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ORIGINAL ARTICLE

In vitro growth and cell wall degrading enzyme production by Argentinean isolates of *Macrophomina phaseolina*, the causative agent of charcoal rot in corn

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KEYWORDS
Macrophomina phaseolina;
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Corn;
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Abstract *Macrophomina phaseolina* is a polyphagous phytopathogen, causing stalk rot on many commercially important species. Damages caused by this pathogen in soybean and maize crops in Argentina during drought and hot weather have increased due its ability to survive as sclerotia in soil and crop debris under non-till practices. In this work, we explored the *in vitro* production of plant cell wall-degrading enzymes [pectinases (polygalacturonase and polymethylgalacturonase); cellulases (endoglucanase); hemicellulases (endoxylanase) and the ligninolytic enzyme laccase] by several Argentinean isolates of *M. phaseolina*, and assessed the pathogenicity of these isolates as a preliminary step to establish the role of these enzymes in *M. phaseolina*–maize interaction. The isolates were grown in liquid synthetic medium supplemented with glucose, pectin, carboxymethylcellulose or xylan as carbon sources and/or enzyme inducers and glutamic acid as nitrogen source.

Pectinases were the first cell wall-degrading enzymes detected and the activities obtained (polygalacturonase activity was between 0.4 and 1.3 U/ml and polymethylgalacturonase between 0.15 and 1.3 U/ml) were higher than those of cellulases and xylanases, which appeared later and in a lesser magnitude. This sequence would promote initial tissue maceration followed by cell wall degradation. Laccase was detected in all the isolates evaluated (activity was between 36 U/l and 63 U/l). The aggressiveness of the isolates was tested in maize, sunflower and watermelon seeds, being high on all the plants assayed. This study reports for the first time the potential of different isolates of *M. phaseolina* to produce plant cell wall-degrading enzymes in submerged fermentation.

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PALABRAS CLAVE
Macrophomina phaseolina;
Podredumbre carbonosa;
Maíz;
Enzimas degradadoras de pared celular vegetal

Crecimiento *in vitro* y producción de enzimas degradadoras de pared celular vegetal de aislamientos argentinos de *Macrophomina phaseolina*, agente causal de la podredumbre carbonosa en maíz

Resumen *Macrophomina phaseolina* es un fitopatógeno polífago, causante de podredumbre carbonosa. Los daños que genera en cultivos de soja y maíz bajo siembra directa en Argentina, en períodos secos y calurosos, se incrementaron por su habilidad para sobrevivir como esclerocios en suelos y restos de cosecha. El propósito del trabajo fue estudiar la producción *in vitro* de enzimas degradadoras de pared celular vegetal (pectinasas [poligalacturonasa y polimetilgalacturonasa]; celulasas [endoglucanasa]; hemicelulasas [endoxilanasa] y la enzima ligninolítica lacasa) de varios aislamientos argentinos de *M. phaseolina* y evaluar la patogenicidad de esos aislamientos, como paso preliminar para establecer el papel de estas enzimas en la interacción *M. phaseolina*-maíz. Se estudió la cinética de crecimiento del hongo y la de la producción de dichas enzimas en medios de cultivo líquidos sintéticos con ácido glutámico como fuente de nitrógeno y con pectina, carboximetilcelulosa (CMC) o xilano como fuentes de carbono. Las pectinasas fueron las primeras enzimas detectadas y los máximos títulos registrados (1,4 UE/ml [poligalacturonasa] y 1,2 UE/ml [polimetilgalacturonasa], respectivamente) superaron a los de celulasas y xilanasas, que aparecieron más tardíamente y en menor magnitud. Esta secuencia promovería la maceración inicial del tejido, seguida luego por la degradación de la pared celular vegetal. Se detectó actividad lacasa en todos los aislamientos (36 a 63 U/l). La agresividad de todos los aislamientos resultó alta en los 3 hospedantes evaluados: semillas de maíz, de girasol y de melón. En este trabajo se investiga por primera vez el potencial de distintos aislamientos de *M. phaseolina* para producir enzimas degradadoras de pared celular vegetal en cultivo líquido.

Introduction

Fungal plant pathogens secrete a wide range of enzymes such as pectinases, xylanases, cellulases and ligninases that are able to depolymerize each cell wall component[38]. These fungi are of interest for the search of new enzyme activities due to their potential application in bioconversion processes[17]. Hydrolytic enzymes play an important role in plant pathogenicity by facilitating tissue colonization of the host[3,13].

Pectin, a heteropolysaccharide defined as galactosyluronic acid-rich polymers, is composed by α-1,4 linked galacturonate chains with high percentage of methyl esterification. Plant pathogenic organisms are capable of degrading pectin by the combined action of several enzymes which produce the breakdown of polygalacturonic acid through two enzymatic processes: lyases split the α-1,4 glycosidic bond between galacturonic acid residues by transelimination, while polygalacturonases catalyze a hydrolytic cleavage[33]. The action of pectinolytic enzymes, and in particular of endopolygalacturonase on cell walls, appears to be a prerequisite for cell wall degradation by other enzymes. Therefore, pectinases are the first enzymes secreted by most fungal pathogens when attacking plant cell walls, followed by hemicellulases and cellulases[35]. A positive correlation has been established between the production of pectinolytic enzymes, virulence and disease symptoms in several pathosystems[16,31].

Cellulose is an unbranched glucose polymer composed of β-1,4-glucose units linked by a β-1,4-D-glycosidic bond. Many plant pathogenic organisms are capable of degrading cellulose by producing a cellulose complex which involves the synergistic action of three main enzymatic complexes, endoglucanase, exoglucanase that releases either glucose or cellobiose, and β-1,4-glucosidase that hydrolyzes cellobiose and cellodextrins to glucose[25,27].

Xylan is the major constituent of hemicellulose. β-1,4-Xylans are heteropolysaccharides with a homopolymeric backbone chain of 1,4-linked β-D-xylopyranose units. *O*-acetyl, α-L-arabinofuranosyl, α-1,2-linked glucuronic, or 4-*O*-methylglucuronic acids are the most frequent substituents on the backbone. Xylan hydrolysis mainly requires the action of endo-β-1,4-xylanase and β-xylosidase. However, the presence of other accessory enzymes is needed to hydrolyze substituted xylans[15].

The complex aromatic polymer lignin, a component of plant secondary cell walls, provides a barrier to fungal entry and to the diffusion of fungal toxins and enzymes into plant cells[8]. The lignin-degrading enzyme, laccase, is a copper-containing enzyme produced by some fungi that catalyzes the oxidation of phenolic substrates. Several phytopathogenic fungi are known to produce lacasse[18,20]. Laccase protects phytopathogenic fungi from the plant defense system activated in response to a microbial infection, by degrading toxic substances such as phytoalexins, tannins and other phenolic compounds[24].

The charcoal rot fungus *Macrophomina phaseolina* (Tassi) Goid, an Ascomycete which belongs to the family Botryosphaeriaceae, is a soil-borne pathogen distributed worldwide. The pathogen has a wide host range, infecting

more than 500 plant species[5]. The fungus attacks a broad spectrum of economically important crops such as maize, soybean, sorghum, sesame, cotton, beans, sunflower or cucurbits[6,10]. In maize it causes stalk rot during hot and dry weather during plant senescence[37]. The symptoms are similar to other fungal stalk rots and the characteristic sign is the production of black microsclerotia in vascular tissues and inside the rind of the stalk. Microsclerotia in crop debris and in soil are the primary inocula of the disease, surviving up to three years in soil[6]. No-till practices are widely adopted in Argentina, and crop debris remains on the soil surface until complete degradation, increasing the amount of the pathogen inoculum. *M. phaseolina* owns a large repertoire of hydrolytic enzymes able to degrade all major components of the plant cell wall and cuticle, including cellulose, hemicellulose, pectin, lignin, and cutin[12]. Moreover, cellulolytic activity of *M. phaseolina* was shown to be significantly higher than that of other fungal species (*i.e.*, *Aspergillus niger* and *Trichoderma reesei*), demonstrating the pathogenic potential of this fungus[15]. More than twenty laccase genes, which might be involved not only in lignin degradation but also in appressorial melanization and pathogenicity[19] have been identified in *M. phaseolina*[12].

In the present work, we (i) explored the *in vitro* production of plant cell wall-degrading enzymes by several Argentinean isolates of *M. phaseolina*, a fungal pathogen associated with corn stalk rot disease, as a preliminary step to establish the role of these enzymes in *M. phaseolina*–maize interaction; (ii) assessed the pathogenicity of these isolates on watermelon (*Citrullus lanatus*), sunflower (*Helianthus annuus*) and maize (*Zea mays*), and evaluated the relationship (if any) with their ability to produce cell wall-degrading enzymes.

Materials and methods

Microorganisms

Isolates BAFC 3591, 3595, 3820, 3821, 3864 and 3865 (Colección de Cultivos de Hongos del Dpto. de Cs. Biológicas, FCEN, UBA) of the anamorphic species *M. phaseolina* were used in these experiments. These isolates were previously isolated from samples of corn stems showing typical symptoms of a natural infection by *M. phaseolina*. These samples were collected from three locations in Buenos Aires province: Luján (isolates 3591 and 3865), Rafael Obligado (isolates 3820 and 3821) and San Antonio de Areco (isolates 3595 and 3864). Microsclerotia of *M. phaseolina* were obtained from stem tissues and placed on potato dextrose agar (PDA) plates. Pure cultures were developed by removing growing hyphal tips from a single microsclerotium colony and culturing them on PDA at 28–30 °C for 5–7 days in darkness[23].

Basal culture medium

Glutamic acid, 9.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; H_2KPO_4, 0.5 g; HK_2PO_4, 0.6 g; $CuSO_4 \cdot 5H_2O$, 0.4 mg; $MnCl_2 \cdot 4H_2O$, 0.09 mg; H_3BO_3, 0.07 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.02 mg; $FeCl_3$, 1 mg; $ZnCl_2$, 3.5 mg; thiamine hydrochloride, 0.1 mg; distilled water up to 1 l. Alternatively different carbon sources were added. In order to test pectinase, cellulase or xylanase production, the basal medium was supplemented either with 10 g of pectin from apple, 10 g of carboxymethylcellulose (CMC) or 10 g of oat spelt xylan, respectively. To evaluate laccase production, glucose 10 g was used as carbon source, and $CuSO_4 \cdot 5H_2O$ as laccase inducer[18] was added to the medium, final concentration 0.2 mM. Final pH was adjusted to 3.5 with either NaOH or HCl 1 N.

Culture conditions

One hundred milliliters Erlenmeyer flasks with 25 ml of medium were inoculated with one agar plug (0.25 cm^2), cut out from a colony grown on Bacto-agar 2%. Incubation was performed at 28 ± 1 °C under stationary conditions. Cultures were harvested every three days during three weeks, filtered through a filter paper using a Büchner funnel and dried overnight at 70 °C. Dry weight of mycelia was then determined. The culture supernatants were used as enzyme sources.

Enzyme assays

Polymethylgalacturonase (PMG) or polygalacturonase (PG) (endo plus exo activities) were assayed by following the release of reducing groups from apple pectin or polygalacturonic acid in 50 mM sodium acetate buffer, pH 4.8 at 37 °C, in accordance with the Somogyi–Nelson method[26]. One unit of enzymatic activity was defined as the amount of enzyme releasing 1 μmol of galacturonic acid per min. Laccase activity was measured with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in 0.1 M sodium acetate buffer (pH 3.5) at 50 °C. Oxidation of ABTS was determined by the increase in A_{420} (420 = 36/mM cm)[4]. Endo-β-1,4-glucanase activity was assayed at 50 °C and endo-β-1,4-xylanase activity was determined at 37 °C by measuring the reducing sugars released respectively from CMC or xylan in 50 mM sodium acetate buffer at pH 4.8, in accordance with the Somogyi–Nelson method[26]. One unit of enzymatic activity was defined as the amount of enzyme releasing 1 μmol of glucose or xylose per min. Enzymatic units (U) were used (μmol/min). Enzyme activity was expressed as EU/ml of culture filtrate. Results are the average of three triplicate experiments with a standard error lower than 5%.

Pathogenicity test

The *M. phaseolina* isolates were grown in potato-sucrose agar (PSA) in Petri dishes and incubated in darkness at 28 °C for 7 days. When dishes were completely colonized by the fungus and covered by microsclerotia, seeds of watermelon, sunflower or maize, previously disinfected with 2% sodium hypochlorite for 2 min and rinsed twice in sterile water, were placed on the plates. Each treatment of six seeds arranged in two plates was replicated three times. Evaluation was done after 5 days, using the following severity assessment key: 0 = healthy seed; 1 = discoloration on a portion of the seedling in contact with the mycelium; 2 = seed teguments invaded by mycelium and microsclerotia but healthy seedling; 3 = seed teguments free from the

fungus but infected seedling; 4 = seed tegument and infected seedling; 5 = infected seed and not germinated[21]. The disease index was calculated by multiplying the number of seeds by the degree of disease severity. Values greater than 3 were considered susceptible. Non-parametric analysis of variance (Kruskal–Wallis) was performed using Infostat software[7].

Results and discussion

Six fungal isolates of *M. phaseolina* were grown in a synthetic medium with several carbon sources alternatively employed to assess the production of different cell wall-degrading enzymes. Figures 1A–C, describe growth and pectinolytic enzyme production in a medium with pectin as carbon source. All isolates were able to grow in this medium, and showed PMG and PG activities having the potential to degrade α-1,4 bonds in pectic substances hydrolytically. The disparity observed in enzyme production among the isolates cannot be attributed to fungal growth, since no major differences were found. Maximal growth values were around 4–4.8 mg/ml of medium, and were usually registered after 11 days of cultivation (Fig. 1A). The peak of PMG activity, which appeared between days 7 and 10 of incubation, preceded, in general, the day of maximal growth (Fig. 1B). On the contrary, PG activity reached its highest level (between day 8 and 14 of culture) after or simultaneously with the growth peak (Fig. 1C), as previously reported in *Botrytis cinerea*[22]. Among the isolates assayed, *M. phaseolina* BAFC 3865 and BAFC 3821 rendered the highest levels of the two enzymes. Isolate BAFC 3820 showed high PG production but low PMG production (Figs. 1B and C). PG titers obtained from *M. phaseolina* isolates (between 0.4 and 1.3 U/ml) were similar to those detected when other phytopathogenic fungi were grown in a medium with pectin. PG-production by *Fusarium oxysporum* f. sp. *niveum* reached a maximum of 0.4 U/ml[36], *Colletotrichum lindemuthianum* produced 0.24 U/ml[11], *Colletotrichum trucatum* rendered 1.08 U/ml[29], while in *Fusarium graminearum* higher levels of PG (5.4 U/ml) than in other phytopathogenic fungi were measured[17]. PMG activities (between 0.15 and 1.3 U/ml) were similar to those obtained for *C. truncatum* (1.2 U/ml)[29] and for *F. graminearum* (1.53 U/ml)[17].

All the isolates tested were able to grow in the media with CMC or with xylan as carbon sources and produced cellulolytic and xylanolytic enzymes; however, their production was much lower than that of pectinases. Cellulolytic (endoglucanase) activity was generally detected after 11 days of cultivation while endoxylanase was observed between day eight and eleven (Fig. 2). Cellulolytic activity detected from *M. phaseolina* isolates was between 70 U/l for isolate BAFC 3864 and 164 U/l for isolate BAFC 3821, while maximum endoxylanase activity was registered in isolate BAFC 3865 (84 U/l) and the minimum in *M. phaseolina* BAFC 3864 (35 U/l) (Fig. 2).

In this work, pectinases were the first extracellular enzymes detected related to the degradation of the main components of a plant cell wall, and the activity obtained was higher than that of cellulases and xylanases, which appeared later and in a lesser magnitude. Similar results were obtained by Kikot et al.[17] when studying cell

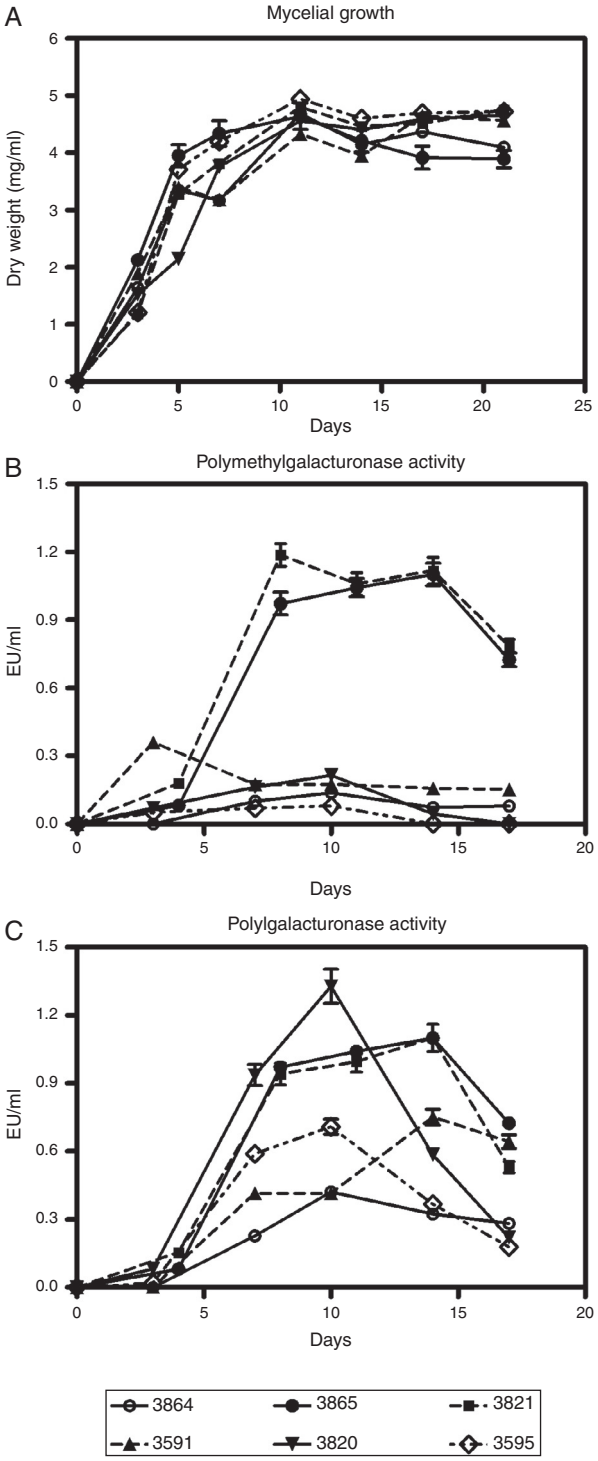

Figure 1 Kinetics of growth (A), polymethylgalacturonase (B) and polygalacturonase production (C) by *M. phaseolina* isolates from different regions in Argentina, in minimum salt medium supplemented with pectin and glutamic acid as carbon and nitrogen sources, respectively. Values represent the mean of three replicates and SEM.

wall-degrading enzymes production by *F. graminearum*. The earlier production of galacturonases during *M. phaseolina in vitro* cultivation coincides with their postulated role in pathogenesis in other phytopathogenic fungi. Ahmad et al.[1] reported that in several pathogens including *M. phaseolina*,

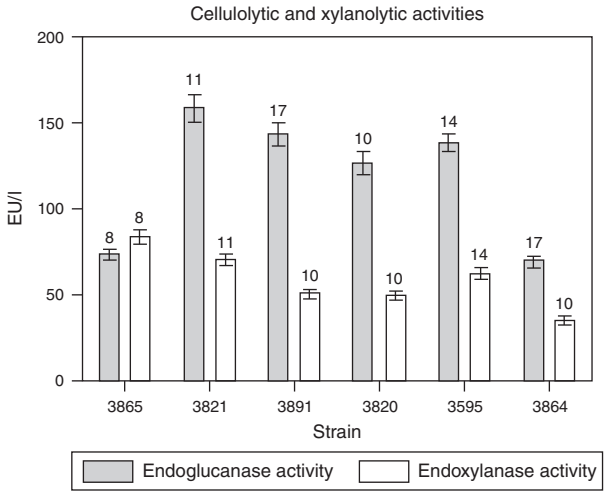

Figure 2 Time courses for the production of endoglucanase and endoxylanase activities by *M. phaseolina* isolates from different regions in Argentina, in minimum salt medium supplemented with carboxymethylcellulose (for cellulases) or xylan (for xylanases) as carbon sources and glutamic acid as nitrogen source. The numbers over the bars indicate the day when the highest value was obtained. Values represent the mean of three replicates and SEM.

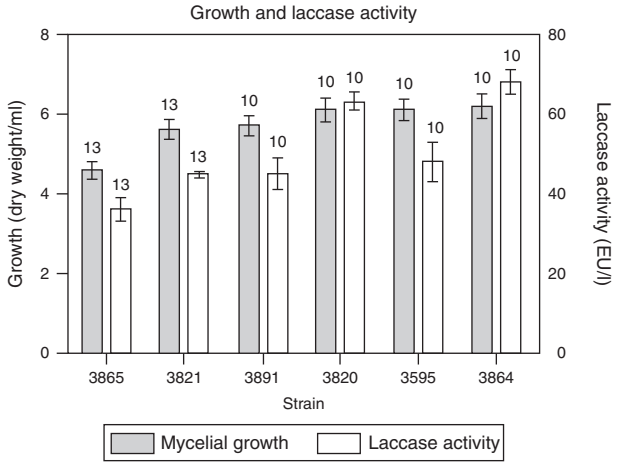

Figure 3 Growth and laccase enzyme production by *M. phaseolina* in minimum salt medium supplemented with glucose and glutamic acid as carbon and nitrogen sources, respectively, supplemented with $CuSO_4$ 0.2 mM. The numbers over the bars indicate the day when the highest value was obtained. Values represent the mean of three replicates and SEM.

pectinase was the chief enzyme while initiating the process of cell wall degradation as it showed the highest activity before cellulases. EndoPG and endoPMG facilitate the entry of the pathogen into the host tissues. In *M. phaseolina*, penetration generally occurs from an appresorium formed over anticlinal walls of epidermal cells or through natural openings. The fungal hyphae grow first intercellularly and then intracellularly through the xylem, producing numerous microsclerotia that plug the vessels[39]. The *B. cinerea* genome contains at least six endopolygalacturonase-encoding (*Bcpg*) genes, strains mutated in genes *Bcpg1* and *Bcpg2* were reduced in virulence[14], which indicates that pectin degradation is necessary for successful infection. Furthermore, in the pathogenic fungus *F. oxysporum*, responsible for causing vascular wilt disease that is characterized by a severe degradation of vascular tissue, the amount of PG activity correlated highly with the development of the disease[2].

Laccase activity was confirmed in the six isolates evaluated, starting in every case before mycelial biomass peaked (*i.e.* it was present in the primary phase growth) (data not shown), and reached its maximum on day 10 coincident with the highest biomass recorded (Fig. 3). Biomass production in all the isolates evaluated varied between 5.2 and 6.2 mg/ml medium. Laccase activity obtained for all isolates assayed was between 36 U/l and 63 U/l. Laccase production was comparable with that of *C. truncatum*[18], when using glucose (41 U/l) or pectin (44 U/l) as carbon sources without the addition of $CuSO_4$. However, laccase production by *F. solani* f. sp. *glycines* under submerged fermentation in a medium supplemented with soybean roots, attained higher values, even exceeding the titers obtained by white rot fungi such as *Schizophyllum commune* cultured under similar conditions[20]. This study reports for the first time the potential of plant cell wall-degrading enzyme production, assayed in submerged fermentation, by different isolates of *M. phaseolina*. Recently, Kaur et al.[15] reported that one of the isolates of *M. phaseolina* was a potential source of several hydrolytic enzymes, such as cellulases, hemicellulase and amylase, with biotechnological applications[28].

The degree of disease severity caused by all *M. phaseolina* isolates tested was high, exceeding degree 3 in the scale described by Manici et al.[21] in every case. The six isolates evaluated invaded and infected all the seeds of the species assayed (Table 1), confirming the polyphagous condition of this phytopathogenic fungus[34]. The comparison of mean values among different plant seed reactions showed no significant differences ($p \leq 0.05$) in the disease index. Manici et al.[21] depicted different degrees of pathogenicity in *M. phaseolina* isolates in a wide variety of crops, such as soybean, sunflower, sorghum, melon and sugar beet, among

Table 1 Pathogenicity of isolates of *Macrophomina phaseolina* on maize, sunflower and watermelon seeds

Seeds	Isolates 3595	3820	3591	3864	3821	3865	*p*
Maize	26 ± 1.73	26 ± 1.73	24 ± 0.58	27 ± 1.0	26 ± 0.58	26 ± 0.58	0.1052
Sunflower	24 ± 1.15	24 ± 0.58	24 ± 1.0	25 ± 0.58	24 ± 0.53	26 ± 1.15	0.0554
Watermelon	26 ± 0.58	28 ± 2.0	29 ± 1.0	28 ± 1.53	27 ± 1.15	28 ± 0.58	0.2076

Severity was recorded according to a 0 to 5 scale. Severity multiplied by the number of diseased seeds = pathogenicity. Values represent the mean of three replications of each species and SEM.

others, but none was pathogenic on maize. High pathogenicity was also observed by Gill-Langarica et al.[9] in soybean crops. Rayatpanah and Dalili[30] studied the pathogenicity of 24 isolates of Iranian *M. phaseolina*; the pathogenicity test demonstrated that no one was pathogenic on maize, while all of them showed pathogenic ability on soybean and sunflower. However, the Argentinean isolates evaluated in this work showed comparable pathogenicity even on maize. Reyes-Franco et al.[32] investigated the pathogenicity of isolates of *M. phaseolina* from different countries, among them, Argentina. Results showed differences between isolates, being the most aggressive those from Mexico, Brazil and Colombia. Considering the results obtained in the present work, the aggressiveness of Argentinean isolates of *M. phaseolina* should be investigated more thoroughly.

Although we could not establish a relationship between differences in cell wall-degrading enzyme production among isolates and pathogenicity, the *in vitro* production of plant cell wall-degrading enzymes by *M. phaseolina* could be related to their production and role *in vivo*: initial pectinolytic production followed by cellulolytic and ligninolytic secretion. This sequence indicates that pectic enzymes are needed to increase the accessibility of cell wall components for degradation by other enzymes, cell lysis and plant tissue maceration. Laccase would contribute not only with tissue disintegration in an advanced phenological stage but would also protect the pathogen against oxidative plant defenses and might be involved in appressorial melanization and pathogenicity.

Ethical responsibilities

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

References

1. Ahmad Y, Hameed A, Ghaffar A. Enzymatic activity of fungal pathogens in corn. Pak J Bot. 2006;38:1305–16.
2. Baayen R, Schoffelmeer EAM, Toet S, Elgersma DM. Fungal polygalacturonase activity reflects susceptibility of carnation cultivars to *Fusarium* wilt. Eur J Plant Pathol. 1997;103:15–23.
3. Belien T, Campenhout S, Robben J, Volckaert G. Microbial endoxylanases: effective weapons to breach the plant cell-wall barrier or, rather, triggers of plant defense systems? Mol Plant Microbe Interact. 2006;19:1072–81.
4. Collins PJ, Dobson ADW. Regulation of laccase gene transcription in *Trametes versicolor*. Appl Environ Microbiol. 1997;63:3444–50.
5. Csöndes I, Cseh A, Taller J, Poczai P. Genetic diversity and effect of temperature and pH on the growth of *Macrophomina phaseolina* isolates from sunflower fields in Hungary. Mol Biol Rep. 2012;39:3259–69.
6. Dhingra OD, Sinclair JB, editors. Biology and pathology of *Macrophomina phaseolina*. Universidade Federal de Vicosa, Minas Gerais, Brazil: Imprensa Universitaria; 1978. p. 166.
7. Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW. InfoStat versión 2011. Argentina: Grupo InfoStat, FCA, Universidad Nacional de Córdoba; 2011.
8. Elstrand M, Sitbon F, Lapierre C, Bottin A, von Arnold S. Altered lignin structure and resistance to pathogens in spi 2-expressing tobacco plants. Planta. 2002;214:708–16.
9. Gill-Langarica HR, Maldonado-Moreno N, Pecina-Quintero V, Mayek-Pérez N. Reacción de germoplasma mejorado de soya [*Glycine max* (L.) Merr.] a *Macrophomina phaseolina* (Tassi) goidanich y déficit hídrico. Rev Mex Fitopatol. 2008;26:105–13.
10. Gupta GK, Sharma SK, Ramteke R. Biology, Epidemiology and management of the pathogenic fungus *Macrophomina phaseolina* (Tassi) Goid with special reference to charcoal rot of soybean (*Glycine max* (L.) Merrill). J Phytopathol. 2012;160:167–80.
11. Hugouvieux V, Centis S, Lafitte C, Esquerre-Tugaye M-T. Induction by α-L-arabinose and α-L-rhamnose of endopolygalacturonase gene expression in *Colletotrichum lindemuthianum*. Appl Environ Microbiol. 1997;63:2287–92.
12. Islam MS, Haque MS, Islam MM, Emdad EM, Halim A, Hossen QM, Hossain MZ, Ahmed B, Rahim S, Rahman MS, Alam MM, Hou S, Wan X, Saito JA, Alam M. Tools to kill: genome of one of the most destructive plant pathogenic fungi *Macrophomina phaseolina*. BMC Genomics. 2012;13:493–508.
13. Jaroszuk-Ściseł J, Kurek E, Słomka A, Janczarek M, Rodzik B. Activities of cell wall degrading enzymes in autolyzing cultures of three *Fusarium culmorum* isolates: growth-promoting, deleterious and pathogenic to rye (*Secale cereale*). Mycologia. 2011;103:929–45.
14. Kars IG, Krooshof CAM, Wagemakers R, Joosten JA, Benen E, van Kan JAL. Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. Plant J. 2005;43:213–25.
15. Kaur S, Dhillon GS, Brar SK, Chauhan VB. Carbohydrate degrading enzyme production by plant pathogenic mycelia and microsclerotia isolates of *Macrophomina phaseolina* through koji fermentation. Ind Crop Prod. 2012;36:140–8.
16. Kikot GE, Hours RA, Alconada TMJ. Contribution of cell wall degrading enzymes to pathogenesis of *Fusarium graminearum*: a review. Basic Microbiol. 2009;49:231–41.
17. Kikot GE, Hours RA, Alconada TMJ. Extracellular enzymes of *Fusarium graminearum* isolates. Braz Arch Biol Technol. 2010;53:779–83.
18. Levin L, Ramos AM, Parisi M, Gally M. Screening of Colletotrichum (Ascomycota) isolates, causal agents of soybean anthracnose, for laccase production. Bol Soc Argent Bot. 2007;42:71–7.
19. Lin SY, Okuda S, Ikeda K, Okuno T, Takano Y. LAC2 encoding a secreted laccase is involved in appressorial melanization and conidial pigmentation in *Colletotrichum orbiculare*. Mol Plant Microbe Interact. 2012;25:1552–61.
20. Lozovaya VV, Lygin AV, Zernova OV, Li S, Widholm JM, Hartman GL. Lignin degradation by *Fusarium solani* f. sp. *glycines*. Plant Dis. 2006;90:77–82.
21. Manici L, Caputo F, Cerato C. Temperature responses of isolates of *Macrophomina phaseolina* from different climate regions of sunflower production in Italy. Plant Dis. 1995;79:834–8.
22. Martínez MJ, Martínez R, Reyes F. Effect of pectin on pectinases in autolysis of *Botrytis cinerea*. Mycopathologia. 1988;102:37–43.

23. Mayek-Pérez N, López-Castañeda C, García-Espinosa R, Martínez de la Vega O, Acosta-Gallegos JA. Variación *in vitro* de aislamientos mexicanos de *Macrophomina phaseolina*. Rev Mex Fitopatol. 1999;17:83–90.
24. Mayer AM, Staples RC. Laccase: new functions for an old enzyme. Phytochemistry. 2002;60:551–65.
25. Moreira FG, Simone R, Costa MAF, Marques de Souza CG, Peralta RM. Production of hydrolytic enzymes by the plant pathogenic fungus *Myrothecium verrucaria* in submerged cultures. Braz J Microbiol. 2005;36:7–11.
26. Nelson N. A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem. 1944;153:375–80.
27. Okunowo WO, Gbenle GO, Osuntoki AA, Adekunle AA, Ojokuku SA. Production of cellulolytic and xylanolytic enzymes by a phytopathogenic *Myrothecium roridum* and some avirulent fungal isolates from water hyacinth. Afr J Biotechnol. 2010;9:1074–8.
28. Pandey A. In: Pandey A, Webb C, Soccol CR, Larroche C, editors. Enzyme technology. New York: Springer Science; 2006. p. 746.
29. Ramos AM, Gally M, García MC, Levin L. Pectinolytic enzyme production by *Colletotrichum truncatum*, causal agent of soybean anthracnose. Rev Iberoam Micol. 2010;27:186–90.
30. Rayatpanah S, Dalili SA. Diversity of *Macrophomina phaseolina* (Tassi) Goid based on chlorate phenotypes and pathogenicity. IJB. 2012;4:54–63.
31. Reignault P, Valette-Collet O, Boccara M. The importance of fungal pectinolytic enzymes in plant invasion, host adaptability and symptom type. Eur J Plant Pathol. 2008;120:1–11.
32. Reyes-Franco MC, Hernández-Delgado S, Beas-Fernández R, Medina-Fernández M, Simpson J, Mayek-Pérez N. Pathogenic and genetic variability within *Macrophomina phaseolina* from Mexico and other countries. J Phytopathol. 2006;154:447–53.
33. Sakal T, Sakamoto T, Hallaert J, Vandamme EJ. Pectin, pectinase and protopectinase: production, properties and applications. Adv Appl Microbiol. 1995;39:2213–93.
34. Su G, Suh SO, Schneider RW, Russin JS. Host specialization in the charcoal rot fungus, *Macrophomina phaseolina*. Phytopathology. 2001;91:120–6.
35. Vallejo Herrera MD, Toro ME, de Figueroa LIC, Vazquez F. Extracellular hydrolytic enzymes produced by phytopathogenic fungi. In: Spencer JFT, Ragout de Spencer AL, editors. Methods in biotechnology: environmental microbiology: methods and protocols. Totowa, New Jersey: Humana Press; 2004. p. 399–421.
36. Vázquez C, Reyes F, Martínez MJ. Comparative study of pectic activities from different formae speciales of *Fusarium oxysporum*. Lett Appl Microbiol. 1993;16:210–3.
37. White DG. Fungal stalk rots. In: White DG, editor. Compendium of corn diseases. St. Paul, Minnesota: APS Press; 1999. p. 128.
38. Wu SC, Halley JE, Luttig C, Fernekes LM, Gutierrez-Sanchez G, Darvill AG, Albersheim P. Identification of an endo-beta-1,4-D-xylanase from *Magnaporthe grisea* by gene knockout analysis, purification, and heterologous expression. Appl Environ Microbiol. 2006;72:986–93.
39. Wyllie TD. Charcoal rot of soybean – current status. In: Wyllie TD, Scott DH, editors. Soybean diseases of the north central region. St. Paul, Minnesota: APS Press; 1988. p. 149.