POLYGALACTURONASE PRODUCTION BY *ASPERGILLUS NIGER*: EXPRESSION IN ONION SEEDS AND POSSIBLE INVOLVEMENT IN VIRULENCE

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SUMMARY

Nine Aspergillus niger isolates differing in virulence were assayed for polygalacturonase (PG) content and isoenzyme pattern in spores, liquid cultures and inoculated onion seeds of cultivar 'Bianca Agostana'. The isolates differed in PG production quantitatively and in terms of isoenzyme pattern. Isolates An1 and An5, characterised by low PG production in liquid culture and low virulence, lacked detectable levels of three PG isoforms (PG3, PG4 and PG5). One acidic PG band (PG6) was detected only during growth in vitro by most of the isolates. Pectin lyase activity was not detected from spores, liquid cultures, or infected seeds. The ability of three isolates differing in virulence, An6, An7 and An14 to colonise five onion cultivars was also tested. The isolates differed in virulence and in quantitative and qualitative PG production. The results suggest that particular PGs from A. niger may contribute to virulence during onion seed colonisation.

RIASSUNTO

POLIGALATTURONASI PRODOTTA DA *ASPERGILLUS NIGER*: SUA ESPRESSIONE NEI SEMI DI CIPOLLA E POSSIBILE IMPLICAZIONE NELLA VIRULENZA. Nove isolati di Aspergillus niger, caratterizzati da diversa virulenza, sono stati saggiati per la loro capacità di esprimere poligalatturonasi (PG) nelle spore e di produrre l'enzima nel liquido colturale e nei semi di cipolla (cultivar "Bianca Agostana") inoculati artificialmente. Gli isolati hanno dimostrato di differire sia per la quantità di PG prodotta, sia per i profili isoenzimatici. Gli isolati An1 e An5, caratterizzati da una bassa produzione di PG, non hanno escreto nel liquido colturale quantità rilevabili di tre isoforme di PG (PG3, PG4, PG5). Una isoforma acidica (PG6) è stata rilasciata dalla maggior parte degli isolati unicamente in vitro. Per tutti gli isolati non è stata rilevata presenza di pectin liasi (PNL) nelle spore, nel liquido colturale e nei semi di cipolla infetti. Per i tre isolati, An6, An7 e An14, è stata confrontata la capacità di colonizzare i semi di cinque cultivar di cipolla; questi isolati hanno dimostrato di possedere, accanto ad una differente virulenza, una diversa capacità di produzione quali/quantitativa di PG. I risultati evidenziano che particolari isoforme di PG prodotte da A. niger possono contribuire alla virulenza durante la colonizzazione dei semi di cipolla.

Key words: Aspergillus niger, onion, polygalacturonase, isoenzymes, virulence.

INTRODUCTION

Aspergillus niger van Tieghem is a seed and soilborne pathogen that causes black mould of onion, one of the most serious disease caused by this fungus. The disease commonly occurs on onions stored at high ambient temperatures (Hayden and Maude, 1992); symptoms of the disease are also severe on young seedlings, with rapid and extensive tissues degradation. Contaminated seeds and soil appear to constitute the principal inoculum source (Kaul, 1973; Gupta et al., 1984; Hayden and Maude, 1992; Hayden et al., 1994; Köycü and Özer, 1997).

Pectolytic enzymes produced by A. niger are used extensively in the food industry as processing aids for extraction, clarification, and maceration. Among these enzymes attention has been focused mainly on the optimisation of industrial polygalacturonase (PG) production (Friedrich et al., 1989; Peričin et al., 1992; Acuña-Argüelles et al., 1995; Berovič and Ostroveršnik, 1997). Commercial preparations of purified PG isoenzymes have also been characterised biochemically (Cooke et al., 1976; Kester and Visser, 1990). Despite these extensive studies, to our knowledge the production of pectolytic enzymes by A. niger during pathogenesis in onion has not been reported.

In the present study we have analysed the content and the isoenzyme pattern of pectolytic enzymes ex-

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pressed in ungerminated spores, in liquid culture and on inoculated onion seeds using nine A. niger isolates differing in virulence. We have also investigated the relationship between the ability of three isolates to infect five onion cultivars and pectolytic enzyme production during seed colonisation.

MATERIALS AND METHODS

Culture of the fungus. Nine isolates of A. niger differing in virulence An1, An5, An6, An7, An9, An10, An11, An12, An14, obtained from onion seeds from different regions in Turkey (Özer and Köycü, 1997) were used. Fungal cultures were maintained on potato-dextrose agar (PDA) (Oxoid Ltd, Basingstoke, England). For enzyme preparation, isolates were surface-cultured in Czapek's liquid medium (pH 5.0) containing 1% (w/v) citrus pectin (Sigma Chemical Co., St. Louis, USA) as sole carbon source. The inoculum was one agar disc (6 mm diameter) cut from the edge of 3 day-old cultures on PDA. The cultures were grown at 25°C in 250 ml Erlenmeyer flasks containing 50 ml medium.

Plant material and inoculation procedure. Onion (Allium cepa L.) seeds of the red cultivars 'Rossa Savonese' and 'Rossa Toscana' and white cultivars 'Bianca Agostana', 'Bianca Musona' and 'Texas Early Grano 502' were obtained from local markets. Ability of the A. niger isolates to colonise different onion cultivars and produce pectolytic enzymes in vivo was determined in two sets of experiments. In the former, 'Bianca Agostana' seeds were inoculated with the nine A. niger isolates to determine their virulence, pectolytic enzyme production and certain biochemical characteristics. In the latter, the response of the onion cultivars to An6, An7 and An14 was assessed. Inoculation followed the procedure described by Hayden et al. (1994) with some modification. Seeds were surface-sterilised by immersion in a solution of sodium hypochlorite, rinsed in sterile distilled water and dried on absorbent paper. Spore suspensions containing 1 x 10^7 spores ml^{-1} were prepared from 7 day-old cultures grown on PDA. To inoculate, seeds of each cultivar were soaked in the spore suspension for 12 h and then dried on absorbent paper. As a control sterilised water was used instead of the spore suspension. Two hundred inoculated seeds of each cultivar were placed in Petri dishes (twenty-five seeds of each lot per dish) on sterilised absorbent paper moistened with sterile distilled water. The dishes were incubated for five days at 25°C in the dark. Colonies that developed on the seeds were examined under a stereo microscope and identified by the appearance of their conidiophores. The incidence of A. niger on inoculated samples was expressed as percentage of seeds colonised. To check for the absence of contaminants, some infected seeds were aseptically placed into Petri dishes containing PDA, but only A. niger grew under these conditions.

Enzyme extraction from fungal cultures and inoculated onion seeds. 3 day-old cultures were collected and the mycelium was removed by filtration using a Büchner funnel. The culture filtrates were centrifuged at 15,000 ***g*** for 15 min at 4°C and the supernatants were dialysed against several changes of distilled water at 4°C. The enzyme preparations from inoculated onion seeds were obtained by grinding infected tissues in an ice-cooled mortar in 0.05 M Tris-HCl buffer pH 7.8 (1 g tissue ml^{-1} buffer) containing 0.1 M KCl, 0.5% (w/v) cysteine and 1% (w/v) insoluble polyvinylpolypyrrolidone (Sigma Chemical Co., St. Louis, USA). The mixture was then strained through three layers of cheesecloth, centrifuged at 15,000 ***g*** for 20 min at 4°C and dialysed against several changes of distilled water at 4°C. The same procedures were applied to control seeds.

Enzyme extraction from spores. Two week-old cultures of each isolate on PDA were flooded with sterile distilled water, and a glass rod was used to remove spores gently from the surface of the cultures. The spores were then collected and twice washed with sterilised distilled water by centrifugation at 3500 ***g*** for 20 min at 4°C. The pellet was suspended in 5 ml of 0.1 M Na-acetate buffer pH 5.0 giving a spore concentration of approximately 5 x 10^7 spores ml^{-1}, ground in an ice-cooled mortar for 15 min and centrifuged at 15,000 ***g*** for 20 min at 4°C. The supernatant was collected, dialysed against several changes of distilled water at 4°C and filter-sterilised through a 0.22 µm membrane (Millipore, Bedford, USA). The supernatant was then quickly frozen and lyophilised. The dried residue was redissolved in a minimal amount of distilled water.

Pectolytic enzyme assays. PG activity was determined as the increase of reducing end-groups over time. Reducing end-groups were measured by the method of Nelson (1944), using D-galacturonic acid (Sigma Chemical Co., St. Louis, USA) (GA) as a standard. Activity was expressed as reducing units (RU). One RU was defined as the amount of enzyme produc-

ing at 35°C 1 µmol of reducing groups min^{-1} from 0.25% (w/v) polygalacturonic acid (PGA) (Sigma Chemical Co., St. Louis, USA) or pectin from citrus (Sigma Chemical Co., St. Louis, USA) in Na-acetate buffer (0.1 M, pH 5.0). PG activity was also measured viscometrically, using Cannon-Fenske viscometers size 200 maintained in a thermostat (Haake F3, Karlsrhue, Germany) at 35°C. Reaction mixtures consisted of 1 ml culture filtrate and 5 ml of 2% PGA in 0.1 M Na-acetate buffer, pH 5.0. Enzyme activity was expressed in viscometric units (VU). 1 VU=1000/t where t is the time in minutes required for a 50% decrease in relative viscosity of the reaction mixture. Mode of action of PG activity was determined according to Bateman and Basham (1976) by measuring end-reducing groups present in the reaction mixture at t time. Controls were always used for each sample. For reaction product determination, samples of 50 µl solution at the end of viscometric assays were chromatographed on silica gel plates (Merck, Darmstadt, Germany) and developed with butanol-formic acid-water (3:2:1) at room temperature. GA was used as a reference compound and comparable reaction mixtures without enzymes were co-chromatographed and compared for the possible presence of free sugars in the reaction mixture. Plates were sprayed with aniline-diphenylamine (Sigma Chemical Co., St. Louis, USA) (Zweig and Sherma, 1972). Products released during enzymatic depolymerisation of PGA appeared as blue spots and they were evaluated as dimer, trimer, or tetramer in comparison to the monomer GA as reference standard.

Pectin lyase (PNL) activity was assayed spectrophotometrically by measuring the increase of absorbance at 235 nm. An increase in absorbance of 1.73 indicated the formation of 1 µmol of unsaturated uronide (Zucker and Hankin, 1970). One unit of enzyme activity catalysed the formation of 1 µmol of unsaturated uronide min^{-1} from 0.25% (w/v) citrus pectin in Tris-HCl buffer (0.1 M, pH 8.5) at 35°C.

Isoenzyme identification. Isoenzyme separation by isoelectric focusing (IEF) was performed horizontally on Multiphor II apparatus (Pharmacia Biotech, Uppsala, Sweden) by using 0.4 mm thick polyacrylamide gels containing 5% (v/v) ampholytes (Pharmacia Biotech, Uppsala, Sweden) covering the pH range 3.5-10.0. The gels were run at a constant power of 5 W for 1.5 h. After IEF, gels were overlaid with ultrathin (0.4 mm) agarose gels prepared as described by Ried and Collmer (1985). For PG isoenzyme detection, 1% (w/v) agarose gel containing 0.1% (w/v) PGA and 10 mM EDTA, buffered at pH 5.0 with 50 mM Na-acetate. The 1% (w/v) agarose gel for PNL detection contained 0.1% (w/v) pectin in 50 mM Tris-HCl buffer, pH 8.5. IEF polyacrylamide gels overlaid with ultrathin agarose gel were incubated at 100% humidity for 30 to 60 min at 35°C. Activity bands were visualised by staining the agarose overlay for 10 min in 0.05% (w/v) ruthenium red (Sigma Chemical Co., St. Louis, USA), followed by rinsing in distilled water. The pI values of pectolytic isoenzymes were estimated from a regression equation of standard proteins (Pharmacia Biotech, Uppsala, Sweden) versus the distance migrated.

RESULTS

Production of pectolytic enzymes from fungal cultures, extraction from spores, from inoculated onion seeds and percentage of seed infection. The nine A. niger isolates produced PG when grown on liquid medium containing pectin as the sole carbon source and, apart from isolate An5, also in infected onion seeds, cv. 'Bianca Agostana' (Fig. 1). PG production from isolates An1 and An5 was lower than that of more aggressive isolates, both when growing on onion seeds and in liquid medium. PG activity was also found in extracts from spores of all isolates (Fig. 2), indicating that it can be expressed in a constitutive manner.

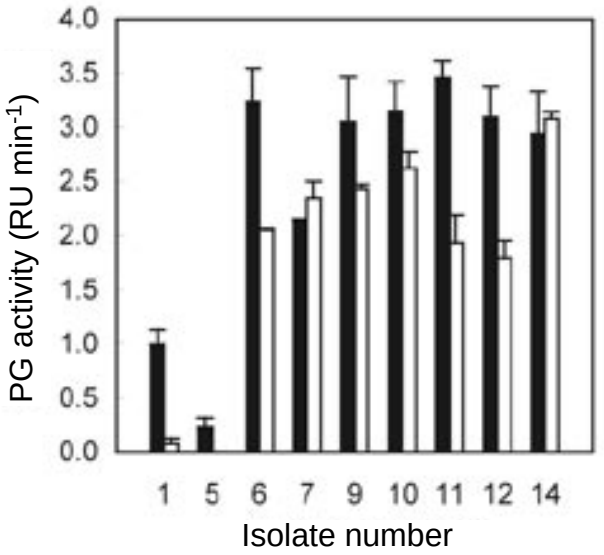

Fig. 1. Polygalacturonase activity produced by the nine A. niger isolates on liquid medium (■) and on inoculated seeds of onion cultivar 'Bianca Agostana' (□). One reducing unit (RU) is defined as the amount of enzyme producing 1 µmol of reducing groups min^{-1} at 35°C from 0.25% (w/v) polygalacturonic acid in Na-acetate buffer (0.1 M, pH 5.0). Data are means of three replicates with vertical bars showing the standard error.

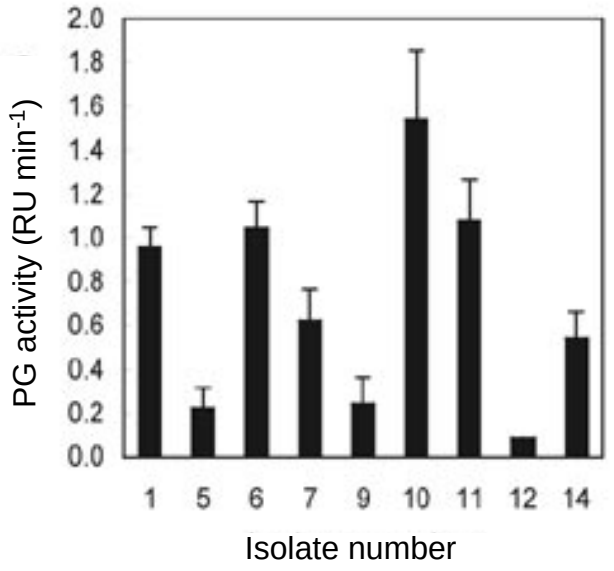

Fig. 2. Polygalacturonase activity detected from spores of A. niger. One reducing unit (RU) is defined as the amount of enzyme producing 1 µmol of reducing groups min^{-1} at 35°C from 0.25% (w/v) polygalacturonic acid in Na-acetate buffer (0.1 M, pH 5.0). Data are means of three replicates with vertical bars showing the standard error.

PNL activity was not detected in spores or culture filtrates or in inoculated seeds.

Virulence of the isolates was determined by inoculating 'Bianca Agostana' seeds as detailed in Materials and Methods. After inoculation at 25°C for 5 days the percentage of seeds colonized was determined. Most of the seeds where the fungus developed were not able to germinate. The percentage of infection of inoculated seeds ranged between 80 and 100, An1 and An5 being the less aggressive. The correlation between percentage of seed infection and PG-producing ability by isolates in liquid culture or on onion seeds was not significant; however, An1 and An5, characterised by the lowest PG producing ability in culture and in vivo, also showed the lowest level of virulence (data not shown).

The ability of three A. niger isolates An6, An7, and An14 to colonise onion seeds of different cultivars, and associated PG production, was also analysed. The cultivars tested were differently susceptible to the three isolates, except that 'Bianca Agostana' was the most susceptible to all three isolates. Susceptibility of other cultivars depended on the isolate inoculated: An6 was more aggressive on 'Texas Early Grano 502', An7 on 'Bianca Musona' and An14 on 'Rossa Toscana'. 'Rossa Savonese' displayed low susceptibility to all three isolates (Table 1). No PG activity was observed from control seeds.

Characteristics of pectolytic activity. PG activity from all isolates was optimal at 40°C and pH 4.0.

The percentage of relative activity on pectin, as compared to PGA of the PG complex differed depending on the isolate tested. The percentage of relative PG activity on pectin was also higher for enzyme extracted in vivo than grown in vitro (Fig. 3).

Data on percentage of end-reducing groups at time t and reaction products showed that isolates grown on liquid culture had typical endo-activity except An7 which displayed exo-PG activity (Table 2). In contrast, all isolates produced exo-PG on inoculated onion seeds (Table 3).

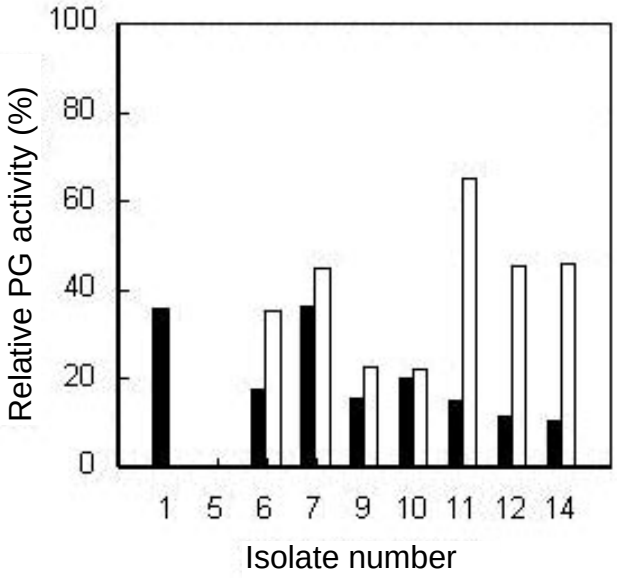

Fig. 3. Percentage of relative polygalacturonase activity on pectin as compared to polygalacturonic acid from A. niger isolates produced on liquid medium (■) and on inoculated seeds of onion cultivar 'Bianca Agostana' (□). Data are expressed as percentage of maximum values.

Polygalacturonase isoenzyme patterns. Extracts of the nine isolates from ground spores, culture filtrates and inoculated onion seeds were separated by thin layer polyacrylamide gel IEF and evaluated for their PG isoenzyme pattern.

Five PG bands, PG1 (pI 6.2), PG2 (pI 5.9), PG3 (pI 5.5), PG4 (pI 5.2), PG5 (pI 4.9) were detected from spores of isolates An6, An7, An9, An10, An11, An12 and An14 (Fig. 4a). PG5 was generally resolved as a faint band. An1 and An5 produced only a faint PG1 band.

Isolates An6, An9, An10, An11, An12, An14 produced two major isoforms PG1, PG2 and an acidic

Table 1. Polygalacturonase activity and percentage of infection (% I) of seeds of different onion cultivars after inoculation with isolates An6, An7, An14 of A. niger. One reducing unit (RU) is defined as the amount of enzyme producing 1 µmol of reducing groups min^{-1} at 35°C from 0.25% (w/v) polygalacturonic acid in Na-acetate buffer (0.1 M, pH 5.0). Values are expressed as means of three replicates.

Cultivar	An6		An7		An14	
	RUs	% I	RUs	% I	RUs	% I
Bianca Agostana	2.07	100.0	2.43	95.0	3.08	85.0
Bianca Musona	0.16	48.2	1.45	80.4	0.32	27.3
Rossa Savonese	2.75	30.5	2.72	28.5	2.88	21.5
Rossa Toscana	3.49	68.0	1.37	55.3	1.95	31.6
Texas Early Grano 502	2.86	78.8	3.19	57.1	1.25	22.9

Table 2. Pattern of hydrolysis and polygalacturonase reaction products produced by the nine A. niger isolates in liquid culture.

Isolates	VU ml^{-1}	Reducing groups %	Reaction products[b]		
			GA	DI-GA	TRI-GA
An1	33.3	2.2	+	+	+
An5	20.0	2.3	+	+	+
An6	50.0	0.9	+	+	–
An7	50.0	3.8	+	–	–
An9	12.5	0.3	+	+	+
An10	12.5	0.7	+	+	–
An11	10.0	0.8	+	+	+
An12	11.1	0.4	+	+	+
An14	12.5	0.7	+	+	+

a: visicometric units: 1000/T where T is the time in min for 50% reduction in viscosity of polygalacturonic acid. – b: reducing groups formed (at the time of 50% loss in viscometry of polygalacturonic acid) 100 being those that would be formed if all the pectic substrate was hydrolysed. – c: reaction products released in reaction mixtures; GA galacturonic acid; DI-GA, dimer; Tri-GA, trimer; –, no spot; +, spot.

Table 3. Pattern of hydrolysis and polygalacturonase reaction products produced by the nine A. niger isolates in infected seeds, cultivar 'Bianca Agostana'.

Isolates	VU ml^{-1}	Reducing groups %[a]	Reaction products[b]		
			GA	DI-GA	TRI-GA
An1	n.d.[c]	n.d.	n.d.	n.d.	n.d.
An5	0.0	4.6	+	–	–
An6	50.0	8.1	+	–	–
An7	66.6	13.9	+	–	–
An9	66.6	8.7	+	–	–
An10	66.6	11.9	+	–	–
An11	33.3	14.8	+	–	–
An12	50.0	10.2	+	+	–
An14	40.0	16.6	+	–	–

a: visicometric units: 1000/T where T is the time in min for 50% reduction in viscosity of polygalacturonic acid. – b: reducing groups formed (at the time of 50% loss in viscometry of polygalacturonic acid) 100 being those that would be formed if all the pectic substrate was hydrolysed. – c: reaction products released in reaction mixtures; GA galacturonic acid; DI-GA, dimer; Tri-GA, trimer; –, no spot; +, spot. – d: not determined.

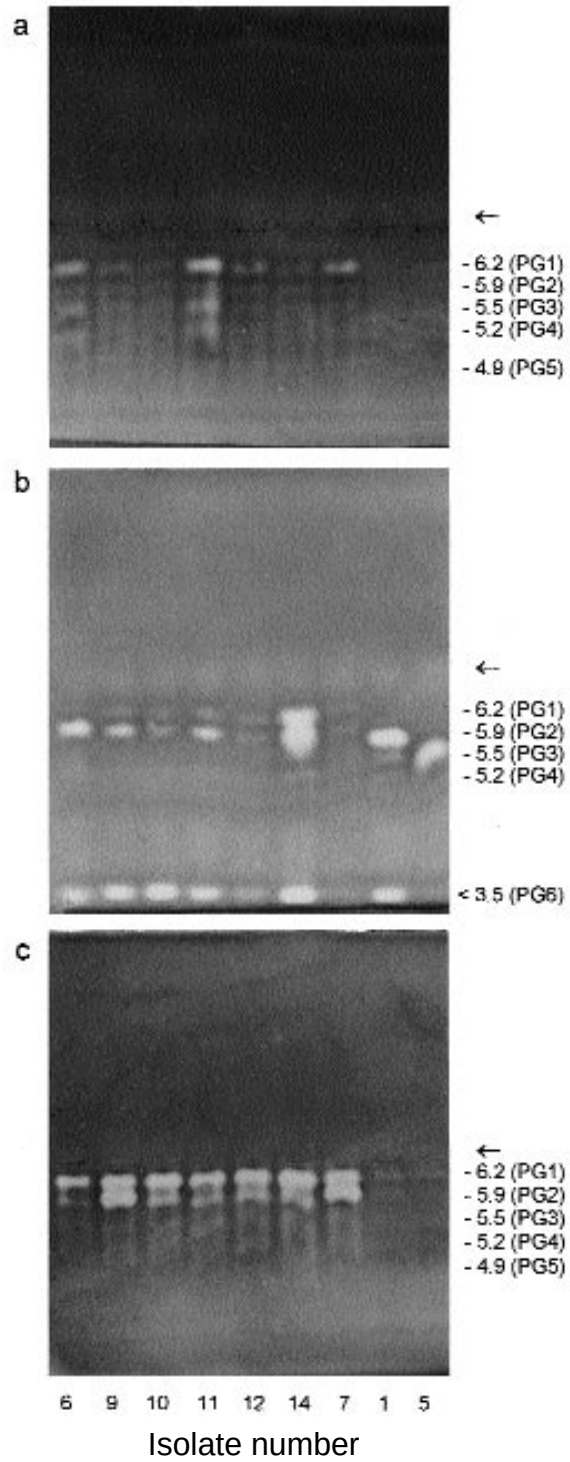

Fig. 4. Polygalacturonase isoenzyme pattern of nine isolates of A. niger from spores (**a**), from liquid cultures (**b**) and from inoculated onion seeds of cultivar 'Bianca Agostana' (**c**). Sample were separated on an isoelectric focusing (IEF) gel (pH 3.5-10.0), followed by agarose overlay activity staining. Position of pI values and polygalacturonase bands is indicated on the right. Arrow indicates where the sample was applied.

extra-band (PG6, pI < 3.5) in liquid culture (Fig. 4b). An14 also expressed PG3, partially superimposed by PG2, PG4 and, very faintly PG5. An1 produced PG2, PG3, PG4 and PG6, An7 PG1 and PG2, An5 only PG3.

PG1 and PG2 were constantly present in extracts from onion seeds of 'Bianca Agostana' inoculated with the nine isolates (Fig. 4c); these isoforms appeared to be the major bands compared to the other bands (PG3, PG4 and PG5) characterised by a similar faint intensity. PG3, PG4 and PG5 were not detectable from seeds infected with An1 and An5.

Inoculation of 'Texas Early Grano 502' with the isolates An6, An7 and An14 did raise the production of 5 PG bands (PG1, PG2, PG3, PG4 and PG5); the same pattern was shown by 'Rossa Toscana' inoculated with An14. In 'Bianca Musona' seeds inoculated with An6, An7 An14 or in 'Rossa Toscana' seeds inoculated with An6 and An7, we observed four PG bands (PG1, PG2, PG3 and PG4), whereas only three bands (PG1, PG2 and PG3) were produced by the three isolates inoculated on 'Rossa Savonese' seeds (data not shown).

The inducible PG6 band that was expressed by most of the isolates in vitro was not found in the in vivo experiments.

PG bands detected in inoculated seeds appeared to be of fungal origin, since no bands were recorded in control tissues.

No PNL isoenzymes were detected from spores, culture filtrates or extracts from inoculated seeds

DISCUSSION

Multiple PG forms have previously been detected from commercial preparations of A. niger (Cooke et al., 1976; Kester and Visser, 1990; Stratilová et al., 1996). In the current report we show that PG isoenzymes are constitutively expressed in spores from isolates of A. niger obtained from naturally infected onions and most of them are produced during fungal invasion of inoculated onion tissues. The presence of PGs in ungerminated conidia suggests the possibility that these enzymes are involved in early stages of infection. One acidic form was also induced in liquid medium from most of the isolates tested. It has been reported that A. niger can produce PNL (Cooke et al., 1976; Acuña-Argüelles et al., 1995). Moreover, studies on the PNL molecular genetics of A. niger indicated the potential to produce PNLs by this fungus (Gysler et al., 1990; Harmsen et al., 1990; Kusters-van Someren et al., 1991). The lack of PNL production from the A. niger isolates tested in the present study might be due to the experimental conditions chosen for the in vitro growth of the isolates. However, the absence of PNL in spores and in extracts from infected onion seeds indicates that this enzyme is not a cell wall-depolymerisating factor during host tissue colonisation.

The isolates tested differed in PG production quantitatively, qualitatively and in virulence. Two isolates, An1 and An5 with the lowest PG production in liquid culture and a low ability to infect onion seedlings, during invasion lacked detectable levels of PG2, PG3 and PG4. These three forms were present in extracts from ungerminated spores and in tissue infected with the more aggressive isolates. This result suggests that these PGs may be determinants for the colonisation of onion tissues. Similarly, it has been found that aggressiveness of A. flavus isolates during infection of cotton bolls was correlated with their ability to secrete a specific PG (Cleveland and Cotty, 1991; Shieh et al., 1997). The isolates tested displayed a PG complex with endo-PG activity during in vitro growth apart from An7, which also exhibited exo-PG activity, as appears from the data on percentage of end-reducing groups and reaction products. Moreover, the isoenzyme pattern of An7 even from the in vitro colonisation lacked acidic PG6; this form may act as an endo-form, not expressed during seed colonisation. Experiments to characterise the purified forms for the catalytic mode of action will elucidate the possible role of PG6 and of the other PGs. In facts, An5 does not produce PG6, but is capable to express in vitro only PG3 with an apparent endo-mode of action.

A. niger is common as saprophyte in soil and on decaying vegetable matter, but occasionally causes crop diseases (Onions, 1966). The less aggressive isolates An1 and An5 with low PG production may be considered as saprophytes with little pathogenicity. The other isolates are more aggressive and secrete more PG, characterised by a more complex isoenzymatic pattern. It has been suggested that multiple forms of an enzyme can allow an organism greater flexibility in its pathogenicity (Magro et al., 1980; Keon et al., 1987). Also, the variation in virulence among different isolates is supported by differences in the PG isoelectric pattern as found in Botrytis cinerea (Di Lenna et al., 1981). It is possible that isolates forming A. niger population differ in virulence depending on the level of production of certain polygalacturonases.

Isolates An6, An7, An14 are able to colonise various onion cultivars and to produce polygalacturonases during invasion. The virulence and PG production of the isolates varied greatly depending on the cultivar, although for the presence of two major forms the isoenzyme patterns were highly similar. Our results demon-

strated different degrees of susceptibility among the cultivars, 'Bianca Agostana' being the most susceptible and 'Rossa Savonese' the most resistant. This finding indicates the possible presence or induction in onion of resistant factors that limit fungal invasion (Favaron et al., 1993; Cammue et al., 1995). Our results show that some PG isoforms produced during tissue invasion may be a pre-requisite for virulence.

ACKNOWLEDGMENTS

The research was supported by the Turkish Scientific and Technical Research Council (TÜBITAK) and by the National Research Council of Italy, Bilateral project on 'Phytopathological studies on the interaction onion-soft rot phytopathogenic fungi' (grant no. 98.02917. CT06).

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Received 1 July 1998
Accepted 6 October 1998

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