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Purification and characterization of a phytoalexin elicitor from spores of the saprobe *Mucor ramosissimus*[1]

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ABSTRACT – (Purification and characterization of a phytoalexin elicitor from spores of the saprobe *Mucor ramosissimus*). Plants accumulate antimicrobial compounds (phytoalexins) in response to a wide variety of microorganisms. *Mucor ramosissimus* Samutsevitsch is a saprobe capable of inducing phytoalexin production in soybean cotyledons and in the leaves of tropical Rubiaceae on whose surface it has been found. In the present study, the elicitor from *M. ramosissimus* was partially purified and the activity compared to that of a glucan elicitor isolated from *Phytophthora sojae*. Optimal isolation of the elicitor (based on fungal growth, yield of spores and elicitor activity) was achieved by autoclaving spores obtained from nine day-old cultures of the fungus. The elicitor was precipitated with ethanol and purified by chromatography on an anion exchange column, which retained the elicitor, and a Concanavalin A-affinity matrix, to which the elicitor did not bind. The purification resulted in a considerable increase (six-fold) in the specific activity of the elicitor. Neutral sugar composition, analyzed by HPLC, revealed the predominance of mannose, followed by glucose and galactose, whereas colorimetric quantification showed the presence of uronic acids. GC-MS analysis of the elicitor revealed the predominance of glucuronic acid and mannose. These results suggest that fragments of mucoran-type polysaccharides are the phytoalexin elicitors present in the spores of the saprobe *M. ramosissimus*. Our results also indicate for the first time that soybean cotyledon tissues can recognize fragments of glucuronic-acid heteropolymers as phytoalexin elicitors.

Key words - elicitor, *Mucor ramosissimus*, mucoran, phytoalexin, saprobe fungus, soybean

RESUMO – (Purificação e caracterização de um eliciador de fitoalexinas de esporos do fungo sapróbio *Mucor ramosissimus*). Plantas acumulam compostos antimicrobianos (fitoalexinas) em resposta a uma grande variedade de microorganismos. *Mucor ramosissimus* Samutsevitsch é um fungo sapróbio capaz de induzir a produção de fitoalexinas em soja e em Rubiaceae nativas, sobre a superfície das quais ele é encontrado. Neste estudo, o eliciador de esporos de *M. ramosissimus* foi parcialmente purificado e sua atividade comparada ao glucano isolado de *Phytophthora sojae*. O ótimo de isolamento do eliciador (baseado no crescimento do fungo, no rendimento dos esporos e na atividade eliciadora) foi obtido a partir de esporos autoclavados de culturas do fungo com nove dias de idade. O eliciador foi precipitado com etanol e purificado por cromatografia de troca iônica, a qual reteve o eliciador, e por uma matriz de afinidade em Concanavalina-A, à qual o eliciador não se ligou. A purificação resultou em considerável aumento (seis vezes) de atividade específica do eliciador. Análises de açúcares neutros por HPLC revelaram a predominância de manose, seguida de glucose e galactose, enquanto que a quantificação por colorimetria mostrou a presença de ácidos urônicos. Análises do eliciador por GC-MS indicaram a predominância de ácido glucurônico e manose. Esses resultados sugerem que fragmentos de um polissacarídeo do tipo mucorano são os eliciadores de fitoalexinas presentes nos esporos de *M. ramosissimus*. Os resultados também indicam, pela primeira vez, que os tecidos de cotilédones de soja reconhecem fragmentos de heteropolímeros de ácidos urônicos como eliciadores de fitoalexinas.

Palavras-chave - eliciador, fitoalexinas, fungo sapróbio, *Mucor ramosissimus*, mucorano, soja

Introduction

The synthesis of antimicrobial compounds named phytoalexins is one of the best-studied defensive responses of plants to pathogens (Dixon & Lamb 1990). Phytoalexin production is observed not only after microbial infection, but also after diverse biotic and abiotic stresses. In addition, cell-free extracts of microbial and plant origin, as well as inorganic and organic substances can trigger the synthesis of these compounds in plant species. The inducing molecules are referred to as elicitors, the term being commonly used to describe molecules capable of stimulating any plant defensive mechanism (Côté *et al*. 1995, Nürnberger 1999).

Different eliciting substances isolated from fungi have been shown to induce phytoalexin accumulation in

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several plant species (Hahn 1996, Shibuya & Minami 2001, and references therein). Among them, the β-1,3 β-1,6 branched glucan isolated from mycelial cell walls or cultures filtrates of *Phytophthora sojae* - a natural soybean pathogen – has been considered the most potent elicitor described (Ayers *et al.* 1976a, Cheong *et al.* 1991, Hahn 1996). Besides glucans, fragments of chitin and chitosan have also been characterized as elicitors of fungal origin (Shibuya & Minami 2001, Agrawal *et al*. 2002). The extent of biochemical information available on elicitor signals and on cellular response resulting in the biosynthesis and accumulation of phytoalexins in soybean make this system particularly attractive for studies of signaling mechanisms in plants (Côté *et al.* 1995).

The phytoalexins from soybean (glyceollins) are pterocarpans derived from the phenylpropanoid pathway and occur as a series of isomers (I-IV) (Paxton 1995). The isoflavone daidzein is the immediate precursor of the glyceollins (Paxton 1995) and genistein (tri-hydroxylated isoflavone) is another anti-microbial isoflavone that is accumulated in soybean tissues in response to fungal elicitors (Rivera-Vargas *et al.* 1993, Graham & Graham 2000). Both daidzein and genistein are present as large pools of pre-formed conjugates in soybean seedling tissues, being released by hydrolysis in response to incompatible interaction with pathogens or treatment with fungal or plant elicitors (Graham *et al*. 1990, Graham 1995).

Some saprobe fungi such as *Heterobasidion annosum* can induce defensive responses in plants even though they are not capable of invading their tissues (Asiegbu *et al.* 1994). Other saprobe fungi have been described as inducers of phytoalexin accumulation in wild dicotyledonous plants as well as in soybean (Braga *et al.* 1986, Braga & Dietrich 1991, Cordeiro Neto & Dietrich 1992), the inducing activity being comparable to that of pathogenic species (Costa & Dietrich 1996, Garcéz *et al*. 2000). Included among these fungi is *Mucor ramosissimus* Samutsevitsch, a filamentous Zygomycete commonly found in soil, litter, or on plant surfaces (Ellis 1997).

Spores of *M. ramosissimus* are found on the leaf surface of Rubiaceae species in tropical environments during the autumn and winter periods (Cordeiro Neto & Dietrich 1992). Live and autoclaved spore suspensions of *M. ramosissimus* were shown to be potent inducers of phytoalexin production in detached leaves of these Rubiaceae and in soybean cotyledons (Cordeiro Neto & Dietrich 1992, Goméz *et al*. 1994, Pelicice *et al.* 2000). Garcéz *et al.* (2000) compared the phytoalexin-eliciting activity of this fungus with a saprobe *Rhizopus* species and observed that only spores of *M. ramosissimus* were capable of inducing phytoalexin response in soybean cultivars susceptible to frog-eye spot and stem canker disease. In spite of its potent phytoalexin-inducing activity, the elicitor isolated from spores of *M. ramosissimus* has not yet been characterized.

In the present work, we report the purification and partial characterization of the elicitor from ungerminated spores of *M. ramosissimus* and the comparison of its phytoalexin-eliciting capacity with that of the widely known β-glucan from *P. sojae*.

Material and methods

Microorganism and culture conditions – *Mucor ramosissimus* Samutsevitsch (URM 3106 - Universidade Federal de Pernambuco, Recife, PE, Brazil) was cultivated in SMA (Synthetic-Mucor-Agar) containing ($g.L^{-1}$): glucose (40.0), asparagine (2.0), K_2PO_4 (0.5), $MgSO_4$ (0.25), thiamine (0.05 $mg.L^{-1}$) and agar (10.0) (Schipper 1973). Cultures were grown in Petri dishes containing 20 mL of SMA medium in darkness at 28 ºC for different periods.

Growth curve and sporulation – Mycelial samples of six mm diameter were taken from ten-day-old actively growing, sporulating cultures of *M. ramosissimus* and placed in the center of Petri dishes (three plates for each day), on SMA medium. The plates were incubated as described above and the diameter of each colony was measured daily. Spores were harvested by adding 20 mL of sterile distilled water to the plate and gently scraping the spores from the culture surface into the solution using a fine brush**.** Samples of the spore suspension were used for spore counting in a Newbauer chamber.

Elicitor extraction – Spore suspensions of *M. ramosissimus* from six to 11-day old cultures obtained as described above were filtered in cheesecloth. After filtration, the suspensions were centrifuged 10 min at 318 *g* and the supernatant was discarded. Spores were resuspended in distilled water and autoclaved for 30 min at 121 ºC at 1.5 atm. The supernatant was collected by centrifugation at 6,000 *g* for 30 min at 5 ºC and stored at -20 ºC. The pellet was discarded. All these procedures were performed under asseptic conditions. The β-glucan elicitor was obtained from mycelium cell walls of *Phytophthora sojae* as described by Hahn *et al*. (1992).

Assay of eliciting activity – The phytoalexin eliciting activity was evaluated by the soybean cotyledon assay (Ayers *et al*. 1976b) using *Glycine max* L. cultivar IAC-18 (Instituto Agronômico de Campinas, SP, Brazil). Soybean seeds were germinated under controlled conditions as described by Pelicice *et al.* (2000). The cotyledons were detached from 9-day-old plantlets and soaked in 10% commercial sodium hypochlorite for 15 min, and subsequently washed

thoroughly with distilled water. A sterilized cork borer of 0.8 cm diameter was used to delimitate the area of the 1 mm-deep well that was created with a sterile scalpel. The wounded surface was treated with 50 µl of one of the following solutions: distilled water (control), elicitor extracted from spores of *M. ramosissimus* (50 µg glucose equivalents.mL^{-1}), or the β-glucan elicitor from mycelial walls of *Phythophthora sojae* (2 µg.mL^{-1}). For all experiments, 2.5 µg glucose equivalents.cotyledon^{-1} were used, except for the dose-response curve in which the amount of the *M. ramosissimus* elicitor ranged from 0 to 10 µg.cotyledon^{-1}. Groups of five cotyledons in triplicate were used per each treatment. The cotyledons were kept in a Petri dish containing water-absorbed filter paper in the dark at 26 ºC for 20 h. The cotyledons were washed with deionized water (1 mL per cotyledon) to collect the diffusates, which were used to estimate phytoalexin production by spectrophotometry at 286 nm and for quantification by High Performance Liquid Chromatography (HPLC) as described below.

Quantification of phytoalexins – Diffusates from the soybean cotyledon assay (15 cotyledons) underwent liquid-liquid extraction with ethyl acetate, as described by Keen (1978). Organic fractions were evaporated to dryness, and the residues then solubilized in methanol and analyzed by HPLC in a Shimadzu chromatograph fitted with a UV-VIS detector. The samples were run on a 4.6 mm × 250 mm Shimadzu CLS ODS C18 column with a linear gradient from 20% to 60% acetonitrile in 0.1% trifluoroacetic acid (0.8 mL.min^{-1}) according to Pelicice *et al.* (2000). Peak area *versus* compound concentration was plotted for various concentrations of available standards. Daidzein and genistein were identified by calibration with authentic standards (Sigma; St. Louis, MO), whereas diffusates from β-glucan elicitor-treated cotyledons of the Williams 82 soybean cultivar (from Illinois Foundation Seeds, Inc., USA) were used as the glyceollin standard. The compounds were monitored at 286 nm.

Elicitor fractionation – The supernatant obtained from autoclaved spore suspensions of 9-day old *M. ramosissimus* cultures (15 × 10^9 spores.mL^{-1}) was concentrated to 1/10 of its original volume and precipitated with 5 volumes of ethanol at 5 ºC for 72 h. The precipitate recovered by centrifugation (10,000 *g*, 5 ºC, 30 min) was washed twice in ethanol, and resuspended in deionized water to 1 mg total sugar per mL, determined by the phenol-sulfuric procedure (Dubois *et al*. 1956). The solution was subjected to Fast Performance Liquid Chromatography (FPLC) anion exchange chromatography in a 1 mL Q-trap column (Pharmacia) and eluted using an initial wash with 10 mM ammonium bicarbonate (10 min), followed by a linear gradient of 10-500 mM ammonium bicarbonate for 50 min, and a final wash with 1 M ammonium bicarbonate for another 10 min at a flow rate of 1 mL.min^{-1}. Fractions of 1 mL were collected and analyzed for carbohydrate and protein (see below) and eliciting activity. The active fractions were lyophilized and resuspended (1 mL) in 100 mM Tris buffer pH 7.4 containing 100 mM sodium acetate, 150 mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$. This solution was applied to a 1 mL column of Con A Sepharose (Pharmacia) previously washed with the same buffer in an FPLC system. The column was eluted with 10 mL of the buffer while collecting 2 mL fractions, and subsequently eluted with 5 mL of 100 mM α-methyl-D-mannopyranoside (Sigma) in the same buffer. The elicitor material without affinity for the Con A Sepharose was quantified for carbohydrate and protein contents (as described below), pooled, and dialyzed (MW cut off of 1,000 Daltons) exhaustively against deonized water. The Con A binding material was also dialyzed under the same conditions. The elicitor material without affinity for the Con A Sepharose was subsequently applied into a 1.0 × 20.0 cm column of Bio Gel P-2 (Bio Rad) equilibrated with distilled water. Maltoheptaose, maltotetraose, and sucrose were used as standards for column calibration. The amounts of total carbohydrates and proteins in each fraction were determined as described below.

Determination of carbohydrate and protein contents – The amounts of total carbohydrates were determined by the phenol-sulfuric acid method (Dubois *et al*. 1956) using glucose as standard. Uronic acid content was determined using a modification of the *m*-hydroxibiphenyl procedure (Filisetti-Cozzi & Carpita 1991) using galacturonic acid as standard. Proteins were monitored by absorbance at 280 nm and quantified by the procedure described by Bradford (1976) with bovine serum albumin as standard.

Analysis of sugar composition and glycosyl linkages – The neutral sugar composition of elicitor-active fractions were determined by hydrolysis in 2 M trifluoroacetic acid at 121 ºC for 2 h. The resulting monosaccharides were separated and quantitated by High Performance Anion Exchange Chromatography with a Pulsed Amperometric Detector (HPAEC/PAD) in a Dionex DX-500 system on a Carbo-Pac PA-1 column using isocratic 12 mM NaOH as eluent. The flow rate through the column was 1 mL.min^{-1}. The elution time of each sugar was compared to those of monosaccharide standards.

The elicitor-active material without affinity for Con A Sepharose was hydrolyzed as described above, trimethyl-silylated (TMS) with Tri-Sil, and analyzed by gas chromatography coupled with mass spectrometry (GC-MS) (York *et al*. 1985). The following temperature conditions were used: 160 ºC for 3 min and increased to 260 ºC at 10 ºC .min^{-1}. Myo-inositol was used as an internal standard.

Periodate and protease treatments – Periodate oxidation of the elicitor was carried out according to the procedure modified from De Wit & Roseboom (1980). Briefly, 50 µg glucose equivalents were incubated with 70 mM sodium periodate (Merck) for 24 h at 30 ºC in the dark. Excess of periodate was eliminated by addition of 100 µl of ethylene glycol. Protease treatment was performed by incubation of the elicitor with protease IV (Sigma) (4.1 units. mL^{-1}) in 10 mM sodium phosphate buffer, pH 7.0 for 24 h at 30 ºC. The reaction was stopped by boiling the extracts for 2 min. Controls were

performed with elicitor without treatments and with samples containing distilled water instead of the elicitor incubated in the same conditions. The eliciting activity was evaluated in soybean cotyledons as described above.

Results and Discussion

M. ramosissimus grew rapidly in SMA medium, the exponential growth phase being observed from 0 to 4 days (figure 1A). After 6 days, the mycelium completely covered the medium surface. Sporulation started on day 4 and maximal spore production occurred from nine to 13 days in culture (figure 1B). The yield of spores on SMA medium was ca.10 times higher than that previously described for the fungus grown on PDA (Potato-Dextrose-Agar medium) (Cordeiro Neto 1992).

Although spore yields were quite similar from four to nine day-old cultures, the carbohydrate content in the elicitor extracts obtained from autoclaved spores increased after six days of culture (figure 1B), indicating that the components of the spores differed from early to late sporulation stages.

The phytoalexin eliciting activity of the *M. ramosissimus* spore extracts, as measured by the soybean cotyledon bioassay, reached a maximum for extracts of spores from eight-nine day-old cultures (figure 1C). Quantification by HPLC also indicated that accumulation of glyceollins is maximal on day 9 and that the precursor daidzein is also induced by the elicitor, while genistein accumulation remains relatively unaffected by elicitor treatment regardless of the age of the fungal culture (figure 1D).

Precipitation of nine-day-old culture extracts with ethanol appeared to enrich for glyceollin-inducing components of the elicitor preparation (figure 2). The dose-response curve performed with the crude

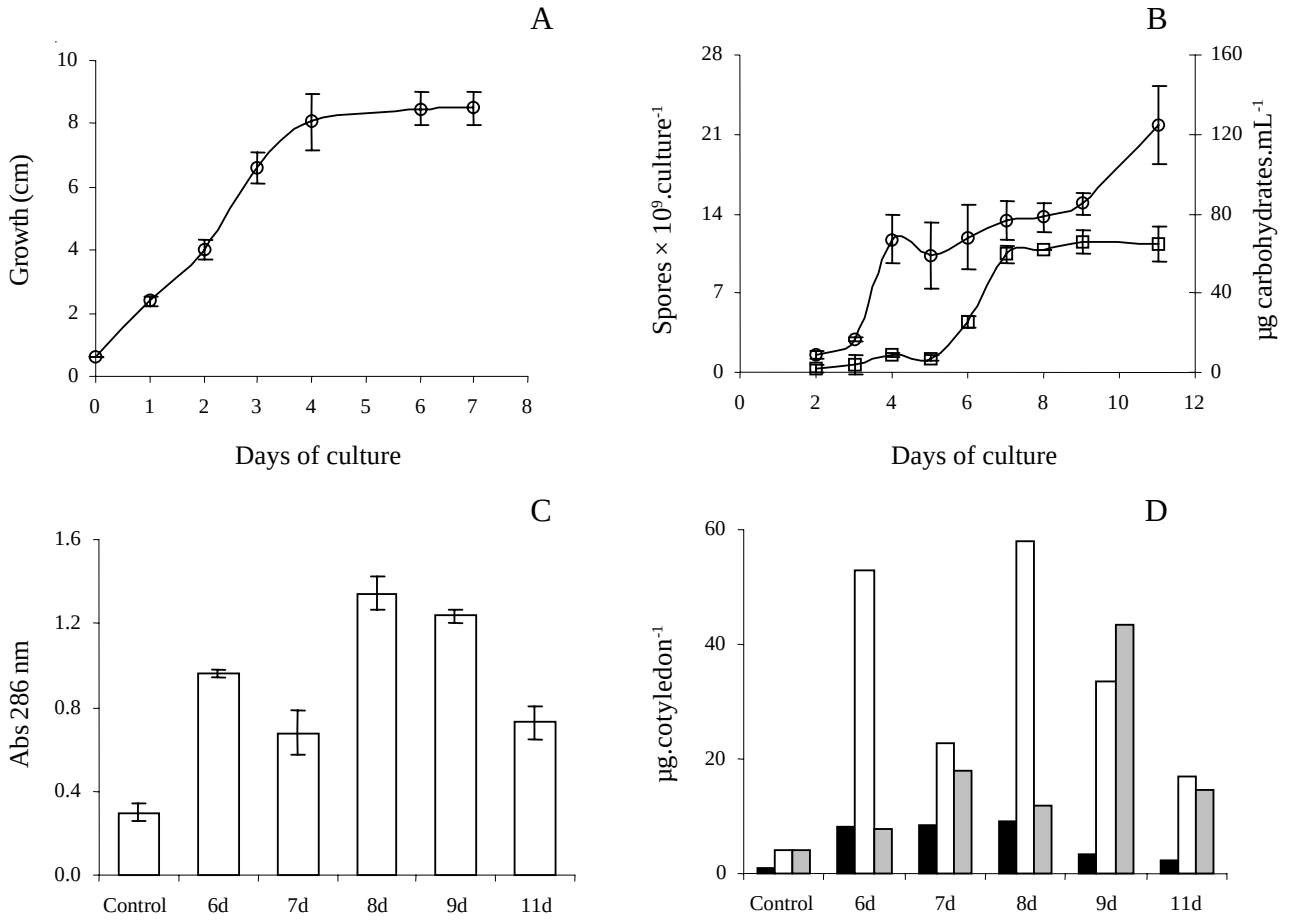

Figure 1. Growth, sporulation, and eliciting activity of *Mucor ramosissimus* grown in SMA medium. (A) Diameter of the fungal colony. (B) Sporulation (O) and carbohydrate content in the autoclaved spore suspension (□). (C) and (D) Eliciting activity of suspensions of autoclaved spores from cultures of different ages measured by absorbance at 286 nm and by HPLC, respectively. (■) Genistein, (□) daidzein and (■) glyceollins in diffusates of soybean cotyledons assayed with distilled water (control) or autoclaved spore suspensions. 2.5 µg of glucose equivalents of the elicitor were applied per cotyledon. Data represent the mean ± standard deviation of triplicates.

precipitated elicitor of *M. ramosissimus* revealed that glyceollin and daidzein accumulations increase as increasing amounts of elicitor are applied per cotyledon (figure 3).

The fractionation of the crude precipitated elicitor using anion exchange chromatography is shown in figure 4A. Most of the carbohydrate-containing material eluted from the Q-trap column in the ammonium bicarbonate gradient. Fractions were pooled based on their carbohydrate contents and assayed on for elicitor activity on soybean cotyledons. Elicitor activity was detected in all bound fractions, although the highest glyceollin accumulations were induced by the fractions designated as G2, G3, G4 and that eluted with 1 M bicarbonate buffer (figure 4B). Neutral sugar composition analysis of the elicitor-active fractions showed the presence of mannose, glucose and galactose and small amounts of arabinose (table 1). Mannose was the major component in all fractions although its proportion decreased with the increased charge of the molecules and their elicitor activity (table 1, figure 4B).

Although the active fractions from the anion exchange column contained Bradford-positive material (ca. 2 μg. mL^{-1}), treatment with protease did not reduce elicitor activity, suggesting that this activity is not attributable to peptide components of the fractions (data not shown). In contrast, the elicitor activity was completely abolished by periodate oxidation indicating that the activity resides in the carbohydrate moiety (data not shown). These results are consistent with the thermal stability of the *M. ramosissimus* elicitor, which was

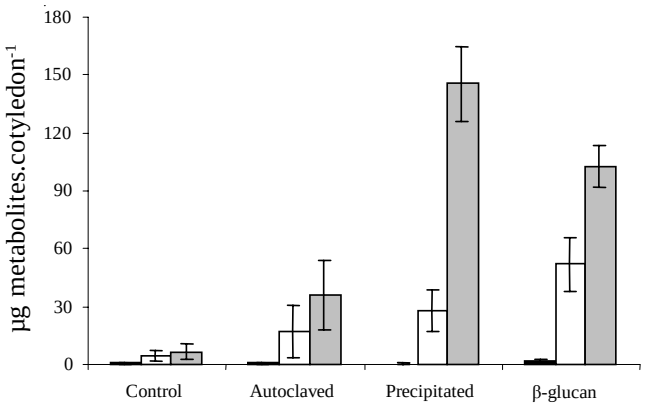

Figure 2. Eliciting activity of autoclaved spores of *M. ramosissimus* and ethanol-precipitated extract on soybean cotyledons. Values represent the amounts of (■) genistein, (□) daidzein and (▩) glyceollins in the diffusates measured by HPLC. 2.5 μg of glucose equivalents of the *Mucor* elicitor and 0.1 μg of the β-glucan of *P. sojae* were applied per cotyledon.

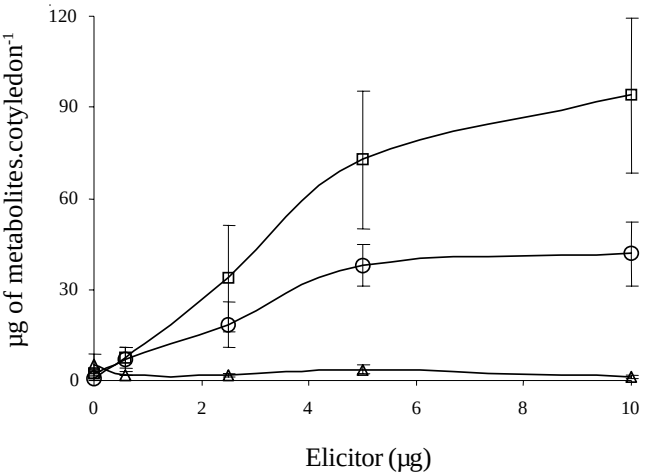

Figure 3. Dose-response eliciting curve of the ethanol-precipitated extract from spores of 9-day-old cultures of *M. ramosissimus*. Values represent the amounts of (△) genistein, (○) daidzein and (□) glyceollins in soybean diffusates measured by HPLC. Data represent the mean ± standard deviation of triplicates.

obtained by autoclaving the spores for 30 min. Similar results were obtained for phytoalexin elicitors obtained from *Colletotrichum* spp., *Phytophthora megasperma* var. *sojae*, *Saccharomyces cerevisae*, and *Hemileia vastatrix* (Ayers *et al*. 1976a, Hahn & Albersheim 1978, Yoshikawa *et al*. 1981, Guzzo & Moraes 1997). The binding of the *M. ramosissimus* elicitor to the ion-exchange column can be attributed to the presence of uronic acids in the extracts (table 2).

Further purification of the *Mucor* elicitor was achieved by Concanavalin-A (Con-A) affinity chromatography. Non-binding material and lectin-binding glycoconjugates were each collected as single fractions (data not shown). About of 55% of the carbohydrate applied to the column remained in the non-binding fraction, which contained elicitor-active components (figure 5). The purification of the elicitor throughout the whole fractionation procedures led to an increase of specific activity from 14.4 to 88.0 and a yield of 0.05% of the initial carbohydrate content (table 2).

Uronic acids and mannose, glucose, galactose, and traces of arabinose were found in the elicitor-active Con-A non-binding fraction (table 2), whereas the bound, inactive fraction was composed mostly of mannose (data not shown).

Preliminary glycosyl linkage analysis of the active fraction indicated the absence of 3- and 3-6 linked glucose. According to Ruiz-Herrera (1992), glucans are not found in mycelial cell walls of Zygomycetes, although they can be present in spores of some species, including

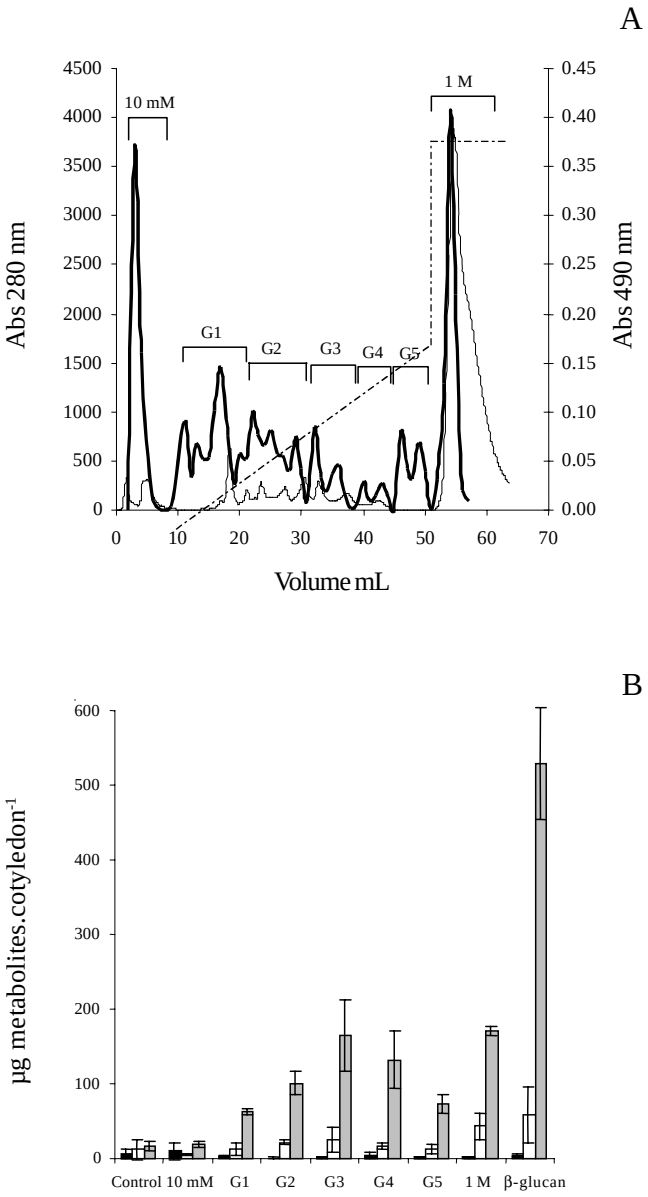

Figure 4. FPLC profile of the precipitated spore extract of *M. ramosissimus* applied to a Hi Trap Q Sepharose column. (A) Elution profile of (—) carbohydrate content measured by phenol-sulfuric method and expressed by absorbance at 490 nm and (–) proteins (absorbance at 280 nm). (-·-·-) Ammonium bicarbonate buffer gradient. Fractions 10 mM (not bound), G1 to G5 (eluted with linear gradient of bicarbonate buffer) and 1 M (eluted with 1 M of bicarbonate buffer) were pooled according to their carbohydrate contents. (B) Eliciting activity of pooled fractions, control (distilled water) and *P. sojae* β-glucan in soybean cotyledons measured by HPLC of (■) genistein, (□) daidzein and (▒) glyceollins in the diffusates. 2.5 µg of glucose equivalents of the *Mucor* elicitor and 0.1 µg of the β-glucan of *P. sojae* were applied per cotyledon. Data represent the mean ± standard deviation of triplicates.

Table 1. Relative proportions of neutral monosaccharides as determined by HPAEC/PAD analysis of the pooled fractions from the Hi Trap Q Sepharose column shown in figure 4.

	Monosaccharide (%)			
Fraction	Arabinose	Galactose	Glucose	Mannose
10 mM	0.5	2.0	16.5	81.0
G1	1.0	3.0	17.0	79.0
G2	1.0	6.0	21.0	72.0
G3	2.0	12.0	21.0	65.0
G4	2.0	12.0	34.0	52.0
G5	1.5	15.5	34.0	49.0
1 M	1.5	20.0	23.5	55.0

Mucorales. However, the presence of mannose and uronic acids and the lack of 3- and 3-6 linked glucosyl residues indicate that the eliciting activity from spores of *M. ramosissimus* cannot be attributed to the presence of branched β-glucans similar to those described for *Phytophthora sojae* (Ayers *et al.* 1976a), *Saccharomyces cereviseae* (Hahn & Albersheim 1978) or *Pyricularia oryzae* (Yamaguchi *et al.* 2000), nor to linear β-1,3 glucan elicitor from the brown algae *Laminaria digitata* that is active in tobacco cell suspension cultures (Klarzynski *et al.* 2000). Furthermore, the *Mucor* elicitor differs from the mannose-containing elicitors found in autoclaved uridiniospores of the coffee rust (*Hemileia vastatrix*) since the latter seems to be composed only of neutral sugars (Guzzo & Moraes 1997). The composition of the Con-A non-binding elicitor fraction determined by silylation (table 2) is consistent with that of a fragment of mucoran, a glucuronic-acid-containing heteropolymer found in *Mucor rouxii* (Bartinicki-Garcia & Reyes 1968, Bartinicki-Garcia & Lindberg 1972). No report of mucoran-like molecules as phytoalexin elicitors has yet been published.

Fractionation of the Con-A non-binding material through a size exclusion column of Bio-gel P2 resulted in two carbohydrate-containing peaks. The activity was detected in a broad peak with apparent molecular mass between 700 and 1,800 Da (data not shown), which would be consistent with a DP of 4-10 for the *Mucor* elicitor. Low molecular mass elicitors have been isolated from fungal cell walls. Sharp *et al.* (1984) characterized a branched hepta-β-glucoside from the phytopathogen *Phytophthora sojae* capable of eliciting phytoalexin accumulation in soybean cotyledons at concentrations of 10^{-8} to 10^{-9} M. Yamaguchi *et al.* (2000) purified a glucopentaose from the rice blast disease fungus that is

Table 2. Steps of purification of the elicitor from *M. ramosissimus* spores.

Procedure	Fraction	Neutral sugar yield mg (%)	Uronic acids yield mg (%)	Specific activity (µg glyceollins/ µg carbohydrate)	Sugar composition * (%)					
					Ara	Gal	Glc	Man	GalA	GlcA	Fuc
Autoclaving	Autoclaved	469.8 (100)	15.0 (100)	14.4	-	-	96.0	4.0	-	-	-
Ethanol precipitation	Precipitated	38.1 (8)	4.0 (26.7)	58.2	0.5	3.0	41.5	55.0	-	-	-
Ion exchange chromatography	Fraction 1 M	1.4 (0.2)	0.7 (4.7)	68.5	1.5	20.0	23.5	55.0	-	-	-
Affinity chromatography	Fraction NB	0.3 (0.05)	0.18 (1.2)	88.0	2.5	26.0	30.0	41.5	-	-	-
CG-MS**					-	6.9	6.8	26.1	10.2	50.0	t

*determined by HPAEC/PAD, ** sugar composition also determined by GC-MS as described in Methods. - = not determined, t = traces.

a highly active elicitor of phytoalexin biosynthesis in cell suspension cultures of rice. A β-1,3-pentaglucan obtained from the algae *Laminaria digitata* was reported as an active elicitor of defense responses in suspension-cultured tobacco cells (Klarzynski *et al.* 2000). Chitin and chitosan oligomers with DP from 4-6 have also been described as potent inducers of the phenylpropanoid biosynthetic pathway in soybean leaves (Khan *et al.* 2003).

Plants have the capacity to recognize and respond to a variety of carbohydrate structures present in microbes to activate plant defense responses regardless of whether they come from a pathogen or a non-pathogen (Hahn & Albersheim, 1978, Asiegbu *et al.* 1994, Costa & Dietrich 1996, Hahn 1996). They are also able to perceive neutral molecules from marine algae (Klarzynski *et al.* 2000) and acidic oligomers obtained from plant cell walls (Hahn *et al.* 1981, Nothnagel *et al.* 1983). In tobacco, neutral glucans derived from brown algae and oligogalacturonides obtained from apple pectin were perceived by suspension-cultured cells as distinct chemical stimuli but had similar elicitor effects (Klarzynski *et al.* 2000). Soybean tissues can also recognize microbial glucans and uronic acid-containing elicitors derived from plants (Hahn & Albersheim 1978, Nothnagel *et al.* 1983) to activate the same defensive responses. Therefore, the *Mucor* elicitor, which contains uronic acids and seems to be structurally unrelated to

Figure 5. Eliciting activity of non-bound (NB) and bound (B) fractions from the FPLC profile in Con A Sepharose column of the 1 M fraction from the Hi Trap Q Sepharose fractionation (figure 4A). (■) Genistein, (□) daidzein and (■) glyceollins contents were measured in soybean diffusates by HPLC. Control (distilled water). β-glucan (elicitor from *P. sojae*). 2.5 µg of glucose equivalents of the *Mucor* elicitor and 0.1 µg of the β-glucan of *P. sojae* were applied per cotyledon. Data represent the mean ± standard deviation of triplicates.

any of the previously described fungal or plant elicitors, represents a new and distinct class of carbohydrate signal molecules.

How plants recognize different elicitors to activate the same defense responses remains to be elucidated. Evidence of synergism between different elicitors in several systems suggest that different but interacting signaling pathways exist in plant cells for several of the carbohydrate elicitors identified to date (*e.g.* Davis & Hahlbrock 1987, Klarzynski *et al.* 2000, Yamaguchi *et al.* 2000, 2002). Further research will be required to determine what receptor(s) in soybean bind the mucoran elicitor fragments, and how the mucoran-induced signaling pathway interacts with the glucan and oligogalacturonide signaling pathways to activate phytoalexin synthesis.

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