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Pectinolytic enzyme production by *Colletotrichum truncatum*, causal agent of soybean anthracnose

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Background: *Colletotrichum truncatum* is the most common pathogenic fungus associated with soybean anthracnose, a prevalent disease in Argentina. Pectinolytic enzymes are involved in the pathogenicity of a wide range of plant pathogenic fungi.

Objectives: To explore pectinolytic enzyme production in Argentinian *Colletotrichum* strains isolated from diseased soybean plants from different geographic locations, as a preliminary step to establish the biological role of the pectinolytic enzymes in the *Colletotrichum* spp.–soybean system, yet unknown.

Methods: Ten strains were screened for *in vitro* pectinolytic enzyme production on a defined medium based on pectin as carbon source.

Results: All isolates were able to grow in this medium and polymethylgalacturonase (PMG), polygalacturonase (PG) and pectin lyase (PL) activities were detected. On the whole, the peak of polygalacturonases activities preceded the day of maximum growth, while PL activity reached its highest level afterwards. Strain BAFC 3097 (from Santa Fe province) yielded high titles of the three enzymes (1.08 U/ml PG, 1.05 U/ml PMG, 156 U/ml PL), after a short incubation period (7–10 days). Low synthesis of polygalacturonases in cultures containing glucose as unique carbon source suggests that these enzymes are constitutive in contrast with PL, which was not detected.

Conclusions: The disparity observed in enzyme production among strains cannot be related to fungal growth, since no major differences in mycelial yield were found; it was not connected with their geographic origin, but might be associated with differences in virulence among strains not yet evaluated.

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Producción de enzimas pectinolíticas por *Colletotrichum truncatum*, agente causal de antracnosis en soja

R E S U M E N

Antecedentes: *Colletotrichum truncatum* es el hongo patógeno más comúnmente asociado con la antracnosis de la soja, enfermedad de alta prevalencia en Argentina. Las enzimas pectinolíticas se relacionan con la patogenicidad de un amplio rango de hongos fitopatógenos.

Objetivos: Investigar la producción de enzimas pectinolíticas por cepas aisladas de plantas de soja enfermas de diferentes regiones de nuestro país, y con ello contribuir a la caracterización fisiológica de dichos aislamientos como paso preliminar para esclarecer el aún desconocido rol biológico de las enzimas pectinolíticas en la interacción *Colletotrichum* spp.–soja.

Métodos: Se investigó la producción *in vitro* de enzimas pectinolíticas, en un medio sintético con pectina como fuente de carbono, de diez aislamientos de *C. truncatum*.

Resultados: Todas las cepas crecieron en dicho medio, detectándose actividades polimetilgalacturonasa (PMG), poligalacturonasa (PG) y pectin liasa (PL). En general, el pico de galacturonas precedió al día de máximo crecimiento, en cambio el de PL se registró posteriormente. La cepa BAFC 3097 (originaria de la Provincia de Santa Fe) produjo altos títulos de las tres enzimas tras 7–10 días: 1,08 U/ml PG, 1,05 U/ml PMG, 156 U/ml PL. *C. truncatum*, cultivado en un medio con glucosa como fuente de carbono, produjo PG y PMG (pero no PL), aunque su síntesis disminuyó marcadamente sugiriendo que estas enzimas son constitutivas.

Palabras clave:

Colletotrichum truncatum

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Conclusión: La disparidad registrada en la producción enzimática entre cepas no puede atribuirse al crecimiento fúngico; tampoco se corresponde con su distribución geográfica; pero podría relacionarse con diferencias en su virulencia, que aún no se han investigado.

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Soybean (*Glycine max*) production in Argentina has increased over the last two decades. More than 90% of the cropped area is located in the northern Pampeana region. This area includes southwest of Cordoba, centre and south of Santa Fe and north of Buenos Aires provinces. Losses have increased due to diseases associated with monocropping, no-till systems and genetic uniformity of cultivars.⁴⁶

Several species of *Colletotrichum* cause plant diseases so-called anthracnoses throughout the world, causing economically significant diseases of cereals, grain legumes, vegetables, forage legumes, fruit crops and perennial crops. Their ability to cause latent or quiescent infections places them amongst the most important post-harvest pathogens.² These imperfect fungi belong to the subdivision Deuteromycotina (form-class Deuteromycetes, form-subclass Coelomycetidae, form-order Melanconiales, form-family Melanconiaceae) with 39 “accepted” species,⁴⁰ which continue to be revised and clarified by molecular taxonomic techniques.

Soybean anthracnose is an economically important disease widely distributed in every soybean area production.³⁷ Even though soybean is susceptible to the pathogen throughout all stages of development, symptoms become more noticeable in crop maturation, and anthracnose is included in the complex commonly known as late season diseases (LSDs). LSDs are presently the most damaging soybean diseases in Argentina, as they affect the plants during the yield generation period.

Although taxonomy and nomenclature in the group is confusing,¹⁸ the species most frequently associated with soybean anthracnose is *Colletotrichum truncatum*^{24,12} (teleomorph *Glomerella truncata*).¹ Other species of *Colletotrichum* involved are *C. coccodes*, *C. destructivum* (teleomorph *Glomerella glycines*), *C. gloeosporioides* (teleomorph *Glomerella cingulata*) and *C. graminicola* (teleomorph *Glomerella graminicola*).²

Cellulose, hemicellulose, lignin and pectin are the main components of plant cell wall. Pectin, a heteropolysaccharide defined as galactosyluronic acid-rich polymers, is composed of α -1-4 linked galacturonate chains with high percentage of methyl esterification. Pectinolytic enzymes, capable of degrading pectin and leading to maceration of plant tissues, are the first enzymes secreted by most fungal pathogens when attacking plant cell walls.^{7,19} Pectin degradation can be attained by the combined action of several enzymes such as pectin methylesterases and pectin depolymerases, including hydrolases and lyases, such as polymethylgalacturonase and pectin lyase. Degradation involves the breakdown of polygalacturonic acid through two enzymatic processes: lyases split the α -1-4 glycosidic bond between galacturonic acid residues by trans-limitation, while polygalacturonases catalyze a hydrolytic cleavage.³⁶

The role of pectin degrading enzymes in causing cell-wall degradation is so important that it determines the virulence of many pathogens.^{8,7,35} In a number of systems, correlations have been established between the presence of pectinolytic enzymes, disease symptoms and virulence.¹⁰

Several *Colletotrichum* species, including *C. truncatum*²⁹; *C. lindemuthianum*²⁷ and *C. destructivum*,²² are intracellular hemibiotrophic pathogens and have a restricted host range, suggesting that host specificity in *Colletotrichum* might be associated with intimate contact between infective hyphae and

living host cells.² *C. truncatum* establishes an initial biotrophic interaction that lasts approximately 24 h followed by a secondary necrotrophic development.^{28,31} Two endopolygalacturonases were characterized in *C. lindemuthianum*: endopolygalacturonase 1 (endoPG1), released during saprophytic growth on pectin medium and induced during necrotrophic colonization, and endopolygalacturonase 2 (endoPG2), not detected in culture fluids, and associated with fungal cell wall components⁵ and wall degradation at the site of penetration. Limited cell wall degradation might be necessary for the beginning and preservation of the biotrophic growth by reducing cellular damage. During the necrotrophic phase, the production of endoPG1 is accompanied by other pectinases such as pectin lyases⁴⁵ and probably pectin methyl esterases, which produce synergist and extensive wall dissolution. The pectinolytic enzymes of *Colletotrichum* species have been investigated in several crops, including avocado (*C. gloeosporioides*),⁴³ bean (*C. lindemuthianum*),^{44,45,16} rubber (*C. acutatum*)¹¹ and pea (*C. truncatum*).²⁹ As far as we know, there is only one previous report on pectinolytic enzyme production by *Colletotrichum* spp. isolated from soybean plants.⁶ Although pectinolytic enzymes are involved in the pathogenicity of a wide range of plant pathogenic fungi, their biological function in the *Colletotrichum* spp.–soybean system is unknown. In the present work, by exploring *in vitro* pectinolytic enzyme production, we wish to contribute to the physiological characterization of Argentinean *C. truncatum* strains isolated from diseased soybean plants from different geographic locations, as a preliminary step to establish the role of the pectinolytic enzymes in the *Colletotrichum* spp.–soybean interaction.

Material and methods

Microorganisms

Strains BAFC 3093–3102 (BAFC: Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires) of the anamorphic species *C. truncatum* were used in these experiments. The isolates were obtained from lesions of stems and pods of symptomatic soybean plants from seven localities in the provinces of Buenos Aires (3099, 3100, 3101), Santa Fe (3093, 3096, 3097, 3098), Chaco (3102), La Rioja (3095) and Misiones (3094). Pieces of symptomatic tissue bearing immature acervuli were surface sterilized in 1.5% sodium hypochlorite for 2 min, rinsed twice in sterile water and incubated in humid chambers at 25–27 °C under 12 h near ultraviolet light (nuv) and 12 h of darkness, until liberation of masses of conidia, which were cultured in potato–dextrose agar (PDA) plates at 25–27 °C in darkness. Hyphal tips were aseptically transferred to PDA and incubated at 25–27 °C under nuv light/darkness (12/12 h) to obtain pure cultures. Stock cultures were maintained on potato–dextrose agar slants at 4 °C.

Basal culture medium

Pectin from apple, 10 g; asparagine monohydrate, 4 g; MgSO₄ · 7H₂O, 0.5 g; H₂KPO₄, 0.5 g; HK₂PO₄, 0.6 g; CuSO₄ · 5H₂O,

0.4 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.09 mg; H_3BO_3 , 0.07 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.02 mg; FeCl_3 , 1 mg; ZnCl_2 , 3.5 mg; thiamine hydrochloride, 0.1 mg; distilled water up to 1 l. Final pH: 3.5. In one assay pectin was replaced by glucose, the initial pH of this medium being 6.2.

Culture conditions

100 ml Erlenmeyer flasks with 20 ml of medium were inoculated with one agar plug (0.25 cm²) and cut out from a colony grown on Bacto-agar 2%. Incubation was carried out at 23 ± 1 °C under stationary conditions. Cultures were harvested at different incubation periods, filtered through a filter paper using a Büchner funnel and dried overnight at 70 °C. Dry weight of mycelia was determined. The culture supernatants were used as enzyme sources.

Enzyme assays

Polygalacturonase activity (endo plus exo activity) was assayed by following the release of reducing groups from 0.1% apple pectin (polymethylgalacturonase—PMG) or polygalacturonic acid (polygalacturonase—PG) in 50 mM sodium acetate buffer of pH 4.8 according to the Somogyi–Nelson method.³⁹ One unit of enzymatic activity was defined as the amount of enzyme releasing 1 μmol of galacturonic acid per min at 37 °C. Enzyme activity was expressed as EU/ml of culture filtrate. Pectin lyase (PL) activity was assayed by the thiobarbituric acid method.³⁰ The reaction mixture contained 3 ml of pectin (1.2% in 0.05 M Tris HCl buffer, pH 8.0) and 2 ml of culture filtrate. The mixture was incubated for 1 h at 30 °C. After incubation, 1.5 ml of 1 N HCl and 3 ml of 0.04 M thiobarbituric acid were added and kept at 100 °C for 20 min. Afterwards, absorbance was measured at 550 nm. In all assays, boiled enzyme and substrate mixtures were used as controls. One unit of lyase activity was defined as the amount of enzyme causing an increase of 0.01 in absorbance during 30 min. Results are the average of three triplicate experiments with a standard error lower than 5%.

Results and discussion

Kinetics of *in vitro* production of extracellular pectinolytic enzymes by ten fungal strains from *Colletotrichum* isolated from soybean plants from Argentina that showed typical anthracnose symptoms was characterized in a synthetic medium based on pectin and asparagine as carbon and nitrogen sources, respectively. Fig. 1 depicts the relationship between their growth and pectinolytic enzyme production. All isolates were able to grow in the mentioned medium and produced a complex of enzymes having the potential to degrade α -1-4 bonds in pectic substances hydrolytically, as well by a trans-eliminative mechanism. The disparity observed in enzyme production among strains cannot be attributed to fungal growth, since no major differences in mycelial yield were found. Maximal growth values were around 50–70 mg/20 ml of medium, and were usually registered after 10–15 days of cultivation. In general, the peak of PG and PMG activities preceded the day of maximum growth. On the contrary PL activity reached its highest level simultaneously with or after the peak of growth. As detected in this work also in *C. lindemuthianum*⁴⁴ and *Rhizoctonia solanii*²³ the PG peak preceded the one of PL, and in *Fusarium oxysporum* f. sp. *melonis*²⁶ and *Botrytis cinerea*²⁵ PG activities peaked during growth, whereas the highest levels of PL were detected during autolysis. The earlier production of galacturonases during *C. truncatum* *in vitro* cultivation coincides

with their postulated role in pathogenesis. Initially, several endoPG isoforms facilitate biotrophic development without causing extensive tissue maceration, while other isoenzymatic forms predominate in the necrotrophic stage. On the contrary PL, which appears later, is more likely to play an important role during the necrotrophic phase of tissue colonization.^{44,15}

Among the strains assayed, *C. truncatum* BAFC 3097 rendered high levels of the three enzymes, after a short incubation period (7–10 days; 1.08 U/ml PG, 1.05 U/ml PMG, 156 U/ml PL). Strains BAFC 3095 and 3100 also demonstrated high production of PG, PMG and PL (Fig. 1).

As shown in Table 1, pectinolytic enzyme production proved to be dependent on medium composition. In a medium where pectin was replaced by glucose *C. truncatum* BAFC 3094 attained greater growth but no PL activity could be measured, and PG and PMG activities noticeably decreased. The low synthesis of galacturonases in cultures containing glucose suggests that these enzymes are constitutive in contrast with lyase, which was not detected. Nevertheless, differences between *in vivo* and *in vitro* production, due to plant–pathogen interactions, cannot be ruled out. In a previous study, *B. cinerea* demonstrated constitutive production not only of endo- and exoPG but also of PL, and their patterns of *in vitro* production were correlated with *in vivo* production during the infection of bean leaves.²¹ Also *Sclerotinia sclerotiorum* produced PG and PMG constitutively in a medium with glucose as carbon source.³⁴ Conversely trace amounts of PL activity were detected when *C. lindemuthianum* was cultivated in a medium with glucose, most probably corresponding to a constitutive enzyme required to start depolymerization of pectin when the organism is confronted with this substrate.¹⁶ Two regulation mechanisms are thought to occur during pectinolytic enzyme secretion by pathogenic fungi: (i) the enzyme is specifically induced by the substrate (i.e. pectin) or (ii) the enzyme is constitutive, but its expression is restricted by the presence of simple sugars (catabolite repression).^{3,13,14,17,38} Nevertheless, PL production may also be affected by the initial pH of the medium. The initial pH of the medium and its development during the incubation period influenced pectin and pectate lyase production in previous studies.^{9,41} In some anthracnoses, the launching factor to disease development is a PL produced by the fungus. High pH levels at the infection area, promoted by ammonia-releasing compounds secreted by the fungus, enhance not only the production and activity of this enzyme, but also the extent of disease.³² In this study an increase of pH of both media during culture time was detected, reaching final pHs of about 8.

PG and PL activities obtained from *C. truncatum* strains fall within the range of other phytopathogenic fungi grown in a medium with pectin. *Thanatephorus cucumeris* produced up to 25 U/ml of PL²⁰ and *F. oxysporum* around 180 U/ml of PL⁴; PG-production by *F. oxysporum* f. sp. *niveum* reached a maximum of 0.4 U/ml⁴² while *C. lindemuthianum* produced 0.24 U/ml.¹⁷

In a previous study a comparison between pathogenic and non-pathogenic strain of *C. lindemuthianum* revealed significant differences in terms of PL production in liquid culture. On 92%-esterified pectin, the pathogenic strain had more PL activity and cell walls isolated from its host (*Phaseolus vulgaris*) induced PL only in the pathogenic strain.¹⁶ These authors hypothesize that differences in pathogenicity may in part reside both in the amount and time-course of PL production. In the present study, the disparity observed in enzyme production among strains cannot be related to fungal growth, since no major differences in mycelial yield were found, nor was it connected with their geographic origin, but might be associated with differences in virulence, not yet evaluated. Recently, when

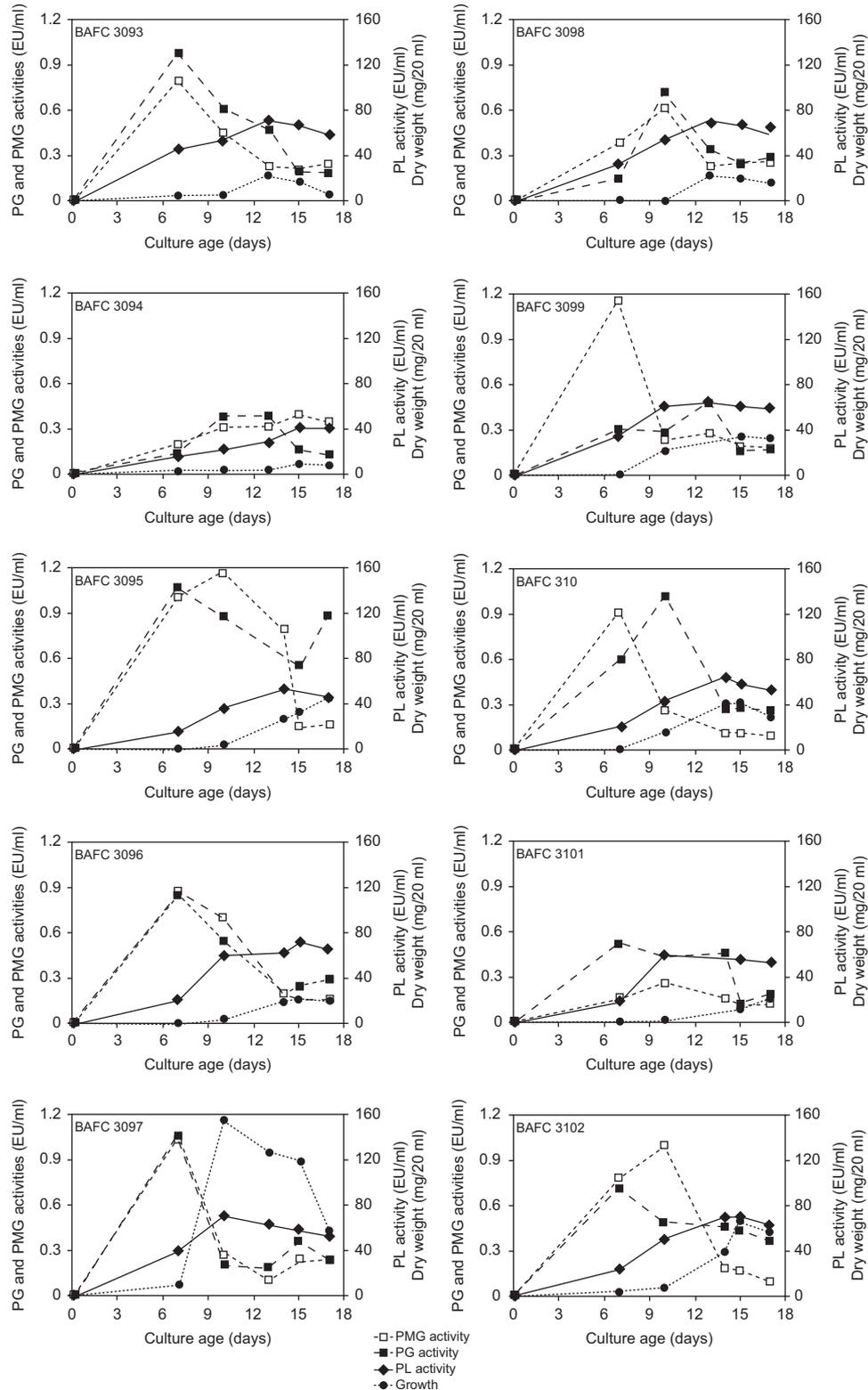


Figure 1. Kinetics of growth and *in vitro* pectinolytic enzyme production on a defined medium based on pectin as carbon source, by ten Argentinean pathogenic strains of *C. truncatum*, isolated from diseased soybean plants.

measuring genetic variability within isolates of *C. truncatum* from Argentina in terms of percentage of polymorphic loci we also found a high degree of polymorphism (up to 90%). With the primer used in the study we did not find any relation between

the grouping of isolates and their geographic origin.³³ The aim of prospect studies will be to test the hypothesis that variations in virulence among strains of *C. truncatum* causing anthracnose in soybean may be related to differences in pectinolytic activity.

Table 1
Growth and pectinolytic enzyme production by *C. truncatum* BAFC 3094 with glucose or pectin as carbon source.

| Carbon source | Dry weight (mg/20 ml) | PMG activity (EU/ml) | PG activity (EU/ml) | PL activity (EU/ml) |
|---------------|-----------------------|----------------------|----------------------|---------------------|
| Glucose | 89 ^{(10)*} | 0.05 ⁽¹⁰⁾ | 0.08 ⁽¹⁰⁾ | 0 |
| Pectin | 42 ⁽¹⁵⁾ | 0.40 ⁽¹⁵⁾ | 0.39 ⁽¹⁰⁾ | 9 ⁽¹⁵⁾ |

* The numbers in parentheses indicate the days when these maxima were achieved.

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