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Pullulans produced by strains of *Cryphonectria parasitica*—I. Production and characterisation of the exopolysaccharides

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Abstract

Studies published during the last four decades indicated that pullulan has mainly α-(1→6) maltotriose subunits, accompanied by α-(1→6) maltotetraose subunits and other anomalies in the polymer chain. The presence and the extent of these anomalies depend on the strain of *Aureobasidium pullulans* used for obtaining the pullulan as exocellular material, and on the culture conditions as well. Besides *A. pullulan*, other fungi produce this polysaccharide. In all cases, the maximum amount of α-(1→6) maltotetraose subunits was believed to be 7%. This paper reports the characterization of pullulan samples, obtained by liquid cultures of a virulent strain (CP159) and two hypovirulent strains (CP263 and CP102) of *Cryphonectria parasitica*. It was found that in all cases pullulan was much richer in α-(1→6) maltotetraose subunits than the pullulan(s) so far investigated. Chestnut blight caused by *C. parasitica* is a disease of worldwide importance in chestnut production. The structure of pullulan could be correlated with the pathogenesis and symptoms expressed by infected plants.

Keywords: *Cryphonectria parasitica*; Exopolysaccharide(s); Pullulan; Structure; Plant pathogenic fungi; Phytotoxicity

1. Introduction

The present paper deals with the production and characterization of pullulans from virulent and hypovirulent strains of *Cryphonectria parasitica* (Murrill) M. E. Barr, formerly called *Endothia parasitica* (Murrill) P. J. Anderson and H. W. Anderson. The work aims at elucidating the primary structure of pullulans and, possibly, at correlating variations in the chemical structure with their biological activity.

1.1. The pullulans

The exopolysaccharide produced by *Aureobasidium pullulans* (de Bary) G. Arnaud, a mitosporic fungus formerly called *Pullularia pullulans* (de Bary) Berkhout (syn.: *Dematium pullulans* de Bary), was isolated and firstly characterised in the late fifties (Bernier, 1958; Bender, Lehmann, & Wallenfels, 1959). It is a linear glucan, named pullulan by Bender et al. (1959). Pullulan can be used as an adhesive. Moreover, it forms fibres and oxygen-impermeable films. Because of these properties, pullulan has many potential applications, especially in the food and pharmaceutical fields. In addition to this, the progressive discovering of subunits of pullulan different from the predominating α-(1→6) maltotriose subunits, made the polysaccharide the object of many studies. Thanks to them, it was stated that the linear chain of pullulan also contains maltotetraose subunits (Wallenfels, Keilich, Bechtler, & Freudenberger, 1965), that should be randomly distributed throughout the molecule (Carolan, Catley, & McDougal, 1983). So far, the maximum extent at which maltotetraose subunits have been detected is 7% (Catley, Ramsay, & Servis, 1986). Moreover, α-(1→3) and even β-(1→3) and β-(1→6) linkages were found in the main chain of pullulan produced by particular strains, in addition to the α-(1→4), highly predominating, linkages (Sowa, Blackwood & Adams, 1963; Fujii, Shinohara, Ueno, & Imada, 1984).

Mainly by digestion methods, other anomalies in the linear chain of pullulan, such as possible branches, were suspected. As a consequence, the glucan named pullulan can be considered as belonging to a family of similar exopolysaccharides (EPS), for which the term 'pullulan' should be replaced by 'pullulans'. Indeed, Catley, Robyt & Whelan (1966) have already suspected that 'there is, perhaps, no unique structure of

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pullulan', as reported by Catley (1970). On the structures of pullulans so far discovered, interesting reviews have been written, the most complete being those by Gibbs & Seviour (1996); Leathers (2002); Shingel (2004).

As recently reported by Leathers (2002), pullulans are also produced by other fungi than *A. pullulans*. In particular, Evidente, Lanzetta, Mancino, Molinaro, & Sparapano (1997); Sparapano & Bruno (1998); Corsaro et al. (1998) reported that pullulans are also produced by *C. parasitica*, the fungal agent of chestnut blight.

1.2. Toxicity of microbial polysaccharides

Toxicity of pullulans against humans or animals was never detected. On the contrary, phytotoxic effects against different plants were exhibited by commercial pullulans and by pullulans obtained in the laboratory.

Toxicity of microbial polysaccharides to plants has been recognized since long time (Hodgson, Peterson & Riker, 1949). Many of them cause occlusions of the xylem elements or systematically distribute throughout the plant, thus inducing the affected plant to wilt as a consequence of water stress. Solutions of plant or microbial polysaccharides with high molecular weight are absorbed only with difficulty by the cuttings or whole plants used in the bioassays, and usually they induce the test plants to wilt. In contrast, absorption of pullulan solutions by detached leaves of chestnut (Evidente, Lanzetta, Molinaro, Mugnai, Parrilli, & Sparapano, 1996; Evidente et al., 1997; Bruno & Sparapano, 2001; Bruno, Delben, Forabosco, Liut & Sparapano, 2001) or grapevine (Sparapano & Bruno, 1998; Sparapano, Bruno & Graniti, 1998; Sparapano, Bruno & Graniti, 2000) or its injection into the shoots or branches of standing chestnut or vine plants was relatively easy. In both cases, collapse and drying of large parts of the leaf lamina resulted.

The flexibility of pullulan(s) chain, that is reflected in the rheological properties of its solutions, may account for the unusually easy transport of these macromolecules in the xylem vessels (Seviour, Stasinopoulos, Auer, & Gibbs, 1992; Crescenzi, 1994). The toxic effect of pullulan(s) to the leaf tissues is possibly due to the formation of thin films in the mesophyll, which are hardly permeable by oxygen. If this hypothesis is correct, drying of the leaf tissue would be due to a physical rather than a chemical effect of pullulan on the plant cells.

As far as *C. parasitica* is concerned, both virulent and hypovirulent strains were found to be high producers of pullulan and low producers of galactan and galactomannan (Evidente et al., 1996). When detached leaves or cuttings or twigs of chestnut absorbed pure polysaccharides of each fungal strain at low concentrations (50-100 $\mu g\ mL^{-1}$) in water, phytotoxic symptoms appeared on the foliar lamina and on petiole, i.e. browning and collapse of the leaf tissues, which soon desiccated (Sparapano & Bruno, 1998; Bruno & Sparapano, 2001). The same type of leaf symptoms was also observed on chestnut cuttings treated with the pullulan by *A. pullulans* grown in the same cultural conditions of the *C. parasitica* strains. Symptoms similar to those observed on cuttings were shown by the leaves of potted plants or of standing plants of chestnut, syringe-injected with the same pullulan concentration (Bruno & Sparapano, 2001). The production of pullulan by *C. parasitica* strains could generate the gelatinous zone seen in advance of the mycelium in cankers (McCarrol & Thor, 1985).

Sparapano et al. (2000) demonstrated that at very low doses (e.g. 50 $\mu g\ mL^{-1}$) pullulans produced by two mitosporic fungi *Phaeomoniella chlamydospora* (W. Gams et al.) Crous and W. Gams and *Phaeoacremonium aleophilum* W. Gams et al., two esca-associated fungi of grapevine, produced on leaves chlorotic interveinal and marginal spots or patches, which slowly became necrotic. Similar symptoms were produced when identical concentrations of pullulan extracted from naturally infected grapevine wood, or of commercial pullulan produced by three different companies, were assayed on grapevine leaves (Sparapano et al., 2000).

1.3. Cryphonectria parasitica as plant pathogen

Chestnut blight, that was firstly detected on American chestnut trees, *Castanea dentata* (Marsh.) Borkh. (Merkel, 1905), spreads by conidia and ascospores produced by *C. parasitica* and dispersed by air-borne dust or released after heavy rains by means of insects and other crawling animals (Rankin, 1912; Rankin, 1914; Shear & Stevens, 1913; Sharf & DePalma, 1981; Russin, Shain & Nordin, 1981). Once it enters the chestnut through wounds, the fungus grows below the bark. Orange coloured fungal stromata pushed though the epidermis and in these, pycnidia and perithecia were found; in moist conditions sticky orange tendrils of conidia were extruded from the pycnidia (Merkel, 1905; Anagnostakis, 1987). The symptoms were bark cankers, wilting of the distal foliage, and the formation of epicormic sprouts directly below the canker.

There is great variation in appearance and virulence among *C. parasitica* strains. Hypovirulence is a disease, or a group of diseases, of the fungus *C. parasitica* that reduces its ability to kill susceptible chestnut tree hosts. It is controlled by genetic determinants in the cytoplasm of the fungus. The determinants are associated with double-strand RNA (dsRNA). Many of the viruses that infect and cause hypovirulence in the chestnut blight fungus belong to the newly described family *Hypoviridae* (Smart, Yuan, Foglia, Nuss, Fulbright & Hillman, 1999). While the virulent strains are lethal for chestnuts within a few years, the hypovirulent strains are not and keep the tree clear of further infections. By inoculating a hypovirulent strain around the edge of a developing lesion, caused by a virulent strain, the recovery of the lesion results from the action of the plant's defence mechanism and to a transformation of the virulent strain into hypovirulent strain.

There is some hope for biological control of chestnut blight by hypovirulence. Its control, which is a goal of many research groups, will be facilitated by knowing the mechanism(s) by which the pathogen attacks its hosts.

Fungal pathogens and their effects on host plants can be indirectly studied through their secondary metabolism. Fungi can be easily grown in liquid culture and then the culture filtrates

can be manipulated for extraction, purification and chemical characterisation of secondary metabolites, that include EPS.

1.4. Different roles of exopolysaccharides

The roles of EPS produced by phytopathogenic fungi differing by their toxicity to plants should be considered. Important roles are attachment to host plant, adhesion as a prerequisite to pathogenesis, protection of spores, ensuring the survival of the micro-organisms. A role of EPS may be to anchor the fungus against the erosive effects of wind and rain. Another role may be a better adhesion and attachment during the interaction hypovirulent/virulent strains to transferring dsRNA by hyphal anastomoses. As for the simple phenomenon of adhesion, very little is known about the chemistry of fungal adhesives, except that they are generally presumed to include glycoproteins and EPS (Nicholson, 1996). A possible implication of pullulan(s) production by *C. parasitica* could be referred to the ecology of the fungus on bark. Finally, Andrews, Harris, Spear, Lau & Nordhein (1994) suggested a possible implication of pullulan production by *A. pullulans* as a prerequisite for spore dissemination and leaf adhesion.

2. Experimental section

2.1. Production of pullulans by C. parasitica: fungal strains, culture medium and growth conditions

C. parasitica strains CP159 (virulent) and CP263 (hypovirulent) were taken from the fungal collection of the Department of Biology and Plant Pathology, University of Bari. Strain CP159 was isolated from a bark sample taken from cankers margin of chestnut trees (*Castanea sativa* Mill.). Strain CP263 was isolated from cankers of recovering stands in Southern Italy (Rionero, Basilicata). Strain CP102, with intermediate virulence, was obtained by segregation of monoconidial culture of hypovirulent strains *in vitro*. It is equivalent to the deeply pigmented hypovirulent strain that Grente & Sauret (1969) called 'jaune régénéré' and Bonifacio & Turchetti (1973) called 'pigmentato'. This cultural variant was never isolated from naturally infected chestnut plants.

The cultures were maintained by monthly transfers to potato-dextrose-agar (PDA) and incubated at 25 °C, or stored on PDA slants in cotton-capped tubes at 5 °C, in the dark.

Two different media were used, containing mineral Czapek medium amended with 2% glucose or 2% sucrose, respectively. Roux flasks (1 L) containing 150 mL of each medium were inoculated with a mycelial suspension (5 mL) obtained by homogenizing three colonies of each fungal strain grown on PDA at 25 °C for 10 days in the dark. The flasks were incubated at 23 °C for 21 days in the dark as a stationary culture.

2.2. Production of pullulans by C. parasitica: extraction and purification of the exopolysaccharides

At harvesting, the mycelial mat was separated from liquid culture by filtration on a Miracloth sheet (Calbiochem Biochemical La Jolla CA USA) and, subsequently through a 0.45 μm Millipore filter (Waters-Millipore Bedford, MA, USA). Dry weight of the fungal mat was calculated after heating it at 70 °C for 4 days in an oven.

The culture filtrates obtained were treated with three volumes of cold absolute ethanol, that produced phase separation, and left at −20 °C for 24 h, following already established procedures (Sparapano & Bruno, 1998). During storage, the phase containing the EPS separated into floating and settling materials, that were called 'upper' (up) and 'lower' (lo), respectively. Each fraction was separated from the liquid phase by centrifugation at 9,000 rpm for 15 min, weighted and stocked after freeze-drying.

In the present work, the following fractions were studied: CP159upG, CP159loG, CP159loS, CP263upG, CP263loG, CP263loS and CP102loS, where G or S denote the presence of glucose or sucrose in the culture medium, respectively.

The raw EPS were suspended in a buffer solution at pH 7.5 (50 mM tris(hydroxymethyl)-aminomethane-HCl, 5 mM $MgCl_2$ and 0.05% NaN_3) (10 g of dried sample suspended in about 500 mL of buffer solution) and left to swell under stirring at room temperature. The suspensions were treated with a Silverson Machines Ltd, England, laboratory mixer emulsifier for 5 min and then left under mechanical stirring at room temperature for about 6 h. The suspensions were then centrifuged at 15,000 rpm for 30 min at room temperature. The supernatant liquids were collected and treated at 37 °C with ribonuclease for 8 h and thereafter with protease for 16 h. CP159loS, CP263loS and CP102loS were also treated with proteinase K, at 37 °C for 3 h. At the end of the treatment, the enzymes were inactivated in a boiling water bath for 5 min. After that, the solutions were dialyzed exhaustively against distilled water, frozen and lyophilized.

2.3. Other materials

Commercial pullulans were Pullulan PI-20, lot 01207, purchased from Hayashibara Co., Ltd (Okajama, Japan) and Pullulan P4516 produced by Sigma (USA), lot 078H1060.

Protease from *Streptomyces griseus*, EC 3.4.24.31, lot 062K1374, proteinase K from *Tritirachium album*, EC 3.4.21.64, lot 90K8604, ribonuclease A from bovine pancreas, EC 3.1.27.5, lot 119H0756, and bovine serum albumin (BSA), code A-3059, were Sigma-Aldrich Chemie GmbH (Germany) products.

All reagents were of analytical grade and, if not differently indicated, were used without purification. 1-Methylimidazole was from Sigma (USA). Acetic anhydride, distilled prior to use, sodium borohydride, trifluoroacetic acid, toluene (HPLC grade) and the sugars used as the standards for gaschromatography (i.e. inositol, glucose, galactose, mannose, fucose, arabinose, ribose, xylose, and rhamnose) were Aldrich GmbH (Germany) products. Sodium chloride was BDH Laboratory (UK). Glacial acetic acid (CODEX), acetone (RPE-ACS), ethyl acetate (RPE-ACS), dimethylsulphoxide, hydrochloric acid (ISO for analysis), methyl alcohol (ACS-ISO for analysis), anhydrous sodium carbonate, and methyl iodide (RPE) were Carlo Erba (Italy) reagents. Chloroform, dichloromethane,

Folin-Ciocalteu's phenol reagent, hexane (puriss. p.a.), perchloric acid 60% (puriss. p.a.), potassium sodium tartrate tetrahydrate (puriss. p.a.), and sodium azide (purum p.a.) were purchased from Fluka Chemie GmbH (Germany). Copper(II) sulfate pentahydrate, potassium hydride with 65% paraffin oil, and tris(hydroxymethyl)-aminomethan were Merck KGaA (Germany) reagents. Magnesium chloride hexahydrate was Riedel-de Haën GmbH & Co. (Germany) product. Deionized, freshly bidistilled water was constantly used for dialysis and preparation of the solutions.

2.4. Methods

The effect of the raw EPS and the purified pullulans was tested on severe twigs or young and olds leaves of *Castanea sativa*. The concentrations ranged from 10 to 100 μg mL^{-1} for the raw EPS and from 1 to 20 μg mL^{-1} for the purified EPS (Sparapano & Bruno, 1998; Corsaro et al., 1998). During the assay, the leaves and the twigs of chestnut were maintained in a growth chamber at 60% relative humidity, 23 °C and at light of 150 μM m^{-2} s^{-1}.

The presence of residual proteins in the purified EPS was determined by the Lowry colorimetric method (Lowry, Rosebrough, Farr & Randall, 1951), using BSA for the calibration curve. The absorbance was measured at 750 nm, using a Varian model Cary 4E spectrophotometer (Australia). By repeated experiments the method was found to be highly reliable. The values found were confirmed by elemental analysis, performed with a Carlo Erba model 1106 Elemental Analyzer (Italy).

The possible presence of starch in the polysaccharide moisture was checked on the sample CP159loG by iodine/iodide. Solutions of CP159loG in the absence and in the presence of added starch were prepared and their absorbance spectra were recorded in quartz cuvettes from 550 to 750 nm by the Cary spectrophotometer. By this method, no starch was detected down to 2%.

The molecular weights of the purified polysaccharides were estimated by gel permeation chromatography (GPC), capillary viscosity and static laser light scattering.

In the GPC analysis, the column (1.5 m) was packed with Sepharose CL 6B and elution was carried out with 0.05 M NaCl, added of 0.05% (w/v) NaN_3. A Waters Associate model R401 refractive index detector was used (Milford, MA, USA). The column was calibrated using dextran standards of known molecular weights, ranging from 10 to 1000 kDa. The signals were read with a Pharmacia-LKB REC-1 recorder (Uppsala, Sweden).

The viscosity measurements were performed in pure water at 25 °C with a Cannon-Ubbelohde 50L764 viscometer, with an initial volume of 2 mL. The automatic viscometer requires an initial volume of at least 12 mL and was not used because the small amount of CP159loG and CP159loS available. The solutions (1 g L^{-1}) were prepared by stirring a weighted amount of the freeze-dried polysaccharide in doubly distilled water for 30 min at 40 °C. They were filtered at room temperature through Millipore-HA filters (0.45 μm) and degassed under vacuum prior to being injected into the viscometer. The viscometer was put into the Schott-Geräte thermostatic bath (Germany) controlled to ±0.02 °C and the measurements were performed not less than 15 min later. For the next dilutions, filtered distilled water was directly introduced into the viscometer to reach the final required concentration and the measurements were performed 10 min after addition of water and accurate mixing. At least seven flow times were performed for each dilution, five of which were used with a deviation of less than 0.3 s. The value of the intrinsic viscosity, $[\eta]$, was taken as the value of $(\eta_r - 1)/C_p$ at the limit of vanishing C_p, where η_r and C_p denote the relative viscosity and the polysaccharide concentration, respectively. The extrapolation was made according to both the Huggins and Kraemer equations.

From the values of $[\eta]$, the (viscometric) average molecular weights were computed using the Mark–Houwink–Sakurada parameters estimated by Kato, Okamoto, Tokuya, & Takahashi (1982); Kato, Tsunehisa & Takahashi (1984); Buliga & Brant (1987a).

The multi-angle static laser light scattering (MALLS) measurements were made at 488 nm with a Coherent instrument (Santa Clara, CA, USA), using an Ar–ion laser. The angular dependence of scattering was determined between 25 and 110° at 25 °C with a Brookhaven Instruments Co. model BI-HV goniometer (New York, USA). The instrument was calibrated with toluene. For the measurements, the solutions were prepared by dilution with 0.05 M NaCl with CP159loG and CP159loS. With Sigma pullulan and CP263upG + CP263loG the solvent was 0.01% (w/v) NaN_3. The concentrations ranged from 0.4 to 4.6 g L^{-1}. The solutions were filtered through Millipore-HA filters (0.45 μm) directly into the measurement cell. For each concentration C_p and angle ϑ, the function $KC_p/\Delta R_\vartheta$ (were K is the Debye constant and ΔR_ϑ is the excess Rayleigh ratio) was plotted against C_p and $\mathrm{sen}^2(\vartheta/2)$. The values were extrapolated to zero angle and zero concentration to obtain M_w^{-1}, were M_w is the weight average molecular weight.

The composition of the polysaccharides was identified by gas chromatography analysis of alditol acetates. The polysaccharide samples were hydrolysed with 2 M trifluoroacetic acid at 100 °C for 16 h and then converted into alditol acetates as described by Blakeney, Harris, Henry & Stone (1983), using inositol as the internal standard. Two instruments were used. They were a Carlo Erba Instruments model GL 8000 (Milan, Italy), with a column OV1701 (25 m × 0.32 mm), and a Perkin–Elmer model Autosystem XL (Norwalk, CT, USA), with a column SP2330 (30 m × 0.25 mm). Both were equipped with a flame ionisation detector (FID). He was the carrier gas. The following temperature programs were used: for Carlo Erba gaschromatograph, 150 °C for 1 min, up to 180 °C at 3 °C min^{-1}, 180 °C for 30 min, up to 200 °C at 3 °C min^{-1}, 200 °C for 10 min and, for Perkin–Elmer gaschromatograph, 200 °C for 1 min, up to 245 °C at 4 °C min^{-1}, 245 °C for 20 min.

For the analysis of the glycosidic bonds, the polysaccharide samples were methylated and then converted into alditol acetates, following the procedure described by Harris, Henry, Blakeney, & Stone (1984). Gas–liquid chromatographic (GLC) analysis of the alditol acetates was carried out by the

Perkin–Elmer gaschromatograph described above, using He as the carrier gas. The temperature program used was: 160 °C for 1 min, up to 210 °C at 2 °C min^{-1}, up to 240 °C at 4 °C min^{-1}, 240 °C for 10 min.

The water content in the commercial pullulans and in the freeze dried polysaccharides was determined with a Perkin–Elmer TGA 7 Thermogravimetric Analyzer (USA). The temperature was raised from 35 to 190 °C at a rate of 10 °C min^{-1}. The weight loss was expressed as a function of the temperature. The water percentages in the polysaccharides ranged from 7.5 to 10.3 and were taken into account for the correct determination of the polymer concentrations.

3. Results and discussion

3.1. Exopolysaccharide production in liquid cultures

The growth rate of CP159 in liquid culture was higher than that of CP263 and CP102, both in the presence of added glucose or sucrose. As a consequence of different growth rates, different amounts of exocellular material were produced by the three strains of *C. parasitica* (Table 1). They produced mycelium, conidia and chlamydospores embedded in viscous material. Simon, Caye-Vaugien & Bouchonneau (1993) showed that swollen cells and chlamydospores of *A. pullulans* were the forms that produced extracellular polysaccharides. When a significant amount of chlamydospores was present, pullulan was always produced, whatever the culture conditions. This could indicate that in our case the EPS are also produced by chlamydospores.

From culture filtrates of CP159 and CP263, two distinct polymeric fractions (upper and lower) were recovered by ethanol precipitation, with the only exception of CP159 in the presence of added sucrose. The upper fractions appeared 'strand-like' and fibrous, while the lower fractions had a gel-like consistency.

The presence of two distinct fractions was reported by Madi, Harvey, Mehlert, and McNeil (1997) for *A. pullulans* strain IMI145194. The fractions were found to be identical, namely linear glucans containing α-(1→4) and α-(1→6) linkages. The authors concluded that both fractions were pullulan and that the difference in their physical behaviour was due to differences in their molecular mass.

Table 1 shows that CP159 was the highest producer of the upper fraction in liquid medium containing glucose (CP159upG) and the highest producer of the lower fraction in liquid medium containing sucrose (CP159loS).

Strain CP102 did not produce any upper fraction. On the contrary, it was the most effective in producing the lower fraction when grown in liquid medium containing glucose (CP102loG).

3.2. Chemical analysis

The composition of the samples studied is reported in Table 2. The following results are worthy of mention.

On passing from glucose to sucrose added to the mineral Czapek medium as the carbon source, the exopolysaccharide content in the raw material obtained from the liquid cultures differed significantly for both CP159 and CP263. In the former case, the yield in polysaccharide material for the 'lower' fraction moved from 11 up to 74%. This difference cannot be attributed to the way through which the polysaccharides were obtained from the raw material, because the same protocol was followed. Rather, it must be recalled that not only the chemical composition of the culture broth, the pH and temperature, but also other environmental parameters, such as light conditions, may alter the growth and the metabolism of many micro-organisms. These strains of *C. parasitica*, grown under conditions very different from those of the natural habitat, with respect to both the nutritional characteristics of the medium and the environmental parameters, could be particularly sensitive to small differences in the culture conditions. This could change the biosynthesis and the accumulation of their secondary metabolites, including polysaccharides.

In all cases, and regardless of the amount of polysaccharides extracted, the polysaccharide fraction contained only, or almost only, glucose. Galactose and mannose may possibly be present in some cases in addition to glucose. The maximum amount of sugars other than glucose was given by CP102, the hypovirulent strain obtained by segregation. Even in this case, however, the total amount of sugars other than glucose did not exceed 7.5%.

As for CP159, this result is in agreement with the previous evidence by Molinaro, Piscopo, Lanzetta & Parrilli (2002).

Table 1
Effect of the substrate on pH, biomass and exopolysaccharide production of three strains of *Cryphonectria parasitica* grown in liquid cultures containing glucose or sucrose and maintained at 25 °C for 21 days in the dark

Strain	Carbon source	pH		Biomass (g L^{-1})[a]	Fractions obtained by ethanol treatment (g L^{-1})[b]	
		Initial	Final		Upper	Lower
CP159 (virulent)	Glucose	5.7	3.0	2.7	2.3	1.0
	Sucrose	5.7	3.6	2.5	0	4.1
CP263 (hypovirulent)	Glucose	5.7	3.6	1.9	1.2	0.9
	Sucrose	5.7	3.4	1.3	1.0	1.1
CP102 (hypovirulent)	Glucose	5.7	3.2	2.3	0	3.2
	Sucrose	5.7	3.1	1.1	0	0.9

[a] Values calculated on the basis of the dry weight of the mycelial mat.
[b] Values calculated on the basis of the dry weight of the ethanol precipitate.

Table 2
Chemical composition of the polysaccharides from *Cryphonectria parasitica* strains

Sample	Weight b.p. (g)[a]	Weight a.p. (g)[b]	Yield (%)[c]	Proteins (%)[d]	Glc: Gal: Man[e]
CP159upG	0.13	0.02	13	n.d.[f]	1: 0.03: 0.02
CP159loG	4.3	0.5	11	6	1: 0: 0.03
CP159loS	5.5	4.1	74	0.5	1: tr: tr[g]
CP263upG	6.5	2.6	39	2	1: 0: 0
CP263loG	7.4	2.6	35	2	1: 0: 0
CP263loS	5.4	3.6	66	<0.5	1: 0: 0
CP102loS	4.6	0.6	13	3	1: 0.03: 0.05

[a] b.p., approximate weight of the raw material containing exopolysaccharides, before purification.
[b] a.p., approximate weight of the polysaccharides, after purification.
[c] Weight percentage of exopolysaccharides in the raw material.
[d] Weight percentage of proteins in the samples.
[e] Molar ratio between glucose (Glc), galactose (Gal) and mannose (Man) in the polysaccharide containing fraction.
[f] n.d., values not determined.
[g] tr, Gal and Man present in traces.

According to these authors, CP159 strain produces both pullulan, as previously found (Corsaro et al., 1998), and another exopolysaccharide, built up by galactose, mannose and, in a minor extent, rhamnose. The latter polysaccharide represents less than 5% of the polysaccharides produced by CP159. According to the evidence by Molinaro et al. (2002), we have also found that the hypovirulent strain CP263 produces only a polysaccharide constituted by glucose alone. The absence of starch in the sample, the literature evidence and the analysis of the bonds between sugars (see below) indicated that this polysaccharide was pullulan.

On the contrary, the strain of *C. parasitica* obtained in laboratory, i.e. CP102, also produced another polysaccharide in addition to pullulan. Because of its composition in sugars (Table 2), very likely it was the polysaccharide characterised by Molinaro et al. (2002). Should this be true, we recommend caution in considering the presence of this polysaccharide as a certain indication of virulence of the fungal strain, as stated by Molinaro et al. (2002), who suggest that the presence of this polysaccharide is an indication of virulence of the fungal strains. The findings here presented do not support this hypothesis. Strain CP102, a cultural variant, has a very low virulence and, in addition, it was never isolated from naturally infected chestnut plants.

The extracellular microbial polysaccharide released into the culture medium might be related to the morphological state, genomic variability, cultural characteristics, and growth conditions. Besides glucose or sucrose, the culture of *A. pullulans* is able to consume mannose, galactose, fructose, and other sugars as carbon source (Ueda, Fujita, Komatsu, & Nakashima, 1963). Pullulan production by virulent and hypovirulent strains of *C. parasitica* follows the same bioconversion of *A. pullulans*. The yield of pullulan strongly depends on the carbon source. The pathways of pullulan production, apart from direct conversion of glucose or sucrose residues into the polysaccharide, may involve polymerization of the carbohydrate precursors stored inside the cells (Shingel, 2004).

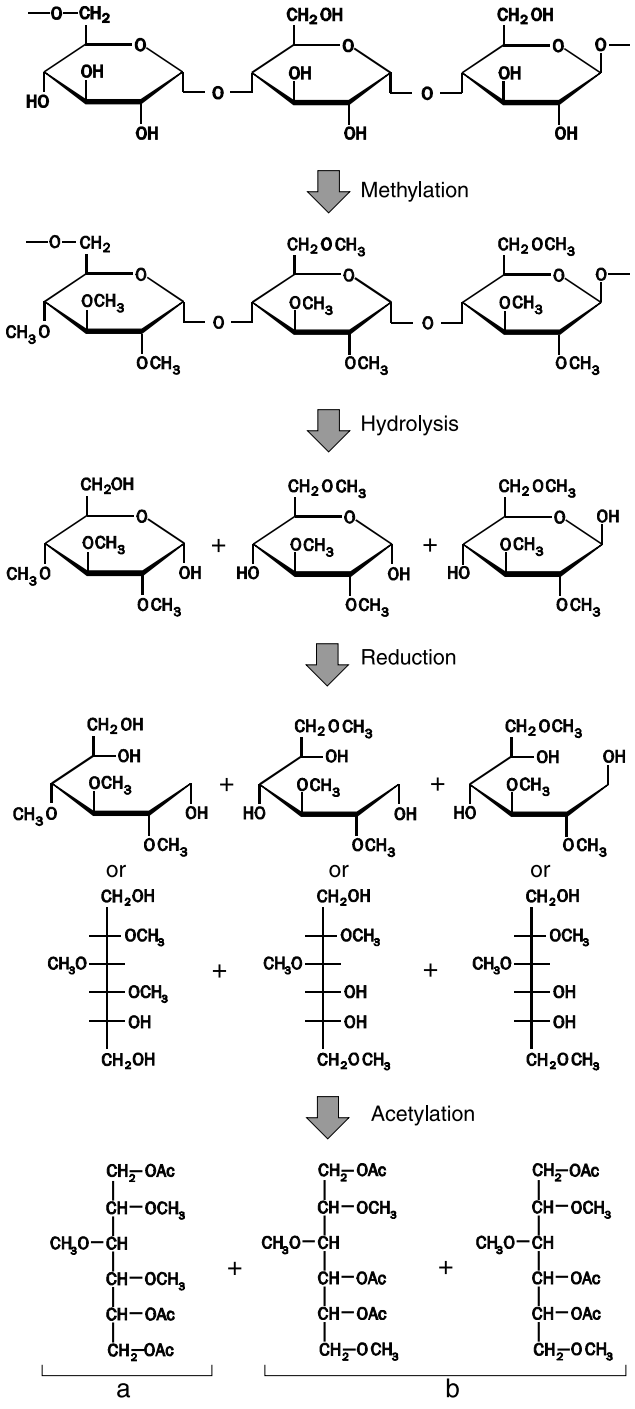
Proteins were found in all samples (Table 2). In turn, this fact deserves two comments. First, the protein fraction was significantly lower than found with EPS produced by other fungal species, such as those responsible of esca syndrome of grapevine (Bruno, Forabosco, Delben, Liut & Sparapano, 2003). Only in CP159loG the percentage of proteins was found to be high (6%). Secondly, it has to be said that for some samples the proteins where also checked before the treatment with hydrolytic enzymes. In all cases, it was found that the treatment with enzymes destroyed about one half of the proteins present in the polysaccharidic fraction. This means that the amount of proteins contained in the polysaccharidic fraction was also low before the treatment with enzymes and, on the other hand, that the strong hydrolytic treatment was unable to free the material from proteins. Therefore, it was suspected that proteic material was covalently bound to the polysaccharides. The presence of proteins bound to the polysaccharidic matrix could be involved in the formation of a glycoprotein which may be associated with the adhesion process of the fungus as it was suggested by Kwon & Epstein (1997) for *Nectria haematococca*, whose microconidia produced a 90 kDa glycoprotein representing a fungal glue. In our case, the role of proteins in the exocellular material was not elucidated so far and is being studied matter.

3.3. Structure of pullulan(s)

In the present work, the polysaccharidic fraction constituted by glucose only, i.e. pullulan, was taken into account for the study of the bonds between the sugars of the chain. This because Molinaro et al. (2002) already elucidated the structure of the 'complex exopolysaccharide' produced by CP159, grown on mineral Czapek medium amended with potato infusion. In addition, the scarce amount of the complex exopolysaccharide available prevented further investigations. However, the presence of particularly high fractions of the tetramers in the structure of the pullulans produced by the three strains of *C. parasitica* was the main reason for our choice.

The analysis of the bonds was made by methylation of the polysaccharide, hydrolysis of the methylated derivative, acetylation of the hydrolysate and subsequent gas chromatographic (GC) analysis of the final products.

Before proceeding with the analysis of our polysaccharide, the protocol just mentioned was applied to maltotriose and to commercial pullulans. The GC analysis of the volatile derivatives of maltotriose allowed us to locate the gaschromatographic peak of 1,4,5-three-*O*-acetyl-2,3,6-three-*O*-methyl exytol, while with the GC analysis of the volatile derivatives of pullulan 1,5,6-three-*O*-acetyl-2,3,4-three-*O*-methyl exytol was also detected. The ratio of the areas under the peaks due to these derivatives should corresponds to the relative amount of α-(1→4) and α-(1→6) linkages (Scheme 1) and should be 2:1 for the commercial pullulan, as confirmed by NMR analysis (Delben, Forabosco, Guerrini, Liut & Torri, in press). As a matter of fact, the ratio was found to be in the range from 2.3 to



Scheme 1.

2.4, and this value divided by 2 was used as the correction factor *f* between the peaks of the named derivatives in the subsequent gaschromatographic measurements.

The same scheme of derivatisation was applied to the pullulan(s) obtained from *C. parasitica*. The values of the ratio by the peaks of 1,4,5-three-*O*-acetyl-2,3,6-three-*O*-methyl exytol and 1,5,6-three-*O*-acetyl-2,3,4-three-*O*-methyl exytol, corrected by *f*, gave the results reported in Table 3. They are of particular interest for two reasons. First, in the case of CP159loG the percentage of the maltotetraose structure in the pullulan sample is 89, that is much higher than the maximum value of 7 so far indicated in the literature (Catley et al., 1986). Secondly, the percentage of maltotetraose strongly depends on the strain of *C. parasitica* and, for the same strain, also varies for different cultures. From our data, it could be stated that the pullulan produced by the virulent strain CP159 was particularly rich in maltotetraose units. However, the percentage of maltotetraose units in the pullulan(s) produced by all three strains of *C. parasitica* was much higher than so far observed in such glucans (Table 3).

Table 3
Methylation analysis of pullulans from *Cryphonectria parasitica*: results of the GC analysis

Sample	Area of peak 1, A1 (arbitrary units)[a]	Area of peak 2, A2 (arbitrary units)[b]	A1/*f*×A2[c]	% of maltotriose sequences
CP159loG	391,724	116,841	2.89	11
CP159loS	620,706	187,706	2.75	25
CP263upG	739,986	254,131	2.42	58
CP102loS	816,673	271,906	2.50	50

[a] Peak of 1,4,5-three-*O*-acetyl-2,3,6-three-*O*-methyl exytol.
[b] Peak of 1,5,6-three-*O*-acetyl-2,3,4-three-*O*-methyl exytol.
[c] *f* is the correction factor (see text).

3.4. Molecular weights

GPC was applied to the pullulans both for testing the weight average molecular weight and the molecular weight distribution, and for separating different fractions for subsequent analysis. The complete set of determinations performed is in Table 4. The following results are worth pointing out.

All the samples studied showed a large distribution of the molecular weights, that, by and large, mostly ranged from 10^4 to 10^6 g mol^{-1}. In all cases, the chromatographic profiles showed one to three small peaks at M_w well below 10^4 g mol^{-1}. The amount of material there contained was too scarce for subsequent analysis. These peaks are not mentioned in Table 4. With the sample CP159loG, the GPC produced an accumulation of proteins and possibly of the complex exopolysaccharide in the fraction named C, i.e. in the fraction containing the material with relatively low M_w. An accumulation of the complex exopolysaccharide was also suspected for CP102loS, because fraction A appeared richer in galactose and mannose than the starting material, described in Table 2. In this case, the possible accumulation was in the fraction at higher molecular weights.

The average molecular weights were also determined with MALLS and with capillary viscosimetry measurements. The MALLS measurements were performed on some fractions obtained by GPC, while viscosimetry was applied to the samples before the GPC separation. Keeping this in mind, a good agreement between the values obtained with the different techniques was obtained (Table 4). Moreover, the molecular weights values were very close for all samples analysed, the viscosimetric value of M_w for CP159loG being the only exception. Indeed, the $[\eta]$ values are the true experimental

Table 4
Characterisation of GPC fractions of exopolysaccharides produced by strains of *Cryphonectria parasitica* and commercial pullulans

Sample[a]	GPC fraction	Weight (mg)[b]	Proteins (%)[c]	Glc: Gal: Man[d]	$[\eta]$ (dL g^{-1})	Average M_w (g mol^{-1}/1000) GPC	MALLS	Viscosity[e]
CP159loG 82.14	A	20.31	0.5	1: 0.02: 0	2.03	700–1000		1036±26
	B	41.34	1	1: 0.01: 0.01		100–500	130±15	
	C	5.78	29	1: 0.3: 0.5		<10		
CP159loS 129.86	A	99.42	<0.5	1: tr: tr[f]	0.65	40–500	70±10	185±3
	B	<0.05				<10		
CP263upG+	A	7.98	1	1: 0: 0	0.80	1000		252±8
CP263loG[g]	B	106.46	1	1: 0: 0		50–500	255±26	
161.40	C	3.82		1: 0: 0		<10		
CP263loS 76.66	A	65.93	<0.5	1: 0: 0	0.36	10–100		75±5
	B	<0.05		1: 0: 0		<10		
CP102loS 160.36	A	39.07	2	1: 0.06: 0.06	0.16	10–30		22±3
	B	<0.05				<10		
Hayashibara pullulan	A		0	1: 0: 0	0.89	800–1000		293±4
	B					50–350		
Sigma pullulan	A			1: 0: 0	0.45	20–200	128±15	104±2

[a] The number below refers to the weight of freeze-dried material analysed (mg).
[b] Weight of the polysaccharide obtained after freeze-drying of the fraction.
[c] Weight percentage of proteins in the fraction.
[d] Molar ratio between glucose (Glc), galactose (Gal) and mannose (Man) in the fraction.
[e] Average values; see text.
[f] tr, Gal and Man present in traces.
[g] For the subsequent characterisations, CP263upG and CP263loG were put together, because the GPC profiles were coincident.

results of the viscosimetric measurements, because the derived molecular weight would be correct only if a good choice of the Mark–Houwink–Sakurada parameters is made. Accordingly, it has to be stated that CP159loG showed a particularly high $[\eta]$ value.

Buliga & Brant (1987b) have shown that the α-(1→6) links between the glucose residues are responsible for the flexibility of the polymer chain of pullulan. This was recently confirmed by Angioletti (2003). A significant enhancement in the rigidity of the chain must be expected as a consequence of lowering the relative number of these bonds along the chain. Being CP159loG exceptionally rich in maltotetraose units, its polymeric chain should be significantly less flexible than for pullulan built up by maltotrialose units only, because this structure, that is the most common for the pullulans, is the richest in α-(1→6) links among all possible structures. This explains the high value of $[\eta]$ found, being our sample particularly poor in α-(1→6) links, and, on the other hand, indicates that the Mark–Houwink–Sakurada parameters used to derive the molecular weights from the values of $[\eta]$ should not be corrected in the case of CP159loG, whose viscosimetric M_w value reported in Table 4 is possibly overestimated.

On passing from CP159loG to CP159loS, the lowering in the fraction of maltotetraose units, reported in Table 4, appears to be very effective in enhancing the chain flexibility, so that the Mark–Houwink–Sakurada parameters found in the literature could be correctly applied to this sample.

Finally, it was observed that the aqueous solutions of CP159loG were not only particularly viscous, but also showed an unusual foaming ability. More interestingly, from 3 g L^{-1} water solution a flexible and appreciably resistant film was obtained.

3.5. Biological activity of pullulan(s)

When detached leaves or cuttings or twigs of chestnut absorbed purified pullulan(s) produced by each fungal strain of *C. parasitica* at low concentrations in water, symptoms appeared on the foliar lamina and on the petiole, i.e. browning and collapse of the leaf tissues, which soon desiccated. The symptoms developed within 10 days on chestnut leaves and within 14–40 days on chestnut twigs (Sparapano & Bruno, 1998; Corsaro et al., 1998). The upper fractions or the lower fractions recovered by liquid culture of strains CP159 and CP263, assayed on leaves or whole seedlings of chestnut, caused similar symptoms, i.e. wilting and dieback. The lower fractions of strain CP102 affected the cuttings or the whole chestnut plants by causing only leaf browning and reddening.

Spores of many plant pathogenic fungi secrete non-specific glues onto the external surfaces of plants before penetration the host. Because many fungal spores are dispersed in water, and most phytopathogenic fungi only germinate in free water, fungal glues produced by spores and germlings must spread on the plant substratum surface and displace water molecules at the interface. However, the glue components are probably exocellullarly modified or polymerised into a water insoluble glue. Firm attachment of fungal pathogens to the host surface is thought to be an essential prerequisite to normal prepenetration development and successful infection. Among plant pathogens, mucilaginous secretion from spores, germ tubes, and appressoria are believed to act as a glue enabling the pathogen to adhere to the plant surface. *C. parasitica* is a wound parasite requiring a penetration to the inner bark as an infection site. Hyphae penetrate the sapwood trough rays, and micelial fans spread in the cambial layer, forming the canker. Conduction in

the xylem is disrupted through tylosis formation by parenchyma adjacent to the vessels. Death of the stem distal to a canker is due to girdling and to translocation of phytotoxic compounds to that site. Pullulans could be the phytotoxic agents.

Pullulans could play a double role in pathogenesis: as toxic compounds, they easily move into the infected plants and cause toxicity, and as mucilaginous substances, they improve fungal attachment to the host tissues.

4. Conclusions

The EPS produced by three strains of the phytotoxic fungus *C. parasitica*, having different grade of virulence, were proved to be built up by almost only glucose, with a possible presence of galactose and mannose in small amount, as already found. No amylose was found in the sample. Linkage analysis revealed that the more abundant polysaccharide had the general structure of pullulans. Contrary to what believed so far, the percentage of tetraose subunits in the pullulan chain ranged from 42 (pullulan from CP263) to 89% (pullulan from CP159).

The higher the virulence of the pullulan producing strain, the higher was the tetraose fraction in the pullulan chain. It is no proved that a correlation between these parameters exists. However, one could suggest to keep this evidence in mind as a possible integration to the interesting hypothesis by Molinaro et al. (2002) about the possible correlation between the kind of polysaccharides produced by different fungal strains and their virulence. Our data, however, showed that the cultural variant and hypovirulent strain CP102, which has been never isolated from naturally infected chestnut plants, also synthesized the exopolysaccharide described by Molinaro et al. (2002). This finding may not support the proposal for correlating the exopolysaccharide production to the virulence of fungal strains. It is more acceptable that the great variety of environmental conditions as well as variability in strain characteristics influences the metabolic pathways of the pullulan(s) formation and affects the structural composition of these biopolymers.

In view of the effect of the structure on the properties of the pullulans here described, studies of their physico-chemical and mechanical properties for special applications in the food industry or biomedical sciences are suggested. In fact, the possible different composition of the pullulans produced by *C. parasitica* corroborates the suggestion by Corsaro et al. (1998) of the industrial production of polysaccharides by these strains of fungi for interesting applications. At authors' knowledge, genetically modified strains of these fungi have not yet applied for industrial productions. Pullulan production by phytopathogenic fungi as *C. parasitica*, *P. chlamydospora* and *P. aleophilum* could be very useful in understanding the role of pullulan by an ecological point of view and in plant pathogenesis and symptom expression of chestnut canker disease and esca syndrome of grapevine (Sparapano & Bruno, 1998; Sparapano et al., 2000).

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