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Phytotoxic extracellular polysaccharide fractions from *Cryphonectria parasitica* (Murr.) Barr[1] strains

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Abstract

Two hypovirulent and one virulent strains of *Cryphonectria parasitica*, the causal pathogen of chestnut blight, produced a large amount of pullulan and a minor polysaccharide fraction containing galactose, mannose and, in one case, also rhamnose. The minor fractions of all strains elicited higher phytotoxicity on chestnut (*Castanea sativa* L.) leaves, twigs and seedlings or on tomato cuttings (*Lycopersicon esculentum* L.) than that given both by pullulan and native extracellular polysaccharide fractions. One of the components of the minor fraction was identified to be a galactan whose structure, on the basis of chemical and spectroscopic methods consisted of the repeat unit: [→ 6)-*β*-D-Gal*f*-(1 → 5)-*β*-D-Gal*f*-(1 →]$_n$. This is the first report on the production of polysaccharides by *C. parasitica* and their phytotoxic activity.

Keywords: Gelatinous zone; *C. Parasitica*; Phytotoxicity

1. Introduction

Cryphonectria parasitica (Murr.) Barr is the causal agent of chestnut blight (Anagnostakis, 1987), which is characterised by a formation of a 'gelatinous zone' (Rankin, 1914; McCarroll and Thor, 1985) beyond the advancing edge of the mycelium. In addition, wilting of distal foliage and the formation of epicormic sprouts appeared directly below the canker.

Toxins produced by the fungus have been widely demonstrated to be responsible for the necrotic effect on chestnut tissues (Bazzigher, 1953, 1958; Sparapano et al., 1989; Sparapano et al., 1990). To test the hypothesis that the 'gelatinous zone' could be made up of polysaccharide material which induce phytotoxicity, we investigated the extracellular polysaccharides (EPs) of three strains of *C. parasitica*, with different levels of virulence.

Phytotoxicity by polysaccharides was recognized since the work of Hodgson et al., 1949. It has been demonstrated that some phytopathogenic fungi like *Phytophthora* spp. produce *β*-1,3-glucans which are phytotoxic and induce wilting symptoms on host and non-host plants (Keen et al., 1975).

2. Materials and methods

2.1. General methods

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 δ relative to internal 1,4-dioxane (67.4 ppm) and TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-d_4), respectively. GLC was performed with a Dani instrument equipped with a flame ionization detector and GLC-MS with a Hewlett-Packard 5890 instrument.

2.2. Fungal cultures

Three strains of *C. parasitica* from the fungal collection of the Department of Plant Pathology of Bari, Italy were

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used: one virulent (CP159) and two hypovirulent strains (CP102 and CP263). Cultures were maintained on potato-dextrose-agar (PDA) at 23°C in the dark.

2.3. Growth and harvest of cultures

Cultures of the three strains of *C. parasitica* were grown on potato-sucrose agar (APS) slants at 25°C for 10 days in the dark and then used as inoculum. The latter consisted of 50–80 mg (dry weight) of mycelium material. Stationary cultures were grown in 150 ml of a Czapek–potato infusion medium in 1 l Roux flasks. The flasks were incubated at 23°C for 20 days, in the dark. At harvest, the mycelium material was removed by filtration through cheese-cloth and then through Whatman filter paper N. 54. The liquid medium, whose pH was 4.1, was lyophilised.

2.4. Preparation of native EPs

The lyophilised culture filtrate (25 g from 7 l) of the CP263 *C. parasitica* strain was suspended in 300 ml of ultrapure Milli-Q water and centrifuged (7000 rpm at 4°C for 45 min). The supernatant liquid brought to 4°C was mixed with three volumes of absolute cold EtOH (900 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 (100 ml) and reprecipitated with absolute cold EtOH (300 ml) as described above. After 24 h, the resulting precipitate was collected by centrifugation, dissolved in the minimal amount of ultrapure Milli-Q water (300 ml) and dialyzed (cut-off 3500 Da) for 2 days against a large volume of water. The content was lyophilised to yield native EPs (1.0 g). When the same procedure was performed on lyophilised culture filtrates of the CP159 (15 g, from 3 l) and CP102 (9.8 g, from 3 l) strains 4.8 and 0.53 g of EPs, respectively, were obtained.

2.5. Purification of native EPs

The native EPs obtained from the CP102 (157 mg), CP263 (4.5 g) and CP159 (950 mg) strains were dissolved in a minimum amount of water and then precipitated with 40% EtOH overnight at − 20°C. The pullulan precipitates were collected by centrifugation at 3600 rpm for 10 min to yield 90 mg for the CP102 strain, 4.3 g for CP263 and 910 mg for CP159, respectively. The supernatant liquids (fraction AB) of CP102 and CP263 strain were further fractionated by precipitation from water with 2-propanol in a 1:2 and 1:4 ratio, respectively. This procedure yielded the soluble fractions A (24 and 105 mg from strains CP102 and CP263, respectively) and, as precipitate, the fractions B (43 and 27 mg from strains CP102 and CP263, respectively). An apparent molecular mass of 50 KDa was found by calibration on a Bio-Gel P-100 column with dextran standards for fractions A from both the strains. The above separation procedure failed on the supernatant liquid (fraction C) from the CP159 (40 mg) strain.

All attempts to further purify the above fractions B, using both further 2-propanol precipitation and chromatography on Bio-Gel P-10 and P-100, failed.

2.6. Methanolysis

Methanolysis was performed on samples (1 mg) with M HCl in MeOH for 16 h at 80°C. The methanolysate was analysed as Me_3Si ethers as follows: the resulting mixture of methyl glycosides was dried over P_2O_5, treated with Sigma-Sil-A (Sigma) for 20 min at 80°C, and subjected to GLC on an SPB-1 capillary column (SUPELCO, 30 m × 0.25 mm i.d., flow rate 1 ml/min, N_2 as carrier gas), with the temperature programme: 160°C for 3 min, 160° → 200°C at 2°C/min, 200° → 260°C at 10°C/min, 260°C for 10 min.

2.7. Acid hydrolysis

Polysaccharide samples were hydrolysed with 2 M trifluoroacetic acid (Albersheim et al., 1967) according to the following procedure: each sample was kept at 120°C up to 1 h, analysing the content in neutral sugars at 15, 30 and 60 min; the molar ratios of the sugars were evaluated 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 using effective response factors (Sweet et al., 1975) and normalising the peak areas with respect to that of *myo*-inositol hexa-acetate used as the internal standard.

2.8. Methylation

Polysaccharide samples were methylated as reported (Sandford et al., 1966). The crude reaction product was filtered on a C-18 Sep-Pak cartridge (Waters) previously washed with EtOH (20 ml), MeCN (2 ml) and water (10 ml). The fractions were eluted with water (50 ml), 4:1 water:MeCN mixture (8 ml), MeCN (2 ml) and EtOH (4 ml). The last two fractions were pooled and evaporated to give the methylated polysaccharide which was hydrolysed with 2 M TFA. The partially methylated products in the hydrolysates 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: 80°C for 2 min, up to 170°C at 30°C/min, 170°C for 0 min, up to 240°C at 4°C/min, 240°C for 10 min GLC of the methylated alditol acetates was carried out on a column identical with that used for GLC-MS (flow rate 1 ml/min, N_2 as carrier gas) with the same temperature gradient.

2.9. Bioassays

Polysaccharide fractions were assayed on host and non-host plants. Severed twigs or leaves of *Castanea sativa* L.

Table 1
Molar ratios among the monosaccharides constituting the native EPs from the CP159, CP263 and CP102 strains evaluated from methanolysis

Monosaccharide	CP159	CP263	CP102
Glc	35.2	19.4	3.1
Gal	2.0	2.0	1.9
Man	2.4	1.0	1.0
Rha	1.0	–	–

and cuttings of tomato (*Lycopersicon esculentum* L. cv. Marmande) were choosen as test plants. Young and old chestnut leaves were treated with aliquots of polysaccharide solutions at 1 to 20 μg/ml for fraction AB, fraction A and fraction B and at 1 to 100 μg/ml for pullulan. For the bioassay on herbaceous plants, the tomato cuttings were taken from young seedlings (1-month old) and then left for 24 h to absorb 3 ml of a toxic solution (20 μg/ml). During the assay the chestnut leaves and twigs and tomato cuttings were maintained in a growth chamber at fairly low levels of relative humidity (60%), temperature (23°C) and light (150 μmol/m^2/s). Symptoms developed within 48 h on the tomato cutting, after 10 days on the chestnut leaves and after 14–40 days on the chestnut twigs, respectively.

3. Results and discussion

It is well known that in a *C. parasitica* population it is easily possible to isolate forms of the blight fungus that appeared different and have reduced virulence. These isolates called 'hypovirulent' can transform virulent strains in hypovirulent types by introducing a ds-RNA into the cytoplasm. In this case the chestnut plants infected by the fungus heal and the canker recovers.

We examined three strains of *C. parasitica*, one virulent (CP159) and two hypovirulent (CP102 and CP263), which in our culture conditions allowed to produce polysaccharides in stationary growth.

The ^{1}H and ^{13}C NMR spectra of native EPs, from all of the strains of *C. parasitica* showed very strong signals for anomeric protons and carbons at δ 5.42, 5.39 and 4.98 brs and δ 101.9, 101.4 and 99.7, respectively. In addition, other minor signals, which are more intense in the case of the CP102 strain, were present in the spectra thus suggesting heterogeneity in the native EPs. Analysis by methanolysis (Table 1) revealed for all of the strains the presence of a large amount of glucose and a minor amount of galactose and mannose, which are in the 2/1 ratio for CP102 and CP263 and in 1/1 ratio for the virulent CP159 strain, this latter contains also traces of rhamnose.

The methylation analysis of the native EPs showed a 2:1 ratio of 2,3,6-tri-O-methylglucose and 2,3,4-tri-O-methylglucose for the glucose component, indicating 4-linked and 6-linked glucopyranose units. These chemical and NMR spectroscopical data (McInTyre et al., 1991) suggested a pullulan structure (Bouveng et al., 1962). Since the glucan was present in very large amount, ranging from 57% up to 95% (Table 2), we exploited the precipitation with 40% EtOH (Waksman et al., 1977) to obtain pure pullulan as precipitate.

The supernatant liquids (fraction AB) was not further purifiable both by EtOH precipitation and by gel chromatography. However, we found that 2-propanol, only in the case of CP263 and CP102 strains, was a convenient co-solvent in order to achieve further purification by precipitation.

The supernatant liquors, so obtained, (fractions A) showed ^{1}H NMR spectra [Fig. 1(a)] which were simpler than that of corresponding precipitates (fractions B) [Fig. 1(b)], indicating that they contain a partially purified component. In particular, in the fractions A spectum [Fig. 1(a)] there were two intense sharp signals at δ 5.25 and 5.04 together with some broad ones. These latter appeared to be more intense in fractions B [Fig. 1(b)]. Both fractions A and B could not be purified either by further precipitation with 2-propanol or by gel-chromatography. Therefore the structural determination, as well as the bioassays, were performed directly on these fractions.

The ^{13}C NMR spectra of fractions A (Fig. 2), showed mainly two sharp anomeric signals at δ 108.7 and 107.8. The chemical shifts of anomeric carbons were diagnostic of β-galactofuranose units (Gorin et al., 1975), suggesting a galactan structure for the major polysaccharide.

Analysis by methanolysis of fractions A showed that it contained a significative amount of mannose (30%) in addition to galactose. This maybe due to presence of the polysaccharide component of fraction B.

The methylation analyses gave, for the galactose counterpart, ca 1:1 ratio of 5-linked and 6-linked galactofuranose units, indicating the presence of a linear polysaccharide as major component of fraction A. Moreover, the presence of only two anomeric signals in the NMR spectra suggested a regular disaccharide structure with the repeat unit as shown

Table 2
Yield and percentage of extracellular polysaccharide production and pullulan in stationary cultures of three strains of *C. parasitica*

Strain	Lyophilized culture filtrate [g/l]	Ethanol precipitate [g/l]	Pullulan [g/l]-(%)[a]
CP 102 Hypovirulent	3.3	0.18	0.103–(57)
CP263 Hypovirulent	3.6	0.14	0.131–(94)
CP159 Virulent	5.0	1.6	1.520–(95)

[a] The percentage is refered to ethanol precipitate fraction

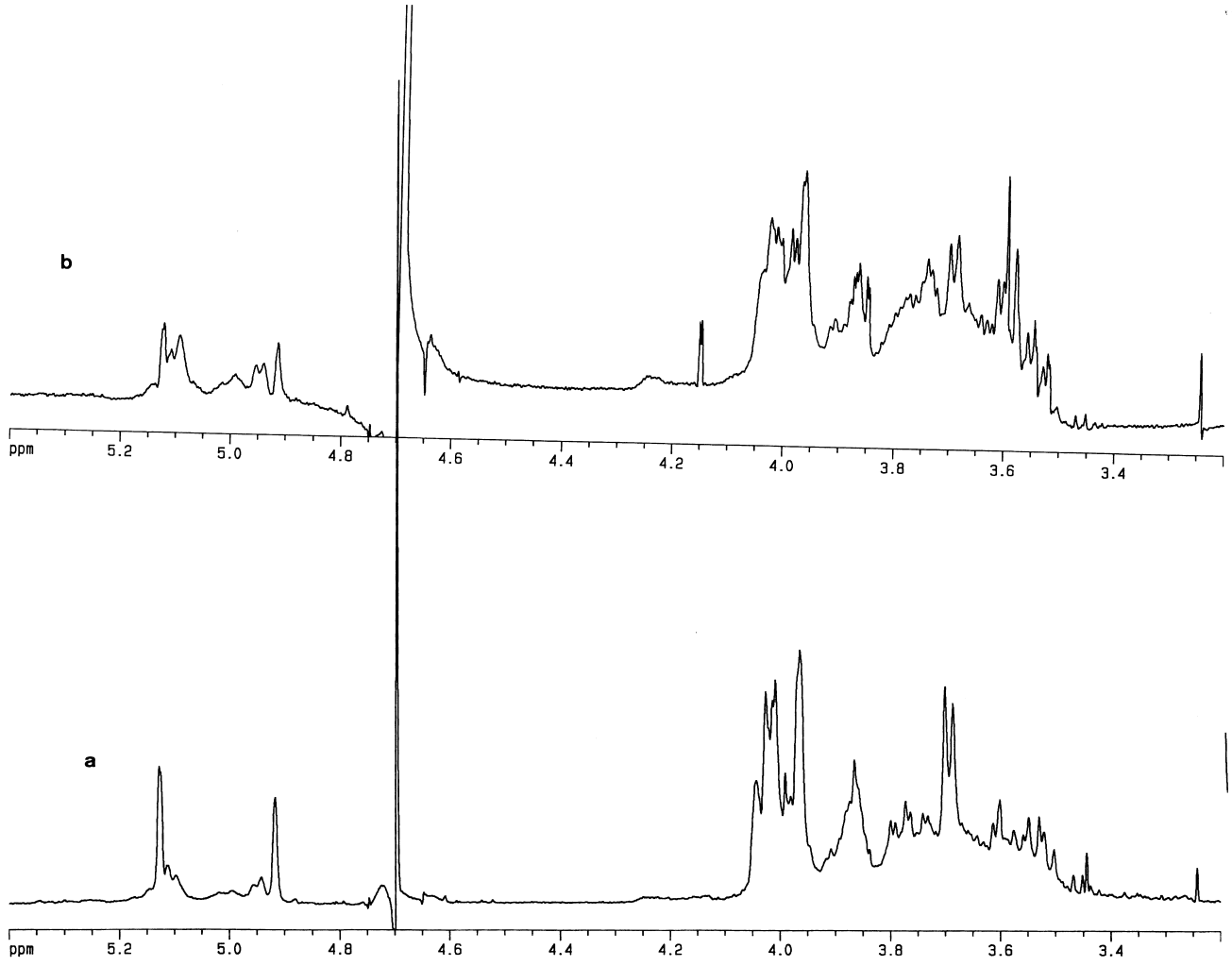

Fig. 1. ^{1}H NMR spectra of fraction A (a) and fraction B (b) in D_2O at 30°C for CP263 strain.

in (1) below

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

This structure represents a new arrangement of galactan which has different ratios of 5-Gal*f* and 6-Gal*f* units compared with those of other galactans (Parra et al., 1994; Leal et al., 1995). It is worth noting that the latter were isolated from the fungal cell walls whereas in our case the galactan arose from the extracellular polysaccharide fraction.

The fraction B, which appeared to be constituted of which could not be separated. Analysis by methanolysis showed the mixture contained mannose and galactose in a 1/1 ratio. Despite its hetereogeneity fraction B was used in the bioassays.

In the case of the virulent CP159 strain, the purification by 2-propanol of the polysaccharide fraction devoid of pullulan (fraction C) failed, in contrast to the hypovirulent CP263 and CP102 strains. This different behaviour suggest a diverse structure for the pullulan accompanying polysaccharide for CP159 compared with the CP263 and CP102 strain. This is substantiated by the different mannose/galactose ratios found in the corresponding native EP fractions (see above). Accordingly, the alditol acetates analysis of fraction C showed that it consisted of mannose, galactose and also rhamnose in the 2.3: 1.0: 1.7 ratio.

Both native EPs and their components fractions from the three strains of *Cryphonectria parasitica* were tested for the phytoxicity biossay on *C. sativa* and *L. esculentum* tissues.

Table 3 shows that the native EPs fractions of each strain of *C. parasitica* induced a toxic effect on chestnut and

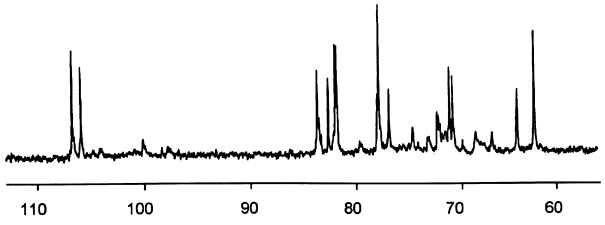

Fig. 2. ^{13}C NMR spectrum of fraction A in D_2O at 30°C for CP263 strain.

Table 3
Symptoms caused by native EPs isolated from culture filtrates of three strains of *C. parasitica* on host and non-host plants

EPs from strain	Host: *Castanea sativa*[a]	Non-host: *Lycopersicon esculentum*[b]
CP102	Browning and necrosis	Necrosis
CP263	Browning and necrosis	Necrosis
CP159	Chlorosis	Chlorosis

[a] Symptoms appeared on severed chestnut twigs after absorption of 3 ml of a solution containing 20 μg/ml of EPs.
[b] Symptoms appeared on tomato cuttings after absorption of 3 ml of a solution containing 20 μg/ml of EPs.

tomato tissues. In both cases, cells died if treated with native EPs of both hypovirulent strains CP102 and CP263. The symptoms caused by that obtained from the virulent strain CP159 appeared less severe.

When each native EP was further purified, the toxicity of the polysaccharide component fractions remained and, indeed, was enhanced. For instance (see Table 4), in the case of CP263 fraction AB, the symptoms (necrosis), which affect only a few leaves or narrow areas of leaves, became more severe (dieback), affecting the whole plant. Pullulan isolated from each strain gave symptoms of wilting and yellowing. Fraction A and B from the CP263 strain showed the highest phytotoxic activity on chestnut tissues. Symptoms appeared 10 days after complete absorption of the toxic solution by single leaves or 14 days later if the toxic solution was absorbed by severed twigs. Tomato cuttings after 48 h of pullulan treatment showed leaflet wilting and necrosis. The treatment of tomato cuttings with fraction A or B caused wilting of the entire explant. The appearance of wilting on test plants recalled the same symptoms shown in nature by infected chestnut plants. This may suggest that all or some of the polysaccharides isolated from the liquid culture of *C. parasitica*, if produced in vivo, could act in the plant as determinants of virulence. We have observed that both host (chestnut) and non host (tomato) were affected by all of the polysaccharides tested. Pullulan appeared to be less toxic than fraction A and B.

The response of *C. sativa* and *L. esculentum* tissues to the native extract or to the component fractions isolated from culture filtrates of *C. parasitica* strains indicates that the concentration threshold when symptoms appear was 100, 20 and 20 μg/ml for pullulan, fraction A and B, respectively.

The importance of cell surface molecules in the specific recognition between a plant and a pathogen is now universally accepted. There is increasing evidence that the expression of plant disease resistance relies on the constant and continuous exchange of information between partners. Thus, molecules exposed at the cell wall play a major role in the biochemical events that determine the outcome of a host–pathogen interaction. However, knowledge of the spatial and temporal distribution of these molecules in infected plant tissues is a prerequisite to a better understanding of their functional specialisation in situ. Extracellular polysaccharides extruded from *C. parasitica* may be regarded as an important virulence factor on host plants. Work is in progress to prove if in the 'gelatinous zone'

Table 4
Symptoms caused by the component fractions of three strains of *C. parasitica* on host and non-host plants

Polysaccharides from	Host: *Castanea sativa*[a]	Non-host: *Lycopersicon esculentum*[b]
Hypovirulent strain CP102		
Pullulan	Reddening and yellowing	Necrosis
fraction AB	Wilting	Yellowing
fraction A	Browning and necrosis	Wilting
fraction B	Browning and necrosis	Wilting
Control (water)	None	None
Hypovirulent strain CP263		
Pullulan	Wilting	Necrosis
fraction AB	Necrosis	Yellowing
fraction A	Dieback	Wilting
fraction B	Dieback	Wilting
Control (water)	None	None
Virulent strain CP159		
Pullulan	Wilting	Wilting
fraction C	Yellowing	Yellowing
Control (water)	None	None

[a] Symptoms appeared after injection of toxin into the cortical tissues of 3-year old chestnut seedlings.
[b] Symptoms appeared on cuttings of tomato plants after polysaccharide absorption.

around the canker in the host plant contains the same polysaccharides as found in the EP fractions.

It is also worth noting that our growing conditions in stationary culture allowed us to produce extracellular polysaccharides from the three strains of *C. parasitica*. Table 2 shows the yields of pullulan produced by each strain of *C. parasitica*. The result showed that the virulent strain CP159 was the highest producer of pullulan (1.52 g/l) corresponding to the 95% of the ethanol precipitate. It is important to underline that this is the first report of *C. parasitica* as a pullulan producer in vitro.

Polysaccharide production by fungi is of evident industrial interest. For instance, pullulan produced by the yeast *Aureobasidium pullulans* is very expensive and it is sold only by two chemical firms in the world (Simon et al., 1993). This may eventually be produced on a large scale for use in several industries, for example as an ingredient in food and drinks, in the plastics industries as nylon-like films and as a component in electric materials (Israilides et al., 1994). The finding that *C. parasitica* produces high amount of pullulan is encouraging for the exploitation of these strains as microorganisms for use in industrial applications. Studies are in progress for dealing with the understanding the role of pullulan in pathogenesis and by improving the substrate composition and culture parameters for yield enhancement.

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