

In vitro toxicity of natural and designed peptides to tree pathogens and pollen

V. Jacobi, A. Plourde, P.J. Charest, and R.C. Hamelin

Abstract: The toxicities of four candidate peptides, which have potential for engineering disease resistance into poplars and conifers, were tested and compared in vitro. Cecropin B, (Ala^{8,13,18})-magainin II amide, and the two synthetic membrane interactive molecules (Peptidyl MIMsTM) D2A21 and D4E1 inhibited germination of spores of the fungal pathogens *Cronartium ribicola* J.C. Fisch., *Gremmeniella abietina* (Lagerberg) Morelet, *Melampsora medusae* Thuem., *Nectria galligena* Bres. in Strass., *Ophiostoma ulmi* (Buisman) Nannf., and *Septoria musiva* Peck. Minimal inhibitory concentrations (MICs) of peptides required to achieve >95% inhibition of germination of conidia and urediniospores ranged from 0.3 to >5 μ M. Permutation analyses on MICs confirmed that peptides significantly reduced germination of fungus spores ($P = 0.0038$) and that MICs of the two synthetic peptides were, on average, lower than those of the two natural peptides ($P = 0.0012$). In contrast, peptides had much less of an effect on the pollen of *Picea*, *Pinus*, and *Populus* species and seeds of white pine; MICs from 10 μ M to >50 μ M of peptide were needed to arrest germination. Peptidyl MIMTM D2A21 combined high antifungal activity with low pollen toxicity. Similar to some antimicrobial peptides from plants, Peptidyl MIMTM D2A21 could not prevent germination of fungal spores in the presence of potato dextrose broth or chloride salts with divalent cations. This may explain why the peptide had only a limited effect on fungal mycelium grown in potato dextrose broth medium.

Key words: antimicrobial peptides (AMP), white pine blister rust, scleroderris canker, poplar leaf rust, Dutch elm disease, septoria canker.

Résumé : Les degrés de toxicité de quatre peptides, possédant un potentiel pour la transformation génétique des peupliers et des conifères pour la résistance aux maladies, ont été testés et comparés in vitro. La cécropine B, la (Ala^{8,13,18})-magainin II amide et deux peptides synthétiques agissant au niveau des membranes (Peptidyl MIMsTM D2A21 and D4E1) ont inhibé la germination des spores des pathogènes suivants : *Cronartium ribicola* J.C. Fisch., *Gremmeniella abietina* (Lagerberg) Morelet, *Melampsora medusae* Thuem., *Nectria galligena* Bres. in Strass., *Ophiostoma ulmi* (Buisman) Nannf. et *Septoria musiva* Peck. La concentration inhibitrice minimale (CIM) requise pour atteindre > 95 % d'inhibition de la germination des conidies et des urediniospores variait de 0,3 à > 5 μ M. Les analyses de permutation ont confirmé que les peptides ont réduit de façon significative la germination des spores ($P = 0,0038$) et que les CIM des deux peptides synthétiques étaient significativement plus basses que les deux peptides naturels ($P = 0,0012$). Cependant, l'effet des peptides sur le pollen des *Picea*, *Pinus* et *Populus* et les graines de pin blanc était moins marqué, avec des CIMs de 10 à > 50 μ M pour arrêter la germination. Le peptide synthétique Peptidyl MIMTM D2A21 combinait une forte toxicité anti-fongique avec une faible phytotoxicité. Cependant, comme d'autres peptides antimicrobiens provenant de plantes, le Peptidyl MIMTM D2A21 perdait son activité toxique sur les spores en présence de PDB ou de chlorures possédant des cations divalents. Ceci pourrait expliquer la faible toxicité de ces peptides sur la croissance mycélienne en milieu de culture.

Mots clés : peptides antimicrobiens (PAM), rouille vésiculeuse du pin blanc, chancre scléroderrien, rouille du peuplier, maladie hollandaise de l'orme, chancre septorien.

Introduction

Small antimicrobial peptides have been identified in a variety of higher organisms (Gabay 1994; Rao 1995). The majority of peptides can be categorized into linear peptides (including the cecropin and magainin families), disulfide-linked peptides (including mammalian and insect defensins as well as plant-derived antimicrobial peptides), and de-

signed synthetic antimicrobial peptides (Rao 1995). Best characterized are the cecropins, inducible peptides found in insects (Boman and Hultmark 1987; Boman et al. 1991), and the magainins, isolated from amphibians (Zasloff 1987; Bevins and Zasloff 1990). Both cecropins and magainins are part of the natural defense system of their respective source organisms and exhibit antimicrobial activities (Boman and Hultmark 1987; Zasloff 1987; Bevins and Zasloff 1990;

Received September 14, 1999.

V. Jacobi,¹ A. Plourde, and R.C. Hamelin.² Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, 1055 du PEPS, P.O. Box 3800, Sainte-Foy, QC G1V 4C7, Canada.

P.J. Charest. Canadian Forest Service Headquarters, 580 Booth Street, Ottawa, ON K1A 0E4, Canada.

¹Present address: BIOTEPP Inc., 177, 71e rue est, Charlesbourg, QC G1H 1L4, Canada.

²Author to whom all correspondence should be addressed (e-mail: rhamelin@cfl.forestry.ca).

Boman et al. 1991). The designed synthetic peptides originate mostly from modifications made in the primary sequences of the original cecropin and magainin molecules (Everett 1994; Maloy and Kari 1995). Analogues with increased antimicrobial activity and pathogen specificity have been identified (Chen et al. 1988; Jaynes et al. 1989; Maloy and Kari 1995).

The antimicrobial activity of linear peptides appears to be closely linked to the ability to form amphipathic α -helices that have a net cationic charge due to the presence of multiple arginine and lysine residues (Maloy and Kari 1995; Rao 1995). This may facilitate an interaction with the anionic phospholipids of the target cell followed by incorporation into the membrane and disruption of the membrane structure (Maloy and Kari 1995). Peptide affinity for bacterial cells over host cells appears to be linked to the composition of cell membranes, with an increased proportion of anionic phospholipids making the pathogen more susceptible (Maloy and Kari 1995; Rao 1995). Antimicrobial activity has predominantly been measured against bacterial pathogens of medical importance (Chen et al. 1988; Maloy and Kari 1995). Nevertheless, peptides that inhibit plant-pathogenic bacteria or fungi in vitro have been identified (Everett 1994; Powell et al. 1995; Reed et al. 1997).

Tree diseases cause serious economic losses in Canadian forests annually (Hall and Moody 1994). For many of these diseases, there are no efficient and economically viable controls. The objective of this study was to test whether or not natural antimicrobial peptides and their synthetic analogues may have the potential to improve the control of some of the most important forest tree diseases in North America, including Dutch elm disease (caused by *Ophiostoma ulmi* (Buisman) Nannf.), white pine blister rust (caused by *Cronartium ribicola* J.C. Fisch.), root rots (caused by fungi like *Cylindrocladium floridanum* Sobers & C.P. Seymour), and tree cankers (caused by fungi such as *Gremmeniella abietina* (Lagerberg) Morelet and *Nectria galligena* Bres. in Strass.). The specific objectives of this study were to (i) assess and compare the in vitro antifungal activities of four peptides, (ii) determine the toxicities of these peptides to tree pollen, and then (iii) select one peptide for gene construction and transformation of poplar and conifer tree species.

Materials and methods

Peptides

Two natural peptides, cecropin B and (Ala^{8,13,18})-magainin II amide, a magainin analogue with increased antimicrobial activity (Chen et al. 1988) (Sigma Chemical Co., St. Louis, Mo.) and two designed synthetic peptides, Peptidyl MIMsTM (membrane interactive molecules) D2A21 and D4E1 (Demeter BioTechnologies, Ltd., Durham, N.C.) were tested. Peptides were aliquotted, stored in lyophilized form at -20°C , and resuspended in sterile water as needed. The amino acid sequences of peptides are provided in Table 1.

Fungal cultures and spore suspensions

Isolates of *Cylindrocladium floridanum*, *Nectria galligena*, *Ophiostoma ulmi*, and *Septoria musiva* Peck were grown on either potato dextrose agar (PDA) (BBL, Cockeysville, Md.) or malt agar (Difco Laboratories, Detroit, Mich.) in standard disposable Petri plates. *Gremmeniella abietina* cultures were maintained on a me-

dium consisting of 30 g/L bacto agar (BA) (Difco Laboratories) containing Campbell's V8 juice (200 mL/L). All cultures were incubated at 18°C for 1–3 weeks and then, with the exception of *C. floridanum*, were exposed to light ($175\text{--}275\ \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) to stimulate spore production. The uredinal stages of the rust pathogens *Melampsora medusae* Thuem. and *Cronartium ribicola* were maintained on poplar (*Populus balsamifera* \times *Populus deltoides*) and *Ribes glandulosum* leaves, respectively. Isolates of fungi used in this study came from various sources (Table 2). Conidia of *G. abietina*, *N. galligena*, *O. ulmi*, and *S. musiva* were harvested from cultures grown in Petri plates. Urediniospores of *M. medusae* and *C. ribicola* were taken from actively growing cultures on poplar and *Ribes* leaves, respectively. All spore suspensions were prepared in sterile deionized water with or without the addition of Tween 20 (0.5 mL/L). Spore concentrations were determined either with a haemocytometer or by dilution plating. Microsclerotia of *C. floridanum* were collected from a mature culture grown on PDA. Mycelium was carefully separated from the medium with a scalpel, transferred into a blender containing 50–100 mL of water, and then ground for 30 s. The resulting crude homogenate was passed successively through 250- and 40- μm mesh filters. The residue on the 40- μm mesh filter, which contained the microsclerotia, was rinsed with water and transferred into 50-mL polypropylene tubes. Microsclerotia were allowed to settle, and the supernatant was decanted. The washing step was repeated until the resulting supernatant appeared colourless, and this preparation was used for in vitro tests.

Fungal propagule germination

Assays were conducted either in sterile 96-well microtest tissue culture plates (Becton & Dickinson Company, Lincoln Park, N.J.) or in disposable Immulon I Removawell strips (Dynatech Laboratories, Inc., Chantilly, Va.). Conidia or urediniospores were challenged with each peptide at final concentrations of 5, 2.5, 1.2, 0.6, 0.3, 0.15, and 0.08 μM in a volume of 100 μL . The solutions were incubated for 60 min at ambient temperature ($22\text{--}24^{\circ}\text{C}$), transferred to 90-mm diameter Petri plates containing BA (15 g/L), and incubated at 18°C . Microsclerotia of *C. floridanum* were treated similarly, except that final peptide concentrations were 50, 10, 5, 2.5, and 1.2 μM . Water controls were included to determine spore germination in the absence of peptide. In addition, conidia of *G. abietina* challenged with 5 μM of each peptide were included to make sure that full peptide activity was maintained throughout all tests. Germination of spores and microsclerotia was assessed 24–96 h after adding suspensions to plates with a Leitz Fluovert FS inverted microscope (Ernst Leitz Wetzlar GmbH, Wetzlar, Germany). Each treatment was replicated three times, and a minimum of 100 spores or 50 microsclerotia were examined for each replicate. Propagules were considered germinated only if the length of the newly formed germ tube or hypha was at least twice the length or diameter of the spore. To compare antifungal activities of peptides, minimal inhibitory concentrations (MICs) were calculated. MIC is defined here as the lowest peptide concentration tested (in μM) that reduced fungus propagule germination by >95%, compared with fungus propagule germination in water without peptide.

Plant pollen and seed germination

Pollen grains from eastern white pine (*Pinus strobus*), white spruce (*Picea glauca*), and Norway spruce (*Picea abies*) (Table 2) were removed from cold storage and transferred into 1.5-mL microtubes without caps and incubated in a moist chamber for 2 h at 4°C and then at ambient temperature for 30 min. Pollen grains were suspended in sterile deionized water at 5×10^3 – 1×10^4 grains/mL and challenged with each peptide at final concentrations of 50, 10, 5, and 2.5 μM . Assay conditions and controls were the same as those described for spore germination tests. Percent pollen

Table 1. Amino acid numbers and sequences of natural and synthetic peptides used in this study.

Name	No.	Sequence
Peptidyl MIM™ D2A21	23	FAKKFAKKFKKFAKKFAKFAFAF
Peptidyl MIM™ D4E1	17	FKLRKIKVRLRAKIKL
Cecropin B	35	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLKEAKAL-NH ₂
(Ala ^{8,13,18})-magainin II amide	23	GIGKFLHAAKKFAKAFVAEIMNS-NH ₂

Note: Abbreviations used in the amino acid sequences are as follows: A, alanine; E, glutamic acid; F, phenylalanine; G, glycine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; R, arginine; S, serine; V, valine; W, tryptophan; -NH₂, indicates terminal amide group.

Table 2. Identification and origins of organisms and types of target structures used in bioassays to determine toxicities of natural and designed peptides.

Organism	Taxonomic rank	Isolate		Target structure
		Number	Source ^a	
<i>Cylindrocladium floridanum</i> Sobers & C.P. Seymour	Fungus, Ascomycota	Q 942018	R.C. Hamelin, LFC	Microsclerotia
<i>Cronartium ribicola</i> J.C. Fisch.	Fungus, Basidiomycota	Mix of isolates	K. Et-touil, LFC	Urediniospores
<i>Gremmeniella abietina</i> (Lagerberg) Morelet	Fungus, Ascomycota	94006 (NA), 900023 (EU)	G. Laflamme, LFC	Conidia
<i>Melampsora medusae</i> Thuem.	Fungus, Basidiomycota	MS13 mono-2	M. Bourassa, LFC	Urediniospores
<i>Nectria galligena</i> Bres. in Strass.	Fungus, Ascomycota	Q 943091	F. Plante, UL	Conidia
<i>Ophiostoma ulmi</i> (Buisman) Nannf.	Fungus, Ascomycota	202, 205	P. DesRochers, LFC	Conidia
<i>Septoria musiva</i> Peck	Fungus, Ascomycota	S 95.04, S 95.03	M.-J. Mottet, GQ-MdF	Conidia
<i>Picea abies</i> [L.] Karst.	Plant, conifer	81419	Pollen bank, LFC	Pollen
<i>Picea glauca</i> [Moench] Voss	Plant, conifer	HUN-10	Pollen bank, LFC	Pollen
<i>Pinus strobus</i> L.	Plant, conifer	78502 825735	Pollen bank, LFC Seed bank, LFC	Pollen Seed
<i>Populus balsamifera</i> L.	Plant, dicot	—	Breeding program, GQ-MdF	Pollen
<i>Populus balsamifera</i> L. × <i>Populus deltoides</i> Marshall	Plant, dicot	—	Breeding program, GQ-MdF	Pollen

^aLFC, Laurentian Forestry Centre, Sainte-Foy, Que.; UL, Université Laval, Faculté de foresterie et géomatique, Sainte-Foy, Que.; GQ-MdF, Gouvernement du Québec, Ministère des Forêts, Service de l'amélioration des arbres, Sainte-Foy, Que.; *Gremmeniella abietina* races: EU, European race, NA, North American race.

germination was assessed 48–72 h after transferring suspensions to pollen germination medium (7.5 g/L BA containing 100 g/L sucrose) in 90-mm diameter Petri plates and incubation in a moist chamber at 28–30°C (Schopmeyer 1994). Each treatment was replicated three times, and 100 pollen grains were counted in each replicate. Pollen grains were considered germinated only if the length of the pollen germ tube was at least twice the length of the grain. Branches of *Populus balsamifera* and *Populus balsamifera* × *Populus deltoides* (Jackii) (Table 2) were collected in March 1997 and forced to shed pollen, which was used immediately after harvest for in vitro tests. Assay conditions were similar to those described for conifer pollen, but incubation on medium was at 24°C.

Seeds of eastern white pine (Table 2) were stratified for 6 weeks at 4°C (Schopmeyer 1994). Groups of 40 seeds each were transferred into individual 35 × 10 mm Petri dishes. Seeds were incubated for 16 h at ambient temperature in either 1 mL of sterile water or in 1 mL of a 50 µM solution of each peptide. Each treatment was replicated twice. The liquids were removed by pipette, and seeds were transferred in groups of 40 onto 90-mm diameter Whatman no. 1 filter paper disks. Disks were placed in Petri plates containing a layer of creped cellulose paper wadding saturated with 10 mL of deionized water. The seeds were incubated in moist chambers for 21 days at ambient temperature. Percent germination was determined at weekly intervals for 3 weeks. Seeds were considered germinated only when the radicle was at least twice the length of the seed. MICs were calculated, and full activity of each

peptide was verified as described for fungus propagule germination tests.

Proteinase K treatment

To confirm that the antimicrobial activities of the peptide preparations used in this study were in fact due to the peptides themselves and not to the presence of some contaminating nonprotein compound, 20 µM of each peptide was digested for 4 h at 37°C with 100 µg/mL of Proteinase K (Boehringer Mannheim Canada, Laval, Que.) in a final volume of 50 µL. An equal volume of *G. abietina* conidium suspension was added to each sample. Conidia were incubated for 60 min in the presence of peptides, and enzyme and suspensions were transferred onto 90-mm diameter Petri plates containing 15 g/L BA. Controls included conidia exposed to each undigested peptide, to sterile water only, and to enzyme only.

Additional tests conducted with Peptidyl MIM™ D2A21

Other assay conditions and control treatments were the same as those for spore germination tests. MIC is defined here as the lowest peptide concentration tested that prevented any detectable bacterial growth.

Aliquots of 2.5 mL of potato dextrose broth (PDB) (Difco Laboratories) containing 0, 10, or 20 µM of Peptidyl MIM™ D2A21 were seeded with mycelium plugs (3 mm diameter) taken from the

periphery of actively growing plate cultures of *C. floridanum*, *N. galligena*, *O. ulmi*, and *S. musiva*. Cultures were shaken at ambient temperature and 60 rpm on an orbital benchtop shaker (Lab-Line Instruments, Inc., Melrose Park, Ill.) for 7 days. Cultures were filtered through two layers of Metrical filters (VWR Scientific, Montréal, Que.), rinsed with sterile deionized water, dried, and weighed. Each treatment was replicated twice.

Equal volumes of *G. abietina* conidium suspension were added to sterile water or different concentrations of PDB liquid culture medium (full-strength; 2-, 4-, and 20-fold diluted), magnesium chloride hexahydrate (at 10, 50, and 500 mM), calcium chloride dihydrate, potassium chloride, and sodium chloride (each at 5, 25, and 250 mM, respectively) containing either 20 μM Peptidyl MIM™ D2A21 or no peptide. Assay conditions and controls were identical to those described for spore germination tests. Each treatment was replicated three times. The effect of salt and PDB diluents on germination of *G. abietina* conidia in the presence of peptide was expressed as threshold concentration, i.e., the highest diluent concentration at which complete inhibition of germination remained unaffected (i.e., a higher concentration allowed germination to occur).

Statistical analysis

A permutation test was used to analyze the effect of peptides on fungal propagule germination expressed as MICs. The six fungi that had been tested against all four peptides were included in the analysis where each fungus was considered as a complete repeat in a randomized block design (Edgington 1987). On the basis of 10 000 permutations and a significance level of $P \leq 0.05$, the following hypotheses were tested: (i) peptides are different, i.e., they do not have the same effect on spore germination of tree pathogens; (ii) synthetic and natural peptides are similar, i.e., the two synthetic peptides have, on average, the same effect as the two natural peptides; (iii) effects of the synthetic peptides are similar, i.e., Peptidyl MIM™ D2A21 has the same effect as Peptidyl MIM™ D4E1; and (iv) effects of natural peptides are similar, i.e., the magainin analogue has the same effect as the cecropin.

Results

All four peptides inhibited germination of conidia and urediniospores of all species of fungi tested but not microsclerotia of *C. floridanum* (Table 3). Variation among the fungi in spore sensitivity to peptide action was observed. MICs against these two types of spores ranged from 0.3 to $>5 \mu\text{M}$ (Table 3). The small and thin-walled conidia of *O. ulmi* and *S. musiva* were more sensitive than the larger spores of *G. abietina*, *M. medusae*, and *N. galligena*. Microsclerotia of *C. floridanum* were the only propagule type that were not affected at peptide concentrations of up to 50 μM , the highest concentration tested (Table 3). Permutation analyses of MICs of the six fungi that were tested against all four peptides demonstrated that (i) the effects of peptides on fungal spore germination differed significantly ($P = 0.0038$); (ii) the two synthetic peptides had, on average, significantly lower MICs than the two natural peptides ($P = 0.0012$) and, therefore, inhibited spore germination better; (iii) the effects of the two synthetic peptides on germination did not differ significantly ($P = 0.75$); and (iv) the magainin analogue has, on average, a higher MIC than the cecropin ($P = 0.0036$) and, therefore, did not inhibit germination as effectively at lower concentrations. Variation within treatment replicates was not observed, i.e., the MICs for each replicate of a specific treatment were identical.

Higher concentrations of peptides were needed to inhibit germination of tree pollen and white pine seeds than to inhibit germination of spores of fungus pathogens (Tables 3 and 4). Poplar pollen was more sensitive to peptide action than conifer pollen (Table 4). Variation within treatment replicates was not observed, i.e., the MICs for each replicate of a specific treatment were identical.

Proteolysis completely eliminated the biological activity of all peptides. Conidia of *G. abietina* exposed to each digested peptide had over 90% germination, as did conidia exposed to water or proteinase K only. Conidia exposed to the corresponding undigested peptides did not germinate.

Complete inhibition of mycelium growth of any of the four fungi tested in PDB liquid cultures was not achieved. In fact, both peptide concentrations had little effect on cultures of *N. galligena*, *O. ulmi*, and *S. musiva* (Table 5). However, at a concentration of 20 μM , Peptidyl MIM™ D2A21 reduced the growth of *C. floridanum* mycelium by more than 50% (Table 5).

Salts and PDB inhibited the effect of peptide on spore germination: conidia of *G. abietina* (NA) did not germinate after exposure to 10 μM Peptidyl MIM™ D2A21 diluted in sterile water, 20-fold diluted PDB, $<10 \text{ mM}$ chloride salts with divalent cations, and $<25 \text{ mM}$ chloride salts with monovalent cations. Above these diluent threshold concentrations, however, conidium germination was observed. In the absence of peptide, germination of *G. abietina* conidia was greater than 90%, independent of diluent.

Discussion

We assessed and compared the in vitro antifungal activities and nonspecific phytotoxicities of two natural and two synthetic designed peptides to evaluate their potential usefulness to augment the natural defense system of poplar and conifer tree species against common pathogens. To our knowledge, this is the first report on antifungal activities of peptides against a variety of fungal propagules (including conidia, urediniospores, and microsclerotia) from a broad range of forest tree pathogens, including members of the Ascomycota and Basidiomycota.

Elimination of biological activities of all peptides used in this study after digestion with proteinase K confirmed that the peptides themselves, rather than any contaminating non-protein substances, were responsible for the observed antimicrobial effects. Fungal propagule germination inhibition tests proved a useful tool to study peptide activities, since the tests were easy to perform and only small amounts of peptides were required. Significant differences in antifungal activities among peptides were observed including stronger antifungal activities by the two synthetic peptides compared with two natural peptides. Differences in sensitivity to peptide action among different fungal propagules also were observed. Spores from a variety of forest tree pathogens were susceptible to peptide action. Only the microsclerotia of *C. floridanum* were unaffected by the two synthetic peptides at the limited range of concentrations tested. However, these are multicellular, thick-walled survival structures and are resistant to environmental extremes as well as to microorganisms. It is, therefore, not surprising that the synthetic peptides did not affect these propagules. It should be men-

Table 3. Toxic effects of four natural and synthetic peptides on germination of propagules of eight selected fungi.

Fungus	Propagule concentration (no./mL)	Germination of untreated spores (%) ^c	MIC (µM) ^{a,b}			
			Magainin	Cecropin	D2A21	D4E1
<i>Cylindrocladium floridanum</i>	2 × 10 ³	86	—	—	>50	>50
<i>Cronartium ribicola</i>	3 × 10 ³	64	5	2.5	0.6	0.6
<i>Gremmeniella abietina</i> (EU)	3 × 10 ⁵	65	5	2.5	1.2	1.2
<i>Gremmeniella abietina</i> (NA)	1 × 10 ⁵	88	5	1.2	0.6	0.6
<i>Melampsora medusae</i>	2 × 10 ⁴	80	5	2.5	2.5	2.5
<i>Nectria galligena</i>	2 × 10 ⁴	88	>5	>5	5	5
<i>Ophiostoma ulmi</i>	2 × 10 ⁴	83	—	—	0.6	0.6
<i>Septoria musiva</i>	3 × 10 ⁴	66	0.6	0.6	0.3	0.3

Note: EU, European race; NA, North American race.

^aValues are means of three replicates for each treatment. Minimum inhibitory concentration (MIC) is the lowest peptide concentration tested that reduced germination by at least 95% compared with germination of propagules in water alone. Highest peptide concentrations tested were 50 µM for *C. floridanum* and 5 µM for all other fungi.

^bResults of permutation analysis on MICs of six fungi tested against all four peptides: (i) peptides affect target organisms differently ($P = 0.0038$); (ii) MICs of synthetic peptides are lower than MICs of natural peptides ($P = 0.0012$); (iii) MICs of synthetic peptides are not different ($P = 0.75$); and (iv) MIC of magainin is higher than MIC of cecropin ($P = 0.0036$).

^cGermination in the absence of peptide. Values are means of three replicates for each fungus.

Table 4. Toxic effects of four natural and synthetic peptides on germination of pollen and seed from selected tree species.

Species	Propagule	Propagule initial concentration (no./mL)	Germination (%) ^b	MIC (µM) ^a			
				Magainin	Cecropin	D2A21	D4E1
<i>Picea abies</i>	Pollen	5 × 10 ³	90	>50	>50	>50	>50
<i>Picea glauca</i>	Pollen	5 × 10 ³	62	>50	>50	>50	>50
<i>Pinus strobus</i>	Pollen	7 × 10 ³	90	>50	>50	>50	50
	Seed	4 × 10 ¹	55	>50	>50	>50	>50
<i>Populus balsamifera</i>	Pollen	1 × 10 ⁴	51	—	10	10	50
<i>Populus balsamifera</i> × <i>Populus deltoides</i>	Pollen	9 × 10 ³	32	—	10	50	50

^aValues are the means of three and two replicates for each treatment of pollen and seed samples, respectively. Minimum inhibitory concentration (MIC) is the lowest peptide concentration tested that reduced germination by at least 95% compared with germination of propagules in water alone. Highest peptide concentration tested was 50 µM.

^bGermination in the absence of peptide. Values are the means of three and two replicates for pollen and seed samples, respectively.

Table 5. Effects of three concentrations (µM) of Peptidyl MIM™ D2A21 on the growth of four fungi in potato dextrose broth.

Fungus	Peptidyl MIM™ concentration (µM)		
	0	10	20
<i>Cylindrocladium floridanum</i>	13.5 ± 0.1	9.9 ± 0.7	5.9 ± 0.7
<i>Nectria galligena</i>	10.4 ± 1.1	11.9 ± 0.4	9 ± 1.7
<i>Ophiostoma ulmi</i>	9.5 ± 0.6	8.9 ± 0.8	10.1 ± 1.2
<i>Septoria musiva</i>	11 ± 1.5	10.4 ± 1.1	8.2 ± 0.6

Note: Values (mycelium dry weight in milligrams) are means ± SD for two replicates for each treatment.

tioned here that our experiments were designed to assess the range of antifungal activities of the different peptides for a variety of fungal species. Follow-up studies could target specific fungus-peptide combinations in order to determine more accurately the toxicity values and effects of different parameters on peptide activities.

A variety of tests have been devised by others to assess the phytotoxicity of peptides (Nordeen et al. 1992; Mills and Hammerschlag 1993; Everett 1994; Powell et al. 1995; Qiu et al. 1995). In our study, inhibition of pollen germination proved to be a simple and sensitive indicator of additional toxic effects of peptides. In general, higher concentrations of peptides were needed to arrest germination of tree pollen

than to inhibit germination of spores of fungal pathogens. Also, none of the peptides used in this study were inhibitory to the germination of white pine seeds at the concentrations tested. Similarly, Powell et al. (1995) designed synthetic magainin analogues that showed greater inhibition of fungal conidium germination than of tree pollen germination. In contrast, membrane interactive peptide D5-C was more toxic to pollen of canola (*Brassica napus*) than to the conidia of two fungal pathogens of canola (Qiu et al. 1995). The fact that the two synthetic peptides exhibited slightly higher antifungal activities than their natural counterparts indicates that it is possible to engineer more active peptides using insights obtained through structure-function analysis. Similarly, oth-

ers have reported on the synthesis of cecropin or magainin analogues with improved in vitro antimicrobial activities (Chen et al. 1988; Jaynes et al. 1989; Maloy and Kari 1995).

Peptidyl MIMTM D2A21 combined high antifungal activity with low pollen toxicity. Interestingly, the peptide was less effective when used to challenge the growth of fungal mycelium in liquid cultures. Two scenarios, not mutually exclusive, could help to explain these results: actively growing fungal mycelium is less sensitive to peptide action than nongerminated fungal spores, or the experimental conditions such as the type of medium used may have prevented or reduced expression of antifungal activity. PDB, the liquid culture medium used in our experiment, interacted in some way with peptide activity, conidia germination, or both. At the higher end of the range of PDB concentrations tested, *G. abietina* conidia germinated even in the presence of 10 µM Peptidyl MIMTM D2A21. This may explain, at least in part, the lack of inhibition of mycelium growth at the 10-µM level. A similar effect was observed in the presence of chloride salts with divalent cations but was less pronounced in the presence of chloride salts with monovalent cations. The composition and ionic strength of the assay environment must therefore be considered as a major factor influencing the results of in vitro tests.

The same mechanisms also may be active in transgenic plants. In an intriguing study (De Bolle et al. 1996), the antimicrobial peptides Mj-AMP2 and Ac-AMP2 from seeds of *Mirabilis jalapa* and *Amaranthus caudatus*, respectively, were expressed in tobacco plants. In previous tests (Broekaert et al. 1992; Cammue et al. 1992), both peptides demonstrated strong in vitro activities against several plant pathogenic fungi and some Gram-positive bacteria. However, none of the transgenic tobacco plants showed enhanced resistance to infection by *Botrytis cinerea* or *Alternaria longipes*, despite verified expression and processing of proteins (De Bolle et al. 1996). Compounds extracted from transformed plants and inhibiting the in vitro antifungal activities of peptides were most likely inorganic cations (De Bolle et al. 1996). Similarly, Terras et al. (1993) observed a decrease in the in vitro antifungal activities of seven cysteine-rich proteins isolated from seeds of Brassicaceae species when inorganic cations were added to the assay medium. In general, divalent cations appeared to be much more effective in reducing peptide activity than monovalent cations. The sensitivity of peach cells and peach protoplasts to cecropin B increased as the concentration of Ca²⁺ ions in the isolation and assay medium was reduced from 10 to 0.1 mM (Mills and Hammerschlag 1993). Similarly, the antifungal activity of polyene macrolides, a group of membrane interactive antibiotics used for treatment of fungal infections in humans, decreases as the ionic strength of the culture medium increases (Kerridge and Whelan 1984). Variations in results of in vitro tests both among studies and also within the same study when using different extraction or incubation media should, therefore, be expected and may not always accurately reflect real differences in antimicrobial activities among peptides.

The broad-spectrum in vitro antifungal activity of Peptidyl MIMTM D2A21 combined with its low pollen toxicity make the gene coding for this peptide a candidate for transformation of poplar and conifer species to improve disease resis-

tance. However, due to the major impact of the assay environment on peptide activity as observed in this and other studies, it would seem that in vitro bioassays cannot predict conclusively the usefulness of these peptides in vivo. We are, therefore, in the process of transforming several poplar lines as well as white pine with a construct containing the gene coding for Peptidyl MIMTM D2A21 to verify its expression and antifungal activity within woody plant tissues.

Acknowledgements

This work was supported by a grant from the Canadian government National Biotechnology Strategy. We thank Manon Gignac and André Dansereau for help with in vitro bioassays and maintenance of fungal cultures, Dr. Jean Beaulieu for providing conifer pollen and white pine seeds, and Serge Morin for collecting poplar pollen. We also thank W.A. Powell and C.M. Catranis for critical reading of the manuscript, P. Cheers for editorial services, and M. Bernier-Cardou for statistical analyses. Demegen provided the two synthetic peptides free of charge.

References

- Bevins, C.L., and Zasloff, M. 1990. Peptides from frog skin. *Annu. Rev. Biochem.* **59**: 395–414.
- Boman, H.G., and Hultmark, D. 1987. Cell-free immunity in insects. *Annu. Rev. Microbiol.* **41**: 103–126.
- Boman, H.G., Faye, I., Gudmundson, G.H., Lee, J.-Y., and Lidholm, D.-A. 1991. Cell-free immunity in *Cecropia*. *Eur. J. Biochem.* **201**: 23–31.
- Broekaert, W.F., Marien, W., Terras, F.R.G., De Bolle, M.F.C., Proost, P., Van Damme, J., Dillen, L., Claeys, M., Rees, S.B., Vanderleyden, J., and Cammue, B.P.A. 1992. Antimicrobial peptides from *Amaranthus caudatus* seeds with sequence homology to the cysteine/glycine-rich domain of chitin binding proteins. *Biochemistry*, **31**: 4308–4314.
- Cammue, B.P.A., De Bolle, M.F.C., Terras, F.R.G., Proost, P., Van Damme, P., Rees, S.B., Vanderleyden, J., and Broekaert, W.F. 1992. Isolation and characterization of a novel class of plant antimicrobial peptides from *Mirabilis jalapa* L. seeds. *J. Biol. Chem.* **267**: 2228–2233.
- Chen, H.-C., Brown, J.H., Morell, J.L., and Huang, C.M. 1988. Synthetic magainin analogues with improved antimicrobial activity. *FEBS Lett.* **236**: 462–466.
- De Bolle, M.F.C., Osborn, R.W., Goderis, I.J., Noe, L., Acland, D., Hart, C.A., Torrekens, S., Van Leuven, F., and Broekaert, W.F. 1996. Antimicrobial peptides from *Mirabilis jalapa* and *Amaranthus caudatus*: expression, processing, localization and biological activity in transgenic tobacco. *Plant Mol. Biol.* **31**: 993–1008.
- Edgington, E.S. 1987. Randomization tests. 2nd ed. Marcel Dekker Inc., New York.
- Everett, N.P. 1994. Design of antifungal peptides for agricultural applications. Natural and Engineered Pest Management Agents. ACS Symposium Series 551, American Chemical Society. Edited by P.A. Hedin, J.J. Menn, and R.M. Hollingworth. Washington, D.C. pp. 278–291.
- Gabay, J.E. 1994. Ubiquitous natural antibiotics. *Science* (Washington, D.C.), **264**: 373–374.
- Hall, J.P., and Moody, B.H. (Compilers). 1994. Forest depletions caused by insects and diseases in Canada 1982–1987. Natural

- Resources Canada, Canadian Forest Service, Forest Insect and Disease Survey, Ottawa, Ont. Inf. Rep. ST-X-8.
- Jaynes, J.M., Julian, G.R., Jeffers, G.W., White, K.L., and Enright, F.M. 1989. In vitro cytotoxic effect of lytic peptides on several transformed mammalian cell lines. *Pept. Res.* **2**: 157–160.
- Kerridge, D., and Whelan, W.L. (Editors). 1984. The polyene macrolide antibiotics and 5-fluorocytosine: molecular actions and interactions. Mode of action of antifungal agents. Cambridge University Press, Cambridge. pp. 343–375.
- Maloy, W.L., and Kari, U.P. 1995. Structure–activity studies on magainins and other host defense peptides. *Biopolymers*, **37**: 105–122.
- Mills, D., and Hammerschlag, F.A. 1993. Effect of cecropin B on peach pathogens, protoplasts, and cells. *Plant Sci.* **93**: 143–150.
- Nordeen, R.O., Sinden, S.L., Jaynes, J.M., and Owens, L.D. 1992. Activity of cecropin SB37 against protoplasts from several plant species and their bacterial pathogens. *Plant Sci.* **82**: 101–107.
- Powell, W.A., Catranis, C.M., and Maynard, C.A. 1995. Synthetic antimicrobial peptide design. *Mol. Plant-Microbe Interact.* **8**: 792–794.
- Qiu, X., Wu, Y., Jaynes, J., Goodwin, P., and Erikson, L.R. 1995. Effect of a membrane interactive peptide on plant cells of canola (*Brassica napus*) and two fungal pathogens. *Plant Cell Rep.* **15**: 115–118.
- Rao, A.G. 1995. Antimicrobial peptides. *Mol. Plant-Microbe Interact.* **8**: 6–13.
- Reed, J.D., Edwards, D.L., and Gonzalez, C.F. 1997. Synthetic peptide combinatorial libraries: a method for the identification of bioactive peptides against phytopathogenic fungi. *Mol. Plant-Microbe Interact.* **10**: 537–549.
- Schopmeyer, C.S. 1994. Seeds of woody plants in the United States. U.S. For. Serv. Handb. 450, U.S. Dep. Agric., Washington, D.C.
- Terras, F.R.G., Torrekens, S., Van Leuven, F., Osborn, R.W., Vanderleyden, J., Cammue, B.P.A., and Broekaert, W.F. 1993. A new family of basic cysteine-rich plant antifungal proteins from Brassicaceae species. *FEBS Lett.* **316**: 233–240.
- Zasloff, M. 1987. Magainins, a class of antimicrobial peptides from *Xenopus* skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc. Natl. Acad. Sci. U.S.A.* **84**: 5449–5453.