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# Chemical structure of two phytotoxic exopolysaccharides produced by *Phomopsis foeniculi* [1]

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**Abstract**

The two main exocellular polysaccharides produced *in vitro* by *Phomopsis foeniculi*, a fungal pathogen of fennel, were isolated and characterized by chemical and spectroscopic methods as a galactan with the known structure [→6)-β-D-Gal*f*-(1→5)-β-D-Gal*f*-(1→5)-β-D-Gal*f*-(1→]$_n$ and a mannan. The latter consists of a backbone of $\alpha-(1\rightarrow 6)$-linked mannopyranose units. Almost all of these are branched at the 2 position with arms containing 2- and 3-linked mannopyranose units. The crude polysaccharide fraction and its components, galactan and mannan, showed phytotoxic effects, i.e. chlorosis, necrosis and/or wilting, on fennel and on two non-host plants, tobacco and tomato. 

*Keywords:* Galactan; Mannan; *Phomopsis foeniculi*; Phytotoxicity; Fungi

## 1. Introduction

*Phomopsis foeniculi* is the agent of the necrosis of stem, leaves and inflorescences of fennel. The disease was observed in France [1] and in Italy [2], where it causes severe losses in fruit production. Studies carried out to improve *in vitro* selection for disease resistance and to gather more information about the toxins produced by the fungus and the mechanism of symptom induction, led to the isolation and characterization of a main phytotoxin named foeniculoxin [3]. However, marked phytotoxic activity remained in the aqueous phase after exhaustive extraction of the culture filtrates with ethyl acetate of the lipophilic phytotoxic metabolites. Therefore, it appeared interesting to isolate

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the hydrophilic phytotoxic metabolites which most probably play a role, together with the lipophilic toxins, in the disease process induced by *P. foeniculi* on fennel plants.

This paper reports the isolation, the chemical characterization and the phytotoxic activity of the two main exocellular polysaccharides (EP) isolated from the culture filtrate.

## 2. Results and discussion

The EP fraction, which was obtained by precipitation from ethanol of the aqueous phase of the cultural filtrate, consisted, on the basis of alditol acetate analysis, of galactose and mannose in a molar ratio of 1.0:2.2, in addition to traces of rhamnose. Absolute configuration determination indicated the D configuration for both mannose and galactose. The EP fraction contained neither phosphorus nor acid sugars as indicated by $^{31}P$ NMR analysis and uronic acid assay [4], respectively. The $^{13}C$ NMR chemical shifts of anomeric carbons occurring in the range 108.7–99.1 ppm revealed the presence of both furanose and pyranose ring forms [5]. Treatment of the EP fraction with Fehling's solution [6] gave, as an insoluble copper complex, a fraction whose acid hydrolysis gave only mannose, while the supernatant liquor yielded mainly galactose together with mannose and rhamnose. However, both fractions proved to contain some copper whose amount, in the range of ppm as revealed by atomic absorption analysis, was incompatible with the bioassays.

Since all the efforts to purify the EP fraction by gel-chromatography were unsuccessful we used a precipitation from 2-propanol [7]. This allowed us to obtain a precipitate of pure mannan (Fraction A), $[\alpha]_D$ +67°, Mw 62–123 kDa, and, from the supernatant a polysaccharide fraction (Fraction B, Mw 13–22 kDa) which gave on acid hydrolysis mainly galactose together with mannose and rhamnose. The molecular weights for Fractions A and B were estimated on Bio-Gel A 0.5 m (calibration with dextran standards).

Methylation analysis of Fraction A (Table 1), constituted of only D-mannose, gave a molar ratio between terminal and nodal units very different from the expected unity and, in particular the amount of 2,6-linked Man*p* was too low. This finding was in agreement with a peculiar acid lability of 3,4-di-OMe-mannose residues as reported by Barreto-Bergter [8].

Table 1
Methylation analysis data of Fraction A

| Residues | Molar ratio |
|---|---|
| 2,6-linked Man*p* | 1.0 |
| terminal-Man*p* | 2.8 |
| 2-linked Man*p* | 0.9 |
| 3-linked Man*p* | 0.5 |
| 6-linked Man*p* | 0.2 |
| 4-linked Man*p* | 0.1 |

The $^1H$ and $^{13}C$ NMR spectra (Fig. 1) of fraction A showed five anomeric broad $^1H$ singlets at $\delta$ 5.30, 5.16, 5.13, 5.10, and 5.06, and three anomeric $^{13}C$ signals at $\delta$ 103.0, 101.4, and 99.0, respectively. The anomeric carbon signals can be assigned to terminal and/or 3-linked, 2-linked and 2,6-linked units, respectively [9,10]. A heteronuclear 2D-NMR experiment allowed us to assign also the anomeric protons: the carbon atom at $\delta$ 103.0 is correlated with the protons at $\delta$ 5.06 and 5.16, that at $\delta$ 101.4 with the proton at $\delta$ 5.30, and that at $\delta$ 99.0 with the protons at $\delta$ 5.13 and 5.10. These NMR data indicate also an $\alpha$ configuration for all of the anomeric centres.

The data above, in agreement with literature [9–11], suggested that the structure of the mannan has a backbone consisting of $\alpha$-(1→6 -linked mannopyranose units almost all branched at the 2 position, whereby the arms are made up of 2- and/or 3-linked units. Further evidence for the latter structural features was obtained from an acetolysis study, whereby preferentially 6-linked sugars are cleaved [12]. The crude reaction mixture was separated on Bio-Gel P-2 to give, besides a fraction in the void volume (14 mg), fractions A (12 mg), B (15 mg), C (18 mg) and D (7 mg) (Fig. 2). Fractions A–C consisted of oligosaccharides and fraction D of mannose.

The oligosaccharide structures were established to be **1**, **2** and **3** for fractions C, B and A, respectively, by a comparison of their NMR data (Table 2) with those reported in literature [13–15], and confirmed by FABMS data of their acetate derivatives.

**1** α-D-Man*p*-(1→2)-D-Man
**2** α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-D-Man
**3** α-D-Man*p*-(1→3)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-D-Man

The acetolysis data gave indications concerning the lengths of branches, which consisted of one,

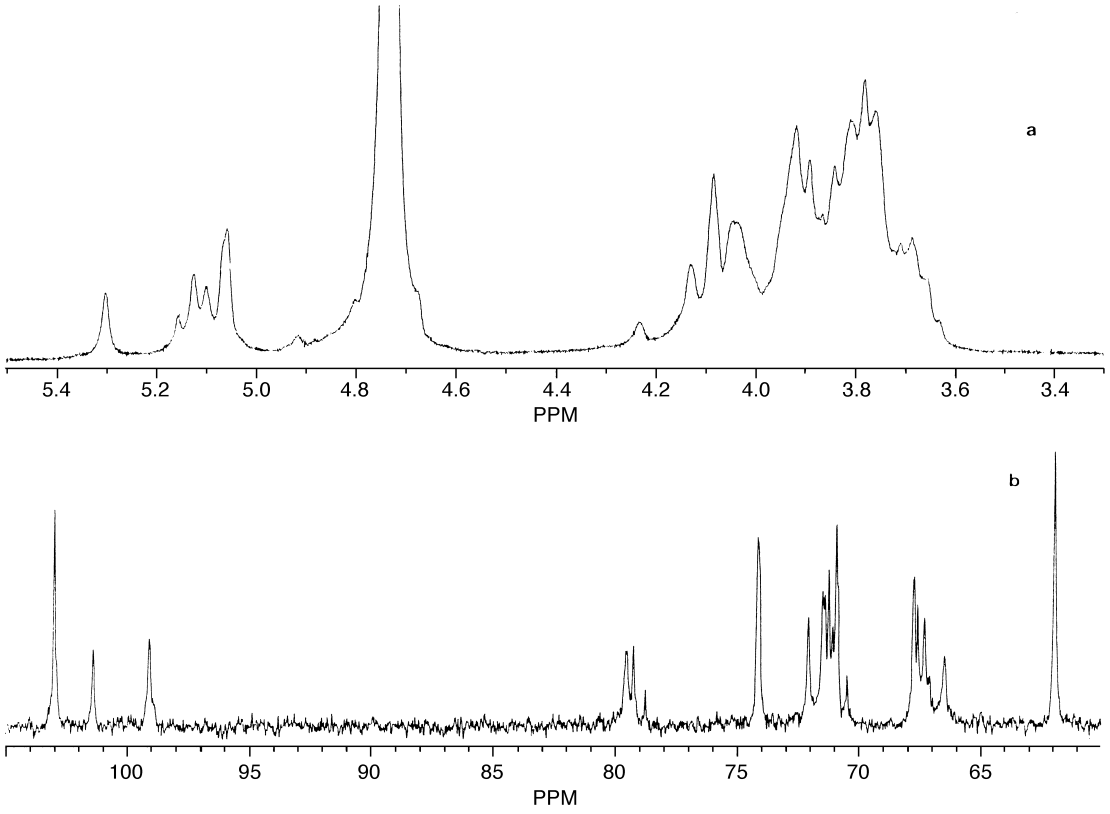


Fig. 1. $^{1}H$ (a) and $^{13}C$ (b) NMR spectra of mannan (fraction A) in $D_2O$ at 30 °C.

two and three units. As far as the meaning of the relative amounts of fractions A–D are concerned, it is important to underline that acetolysis did not completely occur, as proved by the finding of the excluded fraction, and that often it is not highly selective [16]. Therefore, the acetolysis molar ratio of 1.0:1.7:3.0 for compounds **3**, **2** and **1** must be taken carefully in the interpretation of the different-size arm ratios. More reliable structural information could be obtained from the methylation analysis data (Table 1) assuming a value of 3 for both terminal and nodal units. As a consequence an approximate molar ratio of 6:6:2:1 for 2,6-linked Man, terminal-Man, 2-linked Man, and 3-linked Man is found. Thus, it is possible to suggest an average repeating unit as being representative of the greater part of the mannan structure.

$$
\begin{array}{cccccccccccc}
[\rightarrow 6)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow]_n \\
2 & & 2 & & 2 & & 2 & & 2 & & 2 & \\
\uparrow & & \uparrow & & \uparrow & & \uparrow & & \uparrow & & \uparrow & \\
1 & & 1 & & 1 & & 1 & & 1 & & 1 & \\
\alpha\text{-D-Man}p & & \alpha\text{-D-Man}p & & \alpha\text{-D-Man}p & & \alpha\text{-D-Man}p & & \alpha\text{-D-Man}p & & \alpha\text{-D-Man}p & \\
2 & & 2 & & & & & & & & & \\
\uparrow & & \uparrow & & & & & & & & & \\
1 & & 1 & & & & & & & & & \\
\alpha\text{-D-Man}p & & \alpha\text{-D-Man}p & & & & & & & & & \\
 & & 3 & & & & & & & & & \\
 & & \uparrow & & & & & & & & & \\
 & & 1 & & & & & & & & & \\
 & & \alpha\text{-D-Man}p & & & & & & & & &
\end{array}
$$

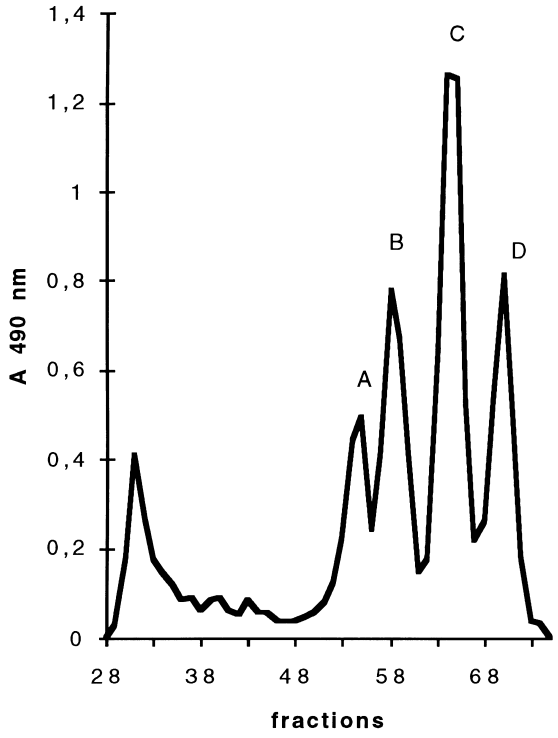


Fig. 2. Gel filtration profile of the acetolysate of mannan (fraction A) on a Bio-Gel P-2 column eluted with water (0.23 mL/min).The amount of carbohydrate was determined by the phenol-$H_2SO_4$ method. A, B, C and D indicate mannotetraose, mannotriose, mannobiose and mannose, respectively.

The mannan structure is not totally branched, because there are also some unsubstituted 6-linked Man*p* units, about one every two or three repeating units. The molar ratio between the 2-linked mannose and all the other mannose units is 1:6.5 for the suggested average repeating fragment. This structure is supported by the measurement of the integral intensity ratio (1:5.8) between the anomeric $^1$H signal at $\delta$ 5.30 (2-linked mannose) and all the other $^1$H signals occurring in the range 5.06–5.16 ppm.

Alditol acetate analysis of fraction B showed that it was constituted of galactose, mannose, and rhamnose in the molar ratio of 4:1.5:1.

The $^1$H and $^{13}$C NMR spectra (Fig. 3), which showed sharp strong anomeric signals at $\delta$ 5.26, 5.22, and 5.05 and at $\delta$ 108.6, 107.9, and 107.8, respectively, were identical to those recently described for a galactan isolated from the cell-wall of *Neosartorya* fungus [17].

These data indicated for the more abundant polysaccharide of fraction B a galactan structure with the repeating unit:

$$[\rightarrow 6)\text{-}\beta\text{-D-Gal}f\text{-}(1 \rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1 \rightarrow 5)\text{-}\beta\text{-D-Gal}f\text{-}(1 \rightarrow]_n$$

Accordingly, the methylation analysis revealed a molar ratio of 1:2 for 6-linked Gal*f* and 5-linked Gal*f* units. No structural information could be obtained for the minor polysaccharide component in fraction B because any attempts to purify it were unsuccessful.

As far as bioassays are concerned, three test plants showed phytotoxicity symptoms after treatment with crude EP, fraction A (mannan) and fraction B (impure galactan) from culture filtrates of *P. foeniculi*. In particular, the necrotic symptoms on leaves of tomato cuttings developed after 48 h with mannan and, even more strongly, with impure galactan and the EP extract. Wilt and basal stem flaccidity symptoms developed on fennel after 24 h with frequent browning of the basal stem; wilting symptoms were even more evident 24 h after the

Table 2
$^1$H and $^{13}$C chemical shifts ($\delta$) of anomeric signals of $\alpha$-D-Man*p* units of oligosaccharides **1–3** and of mannan

| Compound | Terminal unit | Second unit | | Third unit | Reducing unit | Nodal unit |
|---|---|---|---|---|---|---|
| | | 2-linked | 3-linked | 2-linked | 2-linked | |
| **1** | 5.12[a] | | | | 5.44[a] | |
| | 103.1[b] | | | | 93.4[b] | |
| **2** | 5.11[a] | 5.36[a] | | | 5.44[a] | |
| | 103.1[b] | 101.4[b] | | | 93.4[b] | |
| **3** | 5.22[a] | | 5.11[a] | 5.36[a] | 5.44[a] | |
| | 103.0[c] | | 103.0[c] | 101.4[c] | 93.4[c] | |
| Mannan | 5.16[d] | | 5.06[d] | 5.30 | — | 5.13; 5.10 |
| | 103.0 | | 103.0 | 101.4 | | 99.0 |

[a] Ref. 13
[b] Ref. 15
[c] Ref. 14
[d] Interchangeable values

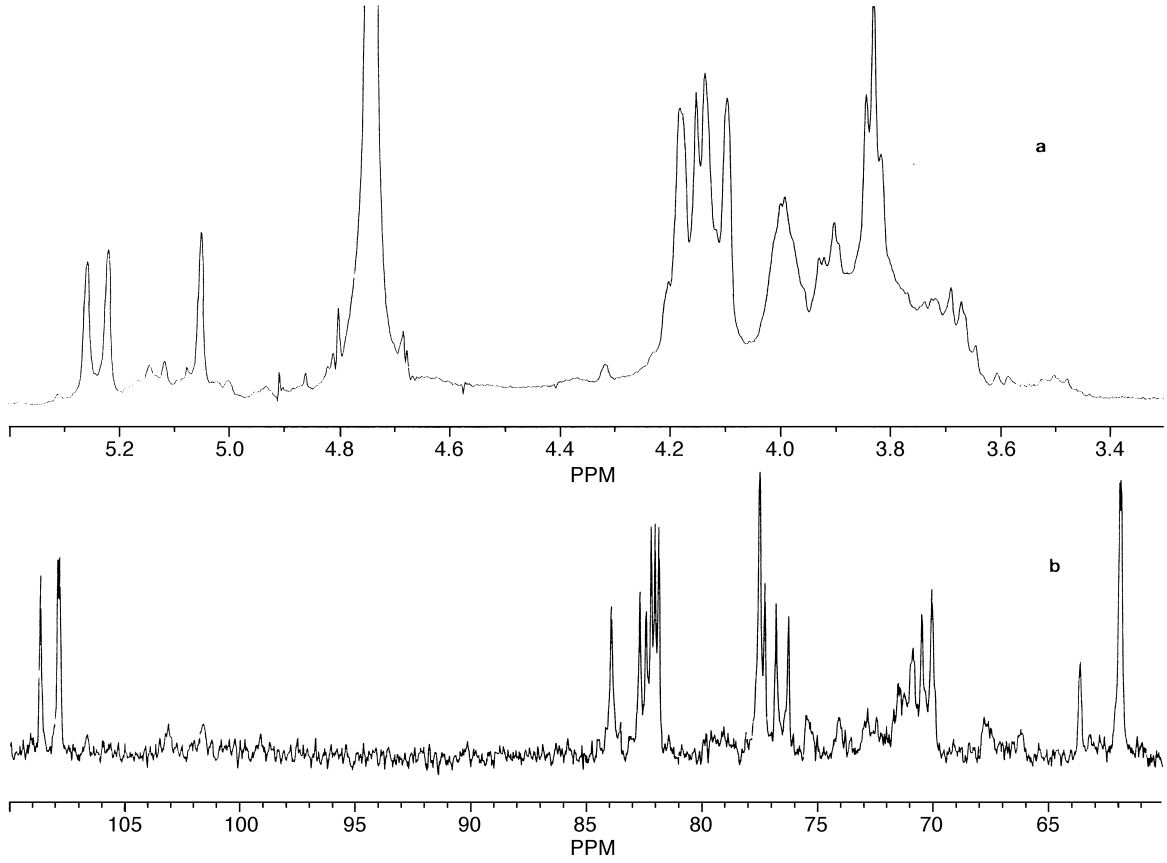


Fig. 3. $^{1}H$ (a) and $^{13}C$ (b) NMR spectra of impure galactan (fraction B) in $D_2O$ at 30 °C.

transfer of cuttings to water. On both plants, symptoms were more evident with concentrations higher than 1 mg/mL of both the EP extract or/and the separated fractions.

Infiltration of tobacco leaves showed to be the most sensitive test, leading to complete necrosis of the infiltrated area after 48 h with all concentrations of the crude extract (Table 3). Both fractions A and B caused necrosis of the infiltrated area and this was often preceded and then surrounded by a large chlorotic halo but more slowly than caused by the EP extract. Using the separated fractions, symptoms started to develop after 48 h and their intensity increased over the following 2–3 days (maximum intensity after 4–5 days) and at increasing concentrations of the substance tested, going from chlorosis and 3–5 mm necrotic area with 0.5 mg/mL to the highest rate (total necrosis of the infiltrated area) using 2.5 mg/mL or more. Fraction B gave slightly more intense symptoms. Infiltration of both polysaccharide fractions together (same ratio as in the EP extract) gave a slightly stronger effect with all the concentrations tested.

Commercial mannan tested as a control on all test plants gave no symptoms on tobacco and later and lighter symptoms on fennel and tomato cuttings.

The involvement of polysaccharidic substances in bacterial and fungal diseases was reported by Hodgson et al. [18–20] but their effective action as phytotoxins has still to be clarified [20,21]. Often comparable damage is caused to the test plant by polysaccharides from nonpathogenic species [22]. Despite this, in several phytopathogenic bacterial species such as *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas* and *Xanthomonas* the production of extracellular polysaccharides proved to be associated with water soaking of leaves and/or wilting symptoms in addition to playing a role in plant colonization by the bacterium [20]. Recently, extracellular polysaccharides from *Xanthomonas campestris* pv. *vesicatoria* were proved to induce phytotoxic effects (chlorosis, necrosis, electrolyte leakage) on the homologous host [23].

The production of polysaccharides inducing phytotoxic effects was also reported for fungal pathogens as recently for *Cryphonectria parasitica* [7] and previously for *Cephalosporium* [24], *Ceratocystis fimbriata* [25], *Ophiostoma ulmi* [26],

Table 3
Intensity of the phytotoxic symptoms (necrosis and chlorosis) induced on tobacco leaves by exopolysaccharide fractions from *Phomopsis foeniculi*[a,b]

| Fraction | Conc. mg/mL | Symptoms developed | | | |
|---|---|---|---|---|---|
| | | After 2 days | | After 5 days | |
| | | Chlorosis | Necrosis | Chlorosis | Necrosis |
| EP | 5.0 | 0 | 3.0 | 4.0 | 4.0 |
| | 2.5 | 0 | 3.0 | 4.0 | 4.0 |
| | 1.0 | 0 | 2.5 | 4.0 | 4.0 |
| | 0.5 | 0 | 2.5 | 4.0 | 4.0 |
| A (mannan) | 5.0 | 3.0 | 0.5 | 4.0 | 2.5 |
| | 2.5 | 1.5 | 1.5 | 2.0 | 3.0 |
| | 1.0 | 1.5 | 0.5 | 2.0 | 2.5 |
| | 0.5 | 1.0 | 0 | 3.0 | 1.0 |
| B (impure galactan) | 5.0 | 1.5 | 1.5 | 4.0 | 3.5 |
| | 2.5 | 0 | 0 | 4.0 | 3.0 |
| | 1.0 | 1.5 | 1.0 | 2.0 | 2.5 |
| | 0.5 | 1.0 | 0 | 2.0 | 2.0 |
| A + B | 5.0 | 3.0 | 1.5 | 4.0 | 3.0 |
| | 2.5 | 2.0 | 2.0 | 2.0 | 3.0 |
| | 1.0 | 1.5 | 1.0 | 2.5 | 2.5 |
| | 0.5 | 1.5 | 0.5 | 2.5 | 1.5 |

[a] Numbers in the table refer to the intensity of each symptom as average of two replicates (0 equals absence of the symptom): necrosis, from 1 (2–3 mm diameter necrosis on the point of injection) up to 3 (necrosis of all the infiltrated area) and 4 (necrosis extended beyond the infiltrated area); chlorosis, from 1 (a paler, hardly visible shade of the infiltrated area) to 3 (intense yellowing of the infiltrated area) and 4 (chlorosis of the area surrounding the necrosis or the infiltrated area).
[b] Water and commercial mannan from Sigma Aldrich were used as control and gave no chlorosis or necrosis.

*Fusarium solani* [27], and different species of *Phytophthora*. Among these, mycolaminarans from cytoplasma of *Phytophthora palmivora*, *P. cinnamomi*, and *P. megasperma* var. sojae [28], and EP from a culture filtrate of *P. cinnamomi*, *P. cryptogea*, and *P. nicotianae* [22] produced severe wilting on several hosts. At the moment not enough is known about the role of these macromolecules; they commonly appear to act by interfering with water movement in plant tissues due to mechanical plugging of the vessels which leads to wilt symptoms. The phenomenon appears to be related to the size of the molecules and their viscosity rather than to their structure [19], though some results on host specificity [29] would suggest a possibly different behaviour in some cases. This could support our finding that commercial mannan [30], with a similar molecular weight to mannan from *P. foeniculi*, showed a very different behaviour on tobacco infiltrated leaves and also on the other test plants. Furthermore, the different phytotoxicity of single fractions with respect to their artificial and natural mixture seems to suggest that in our case there is no relationship between toxicity and the size of polysaccharides. Other ways of symptom induction are suggested, such as stomatal disfunction and the alteration of membrane permeability [21,22] whose activity could also be hypothesized in relation to the necrotic symptoms we recorded in the tobacco-infiltration test.

The data obtained on wilting and necrotic effects on the different test plants by uptake or infiltration of purified mannan and galactan fractions from *P. foeniculi* culture filtrates, which will be more thoroughly investigated, can lead us to hypothesize that the two polysaccharides have a role in disease development. Following stem infection, damage to inflorescences and foliage then occurs, and these long distance effects could in fact be associated with the fungal production of substances with phytotoxic activity.

## 3. Experimental

*General*.—The $^{1}H$ and $^{13}C$ NMR spectra were obtained in $D_2O$ at 400 and 100 MHz, respectively, with a Bruker AM 400 spectrometer equipped with a dual probe, in the FT mode at 30 °C. $^{13}C$ and $^{1}H$ chemical shifts are expressed in $\delta$ relative to internal 1,4-dioxane (67.4 ppm) and TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-$d_4$), respectively. The intensity ratio of proton signals was estimated from an $^{1}H$ NMR spectrum performed with an interpulsed delay of 3 s. $^{31}P$ NMR spectra were measured at 161 MHz in $D_2O$ using 85% $H_3PO_4$ as internal standard. Mass spectra were recorded with a VG ZAB HF instrument equipped with a FAB source. GLC was performed with a Dani instrument equipped with a flame-ionization detector and GLC-MS with a Hewlett–Packard 5890 instrument. Optical rotations were determined with a Perkin–Elmer 141 polarimeter. HPLC was performed with a Varian Vista 5500 instrument equipped with a refraction index Varian RI-3 series detector. The atomic absorption analysis was performed on a Perkin–Elmer 3030 B Atomic Absorption Spectrophotometer. Commercial mannan was purchased from Sigma (M 3640).

*Production of culture filtrate*.—Single spore cultures of *P. foeniculi*, which were freshly isolated from artificially infected fennel, were grown on

potato dextrose agar. After 14 days the colony was flooded with 5 mL sterile distilled water and the surface gently scraped. The resulting conidial and micelial suspension (1 mL) was transferred to Erlenmeyer flasks containing 400 mL modified Czapek Dox Broth (sucrose, 30 g; $K_2HPO_4$, 1 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.01 g; asparagine, 2 g; yeast extract, 2 g; distilled water, 1000 mL; pH 6.7) and incubated in shaken cultures (100 rpm) at 25 °C for 18 days. The cultures were then centrifuged ($10^4$ rpm for 15 min) and the supernatant collected, filtered, and lyophilised.

*Preparation of exocellular polysaccharide (EP).*—The aqueous phase of the extraction (EtOAc) of lipophilic metabolites from cultural filtrates (11.6 L) of *P. foeniculi* was lyophilised. The residue (30 g) was suspended in 300 mL ultrapure Milli-Q water. The suspension was centrifuged (7000 rpm at 4 °C for 45 min), then filtered through a Millipore membrane (0.22 μm), and the filtrate was lyophilised. The residue dissolved in ultrapure Milli-Q water (60 mL) was brought at 4 °C and mixed with 6 vol of absolute cold EtOH (360 mL) and left overnight at −20 °C. The resulting precipitate was collected by centrifugation (7000 rpm at 4 °C for 45 min), dissolved in ultrapure Milli-Q water (50 mL) and reprecipitated with absolute cold EtOH (250 mL) as described above. After 24 h the resulting precipitate was collected by centrifugation as described above, dissolved in a minimal amount of Milli-Q water (300 mL), centrifuged as described above and dialysed (cut-off 3500 Da) for 2 days against a large volume of water. The content was lyophilised to yield the EP fraction (0.9 g)

*Purification of EP.*—The EP fraction (900 mg) was dissolved in 15 mL water, and Fehling's solution was added until the precipitation of the "copper complex" was just complete. The precipitate was collected by centrifugation, washed with $H_2O$, and decomposed by maceration for 1 min at 0 °C with EtOH containing 5% (v/v) conc HCl. The residue was washed with EtOH until the washings gave a negative test for chloride. The final washing with acetone appeared colourless thus suggesting the absence of Cu(II) chloride in the residue. NMR and chemical analysis showed that the residue was a mannan (106 mg).

The supernatant was collected, neutralised with 2 M HCl, and dialysed against tap water for 24 h. The dialysate was freeze dried to yield 643 mg of an amorphous powder which was dissolved again in a minimal amount of water and 4 vol of EtOH were added. The supernatant was collected and freeze dried to yield 110 mg of impure galactan.

Measurements by atomic absorption revealed the presence of copper in the range of ppm in both samples.

In order to obtain the two polysaccharides without residual Cu(II) chloride for the bioassays, another portion of the EP fraction (500 mg) was precipitated using 2-propanol. The EP fraction was dissolved in 10 mL water and cold 2-propanol (8 mL) was added after one night at 4 °C, then the precipitate was collected by centrifugation and lyophylised to yield fraction A (115 mg), consisting of pure mannan, whereas the supernatant gave fraction B (376 mg), constituted of impure galactan.

*Acid hydrolysis.*—Polysaccharide samples were hydrolysed with 2 M $CF_3CO_2H$ at 120 °C for 1 h [31]. The molar ratios of alditol acetates were evaluated using *myo*-inositol as internal standard by GLC on a SP-2330 capillary column (Supelco, 30 m×0.25 mm i.d.; flow rate 1 mL/min, $N_2$ as carrier gas), at 235 °C.

*Methylation analysis.*—Polysaccharide samples were methylated as reported [32]. The crude reaction product was filtered on a C-18 Sep-Pak cartridge (Waters), washed previously with EtOH (20 mL), MeCN (2 mL), and water (10 mL). The fractions were eluted with water (50 mL), 4:1 water–MeCN (8 mL), MeCN (2 mL), and EtOH (4 mL). The last two fractions were pooled and concentrated to give the methylated polysaccharide which was hydrolysed with 2 M $CF_3CO_2H$. The partially methylated products were reduced with $NaBD_4$, acetylated and analysed by GLC-MS on a SP-2330 capillary column (Supelco, 30 m×0.25 mm i.d., flow rate 0.8 mL/min, He as carrier gas) with a gradient temperature as follows: 80 °C for 2 min, up to 170 °C at 30 °C/min, up to 240 °C at 4 °C/min, 240 °C for 10 min.

In order to separate the 3,4,6-tri-OMe-mannitol acetate and the 2,4,6-tri-OMe-mannitol acetate, which have the same retention time under the above GLC conditions, the partially methylated alditol acetates were also analysed by GLC-MS on a SPB-1 capillary column (Supelco, 30 m×0.25 mm i.d., flow rate 0.8 mL/min, He as carrier gas) with a gradient temperature as follows: 100 °C for 2 min, up to 220 °C at 2 °C/min. GLC of the partially methylated alditol acetates was carried out on two columns identical with those used for GLC-MS

(flow rate 1 mL/min, $N_2$ as carrier gas) with the same temperature gradient, using effective response factors [33], and normalising the peak areas with respect to that of *myo*-inositol hexa-acetate used as the internal standard.

*Acetolysis of mannan*.—Acetolysis of 100 mg mannan was performed as described by Kocourek and Ballou [34]. The deacetylated products were applied to a column (1.5×90 cm) of Bio-Gel P-2, and eluted with distilled water at a flow rate of 14 mL/h at room temperature; 1.5 mL fractions were collected. The fractionation yielded five fractions, namely, the exclusion volume (14 mg), the oligosaccharides **1–3** (12 mg; 15 mg; 18 mg), and mannose (7 mg). A sample (1 mg) of each oligosaccharide was acetylated with $Ac_2O$ in pyridine at room temperature overnight. Usually work-up gave the peracetylated products which were analysed by FABMS in the positive-ion mode. Pseudomolecular ion peaks $[M+H]^+$ at $m/z$ 619, 967, and 1255 for di-, tri- and tetra-saccharides, respectively, were measured.

*Determination of the absolute configuration of the monosaccharides*.—The monosaccharides obtained from total acid hydrolysis were purified on a HPLC column (Lichrospher 100 $NH_2$, 10 mm; 3:1 $CH_3CN$–$H_2O$). The configuration of each monosaccharide, identified by $^1H$ NMR spectroscopy, was established by polarimetry to be D for both galactose and mannose units.

*Exopolysaccharides bioassay*.—EP, A and B fractions from *P. foeniculi* and commercial mannan were assayed for their phytotoxic effect on three different plants: the homologous host (uptake by cuttings from two months old plantlets of sweet fennel, cv. Scafati) and two non-hosts: tomato (uptake by four leafed tomato cuttings from about one month old plantlets, cv. Marmande) [35] and tobacco (infiltration of the leaf mesophyll, cv. Samsun) [36]. Lyophilised substances to be tested were dissolved in distilled water and the pH adjusted to 6, where necessary, with HCl or NaOH. All EP preparations were tested at concentration of 0.5, 1.0, 2.5, and 5.0 mg/mL. Distilled water and commercial mannan were used as control in all tests. Fennel and tomato cuttings were left in the test solutions for 24 h, transferred to distilled water and, after 24 h from the transfer to water, were visually scored for symptom development. Chlorotic and/or necrotic symptoms of the infiltrated areas of tobacco leaves were visually scored every day for 1 week.

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