *N. galligena* showed fluorescence, while only a few dispersed macroconidia fluoresced and then, frequently, it was only one cell at the extremity of the conidium (Figs. 9 and 10). It is to be noted that the intense but more diffuse fluorescence observed with *N. galligena* made the precise localization of nuclei generally difficult. With *C. ribicola*, approximately 30–45% of the treated spores exhibited fluorescence, while 7–10% of the control basidiospores did so after their release from *Ribes* leaves. Depending on the position of the spores, it was often possible to observe two nuclei per basidiospore (Fig. 11). Except with *C. ribicola*, at most 5% of the control spores of the other fungi gave a weak fluorescence when treated with SYTOX.

The acridine orange made it possible to easily differentiate the cytoplasm from the nucleus in control spores of *G. abietina* var. *abietina* (Fig. 3). In treated spores, the orange emission from the cytoplasm was weak, even just after 10 min in the presence of the peptide (Fig. 4). In more advanced stages of treatment, it was impossible to distinguish the orange fluorescence inside the spores, but some was occasionally discernible just outside (Fig. 5). The yellow-green fluorescence typical of nuclear material remained visible for some time, although it was more diffuse and less intense than in controls (Fig. 6).

In TEM, most of the control spores showed a simple structure consisting of the plasmalemma, one or many nuclei with their envelopes, numerous ribosomes, and some scattered mitochondria containing only a few well-developed cristae (Fig. 12). Occasionally, a few small vacuoles were also observed. Most of the extracellular sheath usually present around cells of *G. abietina* var. *abietina* had been destroyed by the detergent used to prevent spore adhesion to our test tubes. Some control spores with a disorganized cytoplasm were observed. These might have corresponded with spores unable to germinate that were occasionally observed. In these cases, the cytoplasm was characterized by the presence of rare polymorphic membranous tubules and vesicles (observations not shown).

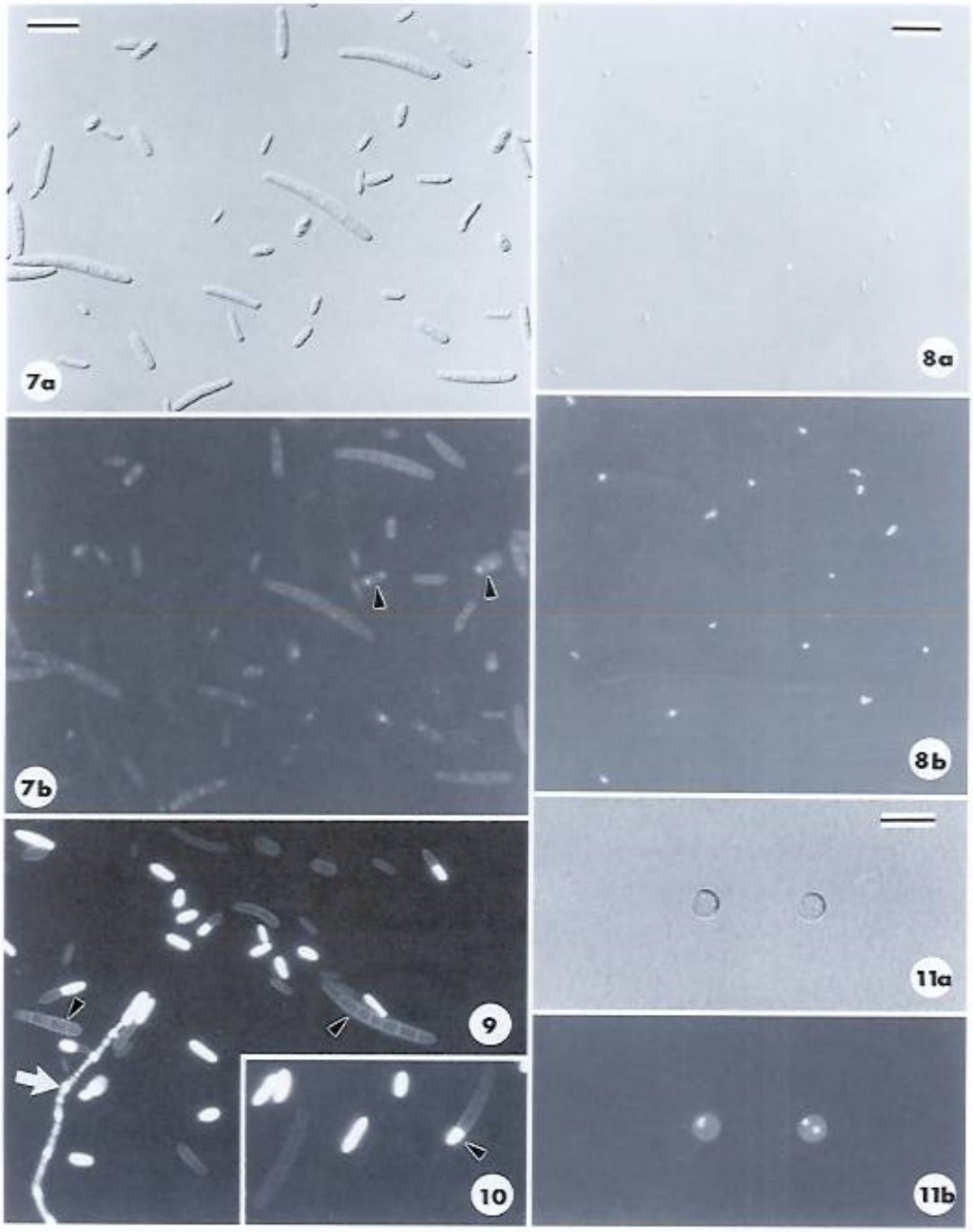
The first ultrastructural change in treated spores of the three fungi studied was that it was difficult to discern the plasma membrane and also at times the envelope around the nucleus and mitochondria (Fig. 13). In other cases, mitochondria and nuclei were misshapen but still discernible owing to the presence of a more or less continuous but swollen envelope (see Fig. 17). Two hours after treatment with 6.25 µM of peptide D2A21, most of the conidia of *G. abietina* var. *abietina* appeared normal, but those of *O. ulmi* already showed some cytoplasmic degeneration with many vesicles at their periphery (observations not shown). After only 10 min exposure to 50 µM of the peptide, many portions of the cytoplasm of these two fungi were more electron-opaque than in untreated spores, and cytoplasm leakage was regularly observed (Fig. 14). Following treatment with higher concentrations of the peptide, or after longer exposure time, the majority of conidia of *G. abietina* var. *abietina* and *O. ulmi* were filled with electron-translucent vesicles accompanied by the scattered presence of electron-opaque material (Fig. 15). It was also noted that at a concentration of 50 µM in the first three experiments, there was an inverse correlation between the number of spores used and the intensity of ultrastructural changes of the conidium cytoplasm with *G. abietina* var. *abietina* and *O. ulmi*.

Both control microconidia and macroconidia of *N. galligena* were similar to those of *G. abietina* var. *abietina* and *O. ulmi*, except that vacuoles were particularly prominent (Fig. 16). While most of the cells of *N. galligena* appeared normal after exposure to 50 µM of the peptide, with 100 µM, many cells corresponding with microconidia in length and diameter were altered. For instance, some of them had a swollen envelope around their mitochondria and nuclei (Fig. 17) or were plasmolyzed and contained no distinct organelles apart from intensely damaged mitochondria and many vesicles (Fig. 18). Most of the macroconidia appeared normal except for some cells located at their extremity (Fig. 19). Electron-opaque material was rarely detected in these microconidia and macroconidia.

Discussion

The observations obtained in light microscopy are in agreement with a recent study showing that the plasma membrane of fungi is rapidly compromised after challenge with small synthetic peptides allowing the passage of the SYTOX green stain through the altered plasma membrane (Reed et al. 1997). This dye has also been used successfully with bacteria to demonstrate their viability following antibiotic treatments (Roth et al. 1997). Although it is possible to underestimate the number of dead cells in tests using SYTOX, as it has been recently reported when the nucleic acids are absent or too altered (Lebaron et al. 1998), the concordance obtained between our results with light and transmission electron microscopy indicated that most of the nucleic acids remained within the spores during our experiments and they were not altered enough to impede the reaction with SYTOX.

With *C. ribicola*, the differences between control and treated basidiospores were not as pronounced as with conidia of the other fungi. A reason for this might be that these basidiospores are very temperature- and moisture-



Figs. 12–15. Conidia of *Gremeniella abietina* var. *abietina*. Fig. 12. A control conidium containing numerous ribosomes, a nucleus with its envelope (arrow) and mitochondria (arrowheads) containing only a few cristae. The plasmalemma is visible on the left side of the figure (curved arrow) but is difficult to discern at the apex of this spore since it was cut obliquely. Scale bar = 0.2 µm. Fig. 13. After exposure to 25 µM of peptide, the plasmalemma as well as the membranes around the nucleus (arrow) and a mitochondrion (arrowhead) are difficult to distinguish. Unusual large vacuoles are also present occasionally containing electron-opaque material and vesicles apparently originating from the invaginating tonoplast (curved arrow). Scale bar = 0.2 µm. Fig. 14. Ten minutes after D2A21 treatment at 50 µM, the altered cytoplasm displays electron-opaque material, some of which has leaked out of the cell (arrowheads). Scale bar = 0.4 µm. Fig. 15. In the most affected conidia 2 h after treatment with 50 µM of peptide, only scattered vesicles (arrowheads) and electron-opaque material can be observed in the cytoplasm. Scale bar = 0.5 µm.

sensitive (Manion 1991). Our in vitro conditions might have hastened their deterioration, as suggested by the detection of 7–10% of control basidiospores reacting with SYTOX. Another reason might be that basidiospores were collected on an agar layer, at the surface of microscope slides, instead of directly in water. Thus, a part of the spore that was embedded in agar might have been inaccessible to the peptide. It is also likely that the numerous inorganic cations present in agar, and particularly divalent ones, reduced the effectiveness of the peptide, as reported elsewhere (Jacobi et al. 2000; Mills and Hammerschlag 1993; Terras et al. 1993). Finally, it is worth mentioning that the surprising frequent presence of two nuclei per basidiospore, which are the results of meiosis, had been already described with fluorescence microscopy by Anikster (1983).

Observations with TEM clearly showed that the plasma membrane, as well as the envelope surrounding mitochondria and nuclei, are rapidly altered after treatment with the peptide. These membranes were often partially or totally missing, and when present they were often swollen. Their degradation appeared to give rise to numerous vesicles that were regularly observed in the cytoplasm of the most disturbed cells. The disruption of nuclear envelopes by small designed peptides, allowing the penetration of SYTOX, has also been noted by Reed et al. (1997). In another study, the effects of the magainin 2 antimicrobial peptide on the structure of fungal hyphae were examined by electron microscopy, and it was found that the treated cells often contained many vacuoles and mitochondria with abnormal morphology (Kristyanne et al. 1997). It has to be noted, however, that they did not describe structural changes of nuclei.

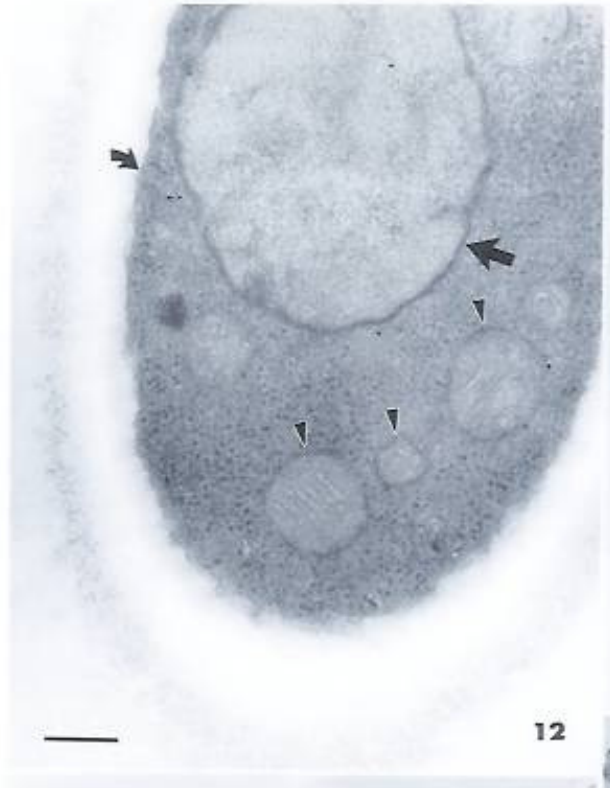
Kristyanne et al. (1997) also found that the fixation was improved with treated hyphae when compared with controls and they mentioned that this was probably caused by a better penetration of fixatives across compromised plasma membranes. The control spores used in the present study are probably more impermeable than hyphae to different substances, and this might explain why some of these cells did not exhibit a clear definition of their fine structure and, in particular, of their various membranous systems after a standard 2- to 3-h period of fixation with glutaraldehyde (observations not shown). However, after such a period of fixation following peptide treatment, likely as a consequence of the greater permeability of the plasmalemma to fixatives, the various types of membranes of the spores always became evident, and the cytoplasm was also frequently more contrasted than in controls. Finally, it is worth noting that the reduced metabolism of control spores might have been partly reflected by the presence of only a few cristae in mitochondria, which is indicative of a low level of respiration (Cheville 1994).

All these results clearly showed that the plasma membrane was rapidly altered after exposure to synthetic D2A21 peptide. At about the same time or very soon afterwards, alteration of the highly compartmentalized internal membranous system was also observed. It is difficult to determine whether these internal bilayers were directly affected by the peptide or indirectly as a consequence of the numerous changes resulting from the compromised plasma membrane. However, it is clear that the capacity of such antimicrobial peptides to form pores in the latter is the primary reason for their effectiveness (Rao 1995). Thus, such peptides might also be called ionophores. By definition, the latter are small hydrophobic molecules that form transmembrane channels greatly increasing the permeability of lipid membranes and they would be common in nature (Alberts et al. 1989). These molecules appear to be regularly produced by most microorganisms to defend themselves against competitors, and many have been used as antibiotics.

Tests to measure inhibition of spore germination of these four pathogenic fungi were carried out to confirm the antifungal activity of the peptide before preparing samples for microscopy. More detailed information about in vitro effects of this peptide against seven tree fungal pathogens, including those of the present study, are described elsewhere (Jacobi et al. 2000). In this article, it is reported that the peptide was effective at lower concentrations than those mentioned in the present study. The reason for this difference is probably that they used less concentrated spore suspensions for their tests. This is supported by the fact that for higher concentrations of fungal cells treated with the peptide in the present study, cytoplasm ultrastructure appeared less altered when compared with samples prepared with a lower number of spores exposed to the same concentration of peptide. More spores have been exposed to D2A21 peptide in our study in order to obtain a satisfactory number to examine in microscopy.

Although the sensitivity to the peptide varies according to the fungal species tested, it seemed that the smaller the spores, the more likely they were to be affected by the treatment. In accordance with the results of Jacobi et al. (2000), the small conidia of *O. ulmi* were more affected by peptide action than the bigger conidia of *G. abietina* var. *abietina* and *N. galligena*. Similarly, after exposure to 100 µM of D2A21 peptide, the structure of *N. galligena* microconidia was altered while that of macroconidia was generally not impaired. These results tend to suggest that a greater surface/volume ratio would be more prejudicial to the fungal spores after initial interactions with the peptide molecules.

The small designed D2A21 peptide possesses interesting antifungal activities. This feature, coupled with the fact that it shows little phytotoxicity (Jacobi et al. 2000), makes it a



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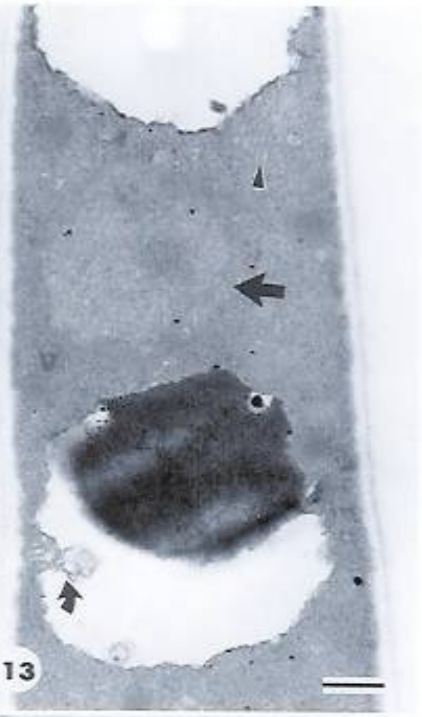

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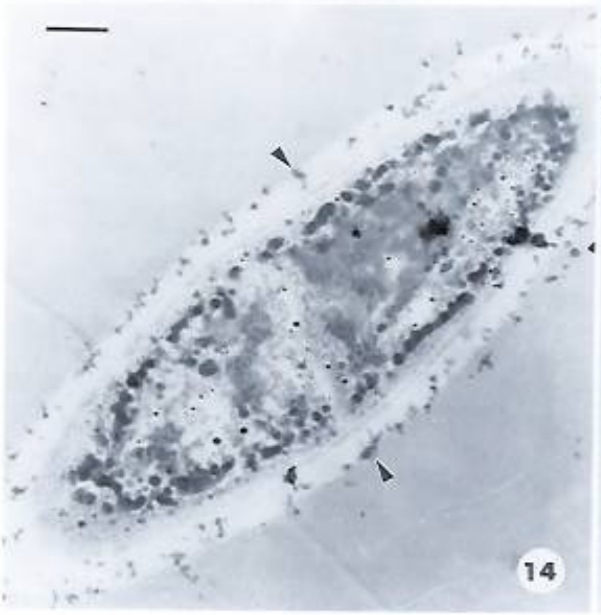

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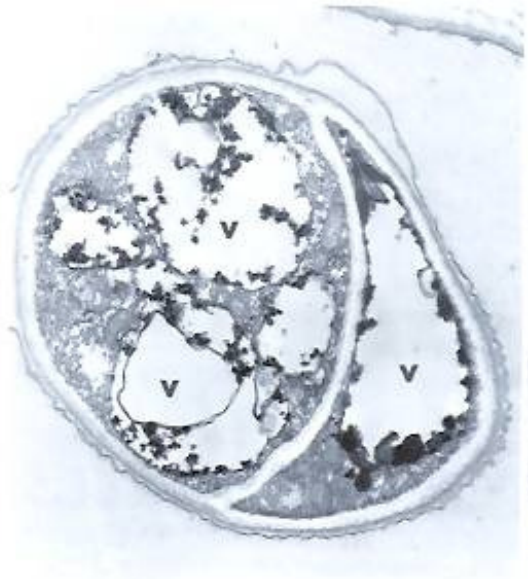

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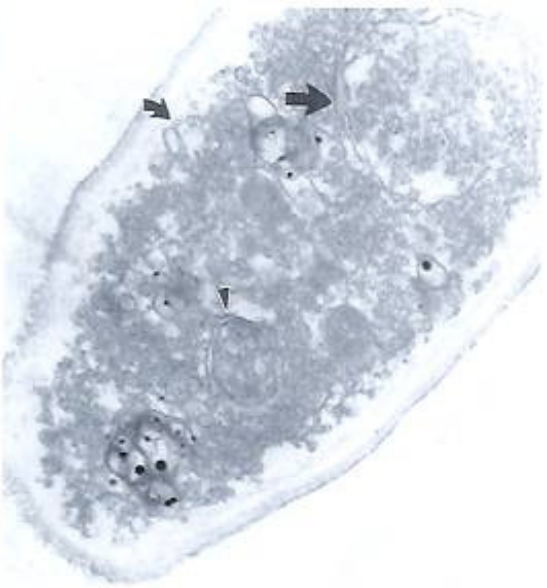

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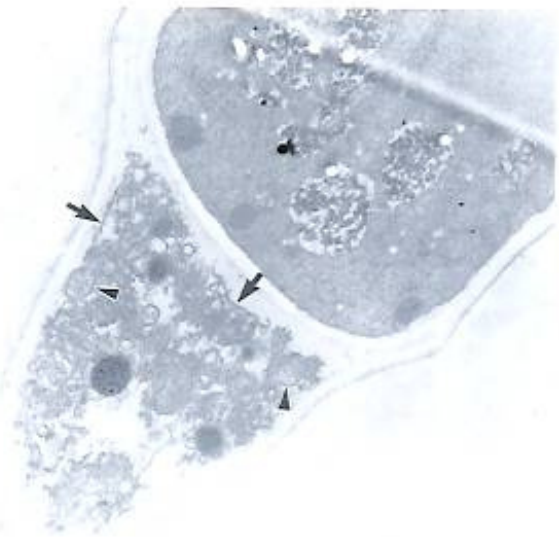

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Figs. 16–19. Conidia of *Nectria galligena*. Fig. 16. Oblique section of a control conidium showing prominent vacuoles (V). Scale bar = 1 µm. Fig. 17. A microconidium of *N. galligena* exposed to 100 µM of peptide that shows incipient structural alteration. The plasmalemma is hardly distinguishable, and many vesicles can be seen at the cytoplasm periphery (curved arrow). The nuclear envelope is more or less continuous (arrow) and a mitochondrion (arrowhead) has a swollen appearance. Scale bar = 0.3 µm. Fig. 18. This microconidium is greatly altered after exposure to 100 µM of peptide. Numerous vesicles (arrowheads) in the retracted cytoplasm are shown and some of them with a double membrane might represent greatly affected mitochondria (arrow). Scale bar = 0.4 µm. Fig. 19. While most of the cells of this macroconidium appeared normal, one cell at its extremity shows signs of alteration. The plasmalemma was observable only in places (arrows) and some mitochondria were still discernible in the cytoplasm (arrowheads). Scale bar = 0.3 µm.

good candidate for genetically engineered resistance to tree fungal pathogenic agents. Trees containing gene constructs to express this peptide have been recently produced (Séguin 1999) and are currently being tested in response to different diseases. Efforts have also been undertaken to obtain an antibody raised against this peptide to be able to follow the expression and the localization of this molecule in transformed trees concomitantly with examination of pathogen development.

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