Intraspecific variability in growth and in vitro production of plant cell wall-degrading enzymes among Argentinean isolates of *Colletotrichum graminicola*, a maize pathogen

Variabilidad intraespecífica en el crecimiento y la producción in vitro de enzimas degradadoras de pared celular vegetal entre aislamientos argentinos de *Colletotrichum graminicola*, un patógeno de maíz

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Resumen

Introducción y objetivos: *Colletotrichum graminicola* (Glomerellaceae, Glomerellales) el agente causal de la antracnosis del maíz es dependiente de la actividad de enzimas degradadoras de la pared celular vegetal, para penetrar en su hospedante. La producción de estas enzimas se considera un factor de virulencia. El objetivo del presente trabajo fue investigar si existe variabilidad entre aislamientos en la capacidad de crecimiento y producción *in vitro* de diversas enzimas involucradas en la degradación de pared celular vegetal.

M&M: Se evaluó la habilidad de ocho aislamientos de *C. graminícola* para crecer y sintetizar enzimas con actividad poligalacturonasa, polimetilgalacturonasa, β-glucosidasa y lacasa en cultivos líquidos utilizando dos medios de diferente composición.

Resultados: La producción de poligalacturonasa, polimetilgalacturonasa y β-glucosidasa difirió marcadamente entre aislamientos y medios de cultivo. Se detectó actividad lacasa sólo en tres de los aislamientos. Los máximos títulos enzimáticos obtenidos fueron respectivamente de 250, 280, 45 y 63 U/l. La variabilidad intraespecífica registrada en la producción enzimática es consistente con la alta variabilidad intraespecífica observada a nivel genético cuando se evaluaron marcadores moleculares ISSR.

Conclusiones: Los aislamientos de *C. graminicola* investigados mostraron notables diferencias en cuanto a la producción de enzimas degradadoras de pared celular vegetal, no asociadas a su capacidad de crecimiento. Esto indica una importante variabilidad intraespecífica que debería tenerse en cuenta al seleccionar un método para combatir a este patógeno.

Palabras claves

Antracnosis, *Colletotrichum graminicola,* enzimas degradadoras de pared celular, maíz.

Summary

Background and aims: *Colletotrichum graminicola* (Glomerellaceae, Glomerellales), the causal agent of maize (Zea mays) anthracnose, as many other fungal pathogens, relies on its battery of cell wall degrading enzymes (CWDEs) to make its way through the cell walls of the host, and thus the production of these enzymes is considered a virulence factor. The aim of this work was to investigate if there is intraspecific variability in growth and in vitro production of several extracellular CWDEs among Argentinean fungal isolates of *C. graminicola*.

M&M: Eight isolates of *C. graminicola* were tested *in vitro* to evaluate growth capacity and polygalacturonase, polymethylgalacturonase, β-glucosidase and laccase production, using two different liquid culture media.

Results: Polygalacturonase, polymethylgalacturonase and β-glucosidase production greatly varied among isolates and culture media. Laccase activity was detected only in three isolates. Utmost enzymatic titres attained were respectively 250, 280, 45 and 63 U/l. The observed intraspecific variability in CWDEs in vitro production is consistent with the high variability found at genetic level when assessing ISSR markers.

Conclusions: The isolates of *C. graminicola* evaluated showed notable differences in CWDEs production, not associated with a differential growth. This indicates a large intraspecific variability, which might be considered when choosing a method to deal with this pathogen.

Key words

Anthracnose, cell wall degrading enzymes, *Colletotrichum graminicola,* maize.

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Introduction

The prevalence of infections in crops has increased considerably lately, affecting harvests around the world. Among all biotic threats, fungi and oomycetes are the ones that pose the greatest risk to global food security. Diverse factors are involved in this phenomenon, which include changing climate conditions and agricultural practices. Higher temperatures in mild winters allow pathogens survival and expansion to new areas, and accelerate their life cycle (Bebber & Gurr, 2015). Many of these tropical fungal pathogens are expanding to subtropical areas, setting new threats to agricultural production.

The Ascomycete *Colletotrichum graminicola* (Ces.) Wils. (Glomerellaceae, Glomerellales) is recognized in Argentina as an important pathogen in maize (*Zea mays* L.), causing anthracnose and stalk rot (Díaz *et al*., 2012). This disease is relatively new in the country: during the 1970's was considered only as a sorghum pathogen (Fernández-Valiela, 1979). *C. graminicola*, as initially described by Wilson, was a single cosmopolitan species with a very wide host range among grasses. However, improved understanding of *C. graminicola* taxonomy has confirmed that the fungus is specific to *Z. mays* (Belisário *et al*., 2022). According to Dean *et al.* (2012), virtually every crop grown is susceptible to at least one species of *Colletotrichum* spp. These fungi cause anthracnose spots and blights on aerial plant parts and post-harvest rots, generating major losses to economically important crops. Anthracnose in maize causes important yield losses, because it prevents the kernel´s development or damages the stems before harvest. No-till farming techniques without a proper crop rotation have also increased the incidence and severity of maize anthracnose in both North and South America (da Costa *et al*., 2014). Estimates of yield reductions are as high as 10 to 20% worldwide (Belisário *et al*., 2022). In nearby countries such as Brazil, this disease has been responsible for most yield losses of maize (Sukno *et al*., 2008). *C. graminicola* has been reported as one of the most frequently found fungal pathogens in Argentina recently; specially affecting second season maize crops (De Rossi *et al*., 2016).

Cell wall degrading enzymes (CWDEs) allowed host penetration and colonization, and are utilized to obtain nutrients from plant polymers. These enzymes include ligninases, pectinases, cellulases, hemicellulases and various other hydrolases that target the cell wall polymers (Kubicek *et al*., 2014). The amount and variety of enzymes released depends on the pathogen's lifestyle. Most necrotrophs discharge copious amounts of enzymes, in an attempt to degrade as much as possible before the plant is able to stand an effective defence; while biotrophs secrete relatively few CWDEs, thus operating by stealth and minimizing host damage. This difference in enzyme production is also reflected in the pathogen's genome: biotrophs encode less CWDEs when compared to other necrotrophic pathogens (Wang *et al*., 2022). *C. graminicola,* like most *Colletotrichum* spp. species, has a hemibiotrophic lifestyle. During the first stages of the disease, it lives as a biotrophic pathogen, spreading through the plant tissue while inflicting minimum damage to the host, before shifting to a more aggressive necrotrophic stage, where extended areas of the host tissue are killed (Münch *et al*., 2008; Torres *et al*., 2016). CWDEs are required for virulence in many phytopathogenic fungi (Wijesundera *et al*., 1989; Paccanaro *et al*., 2017; Ma *et al*., 2019). Several studies have demonstrated the importance of pectinolytic enzymes in plant invasion and symptom development (Reignault *et al.*, 2008; Armesto *et al*., 2019). Ramos *et al*. (2010) showed that the disparities in the production of polymethylgalacturonase, polygalacturonase and pectin lyase activities by *C. truncatum* isolates (the causal agent of soybean anthracnose) were not related with fungal growth or geographical origin, but instead were associated with differences in virulence among isolates. Ligninolytic oxidative enzymes such as laccase also play an important role in tissue maceration, as lignin is a complex aromatic polymer that hinders enzyme diffusion and can alter the extent of the enzymatic degradation of other cell wall polymers. These enzymes also show a protective effect on the phytopathogen, as they are able to detoxify phytoalexins and other phenolic compounds that are involved in host's defence mechanism (Vetchinkina *et al*., 2022).

Considering that maize anthracnose is a relatively new disease in Argentina, a better understanding of the physiological behaviour of local isolates of this fungal pathogen is key to prevent and/or treat

the disease, and will contribute to an ecologically sustainable integrated management. In this study, we analysed the intraspecific variability of eight native isolates of *C. graminicola* (obtained from symptomatic maize plants collected in different production sites in Argentina), related to growth and activity of different extracellular CWDEs putatively involved in fungal penetration and colonization.

Materials and Methods

Fungi

The isolates of *C. graminicola* were obtained from lesions of symptomatic maize plants. They were collected from eight Argentinean localities in the provinces of Buenos Aires [CG 4(6) (Arrecifes 34° 04′ 00″ S, 60° 07′ 00″ W), CG20 (Rojas 34° 11′ 00″ S, 60° 44′ 00″ W), CG21 (Bolivar 36° 15′ S, 61° 06′ W) CG22(1) CG22(2), CG23(4) and CG27 (Pergamino 33° 53′ 01″ S, 60° 34′ 01″ W)] and Santa Fe [CG25(5) (Caseros 33° 03′ 00″ S, 61° 10′ 00″ W)]. The isolates are deposited at the Phytopathology Department, Agronomy Faculty, University of Buenos Aires, Buenos Aires, Argentina.

Culture media

Pectin culture (PEC) and Galhaup (GAL) media were used. Both media shared a basal medium consisting of: $MgSO_4.7H_2O$, 0.5 g; HK_2PO_4, 0.6 g; H_2KPO_4, 0.5 g; $CuSO_4.5H_2O$, 0.4 mg; $MnCl_2.4H_2O$ 0.09 mg; H_3BO_3, 0.07 mg; $NaMoO_4.2H_2O$, 0.02 mg; $ZnCl_2$, 2.5 mg; $ZnCl_2$ 2.5 mg; $FeCl_3$ 1.0 mg; biotin 5 µg; thiamine 100 µg; distilled water up to 1000 ml. For PEC, pectin from apple (10 g/l) and asparagine monohydrate (4 g/l) were added as carbon and nitrogen sources respectively, and additionally 0.5 g/l of $MgSO_4$ and 0.4 mg/l of $CuSO_4$ were supplemented to the basal culture medium (Ramos *et al*., 2010). For GAL, glucose (40 g/l) was used as carbon source, and 5 g/l of yeast extract, 5 g/l peptone, 0.25 g/l $CuSO_4$ and 1 g/l SO_4Mg were added to the basal culture medium (modified from Galhaup *et al*., 2002).

Culture conditions

Erlenmeyer flasks (125 ml) with 25 ml of medium were inoculated with one agar plug (0.25 cm^2) cut out from a colony grown on Malt Extract Agar (MEA). Incubation was carried out at 23 ºC under stationary conditions. Cultures were harvested at different incubation periods, filtered through a filter paper using a Buchner funnel and dried for 24 h at 70 ºC. Dry weight of mycelium was then determined. The culture supernatants were used as enzyme sources.

Enzyme assays

Polymethylgalacturonase (PMG) and polygalacturonase (PG) (endo and exo activities) were assayed using 0.1% apple pectin or polygalacturonic acid respectively, in 50 mM sodium acetate buffer (pH 4.8) at 30 ºC. Liberated reducing sugars were quantified by the Somogyi-Nelson method (Somogyi, 1952). One unit of enzymatic activity was defined as the amount of enzyme releasing 1 µmol of galacturonic acid per min. Laccase activity was measured using 2,6-dimetoxyphenol (DMP) 5 mM in 0.1 M sodium acetate buffer (pH 3.6) at 30 ºC (Paszczynski & Crawford, 1991). Oxidation of DMP was determined by the increase in absorbance at 469 nm (ε_{469} = 27 mM·cm^{-1}). β-glucosidase was determined in 50 mM sodium acetate buffer (pH 4.8) at 30 ºC, by measuring *p*-nitrophenol released from 0.02% *p*-nitrophenyl β-D-glucopyranoside (Wood & Bhat, 1988). Enzyme activities were expressed in International Units (U) as the amount of enzyme required to release 1 µmol of product in 1 min.

Statistical analysis

The data presented in graphics was analysed using Graph Pad Prism 9.4 software. Two way ANOVA tests were performed and the differences between treatments were compared by Tukey's multiple comparison tests (p<0.05).

Results and Discussion

Kinetics of growth (Fig. 1) and *in vitro* production of several extracellular CWDEs (Fig. 2) by eight fungal isolates of *C. graminicola* was characterized in two liquid culture media (PEC and GAL) based on pectin and glucose as carbon sources, respectively. A two-way ANOVA analysis revealed a significant effect of isolate, medium and interaction between factors (p<0.05) for each enzyme studied.

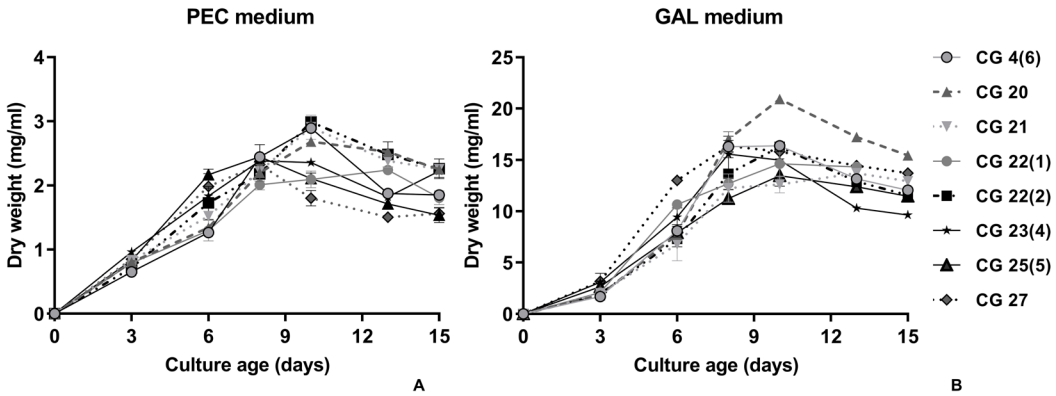

Fig. 1. Kinetics of growth of *C. graminicola* isolates. **A**: in PEC media. **B**: in GAL media. Each point represents the mean of three replicate experiments, error bars denote SEM.

All isolates were able to grow in both media, showing maxima between 8 and 10 days. More biomass was obtained in GAL (Fig. 1B) than in PEC medium (Fig. 1A) (approx. 21 mg/ml vs. 3 mg/ml), probably due to the more easily assimilable carbon source. Values of biomass yield attained in PEC medium were similar to those obtained when culturing *C. truncatum* in the same medium (Ramos *et al*., 2010).

No pectinolytic activity production was detected in PEC medium for some strains (Fig. 2A-B). Among CWDEs, those with pectinolytic activity have been the most extensively studied for their role in host-pathogen interactions and disease development. Pectinases in their multiple forms have proved to be important for the infection process, since they are the first polysaccharidases to be induced when fungi are grown on isolated plant cell walls, and are the first produced in infected tissue (Kikot *et al.,* 2009; Armesto *et al*., 2019). The disruption of pectinase genes reduces the virulence of fungi such as *Botrytis cinerea, Nectria haematococca, Penicillium digitatum* and *Aspergillus niger,* thus hampering their pathogenicity (Have *et al.*, 1998; Rogers *et al.*, 2000; López-Pérez *et al*., 2015; Liu *et al*., 2017). In this study, seven out of the eight isolates showed PMG activity in GAL medium, while only four isolates presented PMG activity in PEC medium (Fig. 2A). These isolates revealed PMG activities earlier in PEC than in GAL medium. In addition, isolates CG20 and CG22(2) showed PG activities formerly in PEC than in GAL medium. These findings might be attributed to catabolite repression by the abundant reducing sugars detected in GAL medium during the first days of incubation (data not shown). PMG activity was not detected in isolate CG22(1). Most isolates exhibited the highest activity after the day of maximum growth (up to 0.28 U/ml), on the contrary in *C. truncatum*, the peak of PMG activity preceded this day (Ramos *et al.*, 2010). All isolates showed PG activity in GAL medium, while only five of them showed PG activity in PEC medium (Fig. 2B). The highest activity (0.25 U/ml), was reached in GAL medium by isolate CG22(2). Most *C. graminicola* isolates tested here, showed the peak of PG activity after the day of maximum growth, as it was previously observed in isolates of another phytopathogenic ascomycete *Macrophomina phaseolina*, grown in similar culture and medium conditions (Ramos *et al.*, 2016). PG titres obtained by *C. graminicola* cultures in this work, resemble those attained by *C. lindemuthianum* (0.24 U/ml) (Hugouvieux *et al.*, 1997), and *Fusarium oxysporum* f. sp. *niveum* (0.4 U/ml), while *C. truncatum* and *F. graminearum* showed higher levels of PG (1.08 U/ml and 1.53 U/ml) (Kikot *et al.*, 2010; Ramos *et al.*, 2010).

All isolates showed β-glucosidase activity in both GAL and PEC media (0.01-0.045 U/ml) (Fig. 2C). As in other phytopathogenic fungi, such as *Fusarium* spp. (García-Maceira *et al*., 2000), cellulases secretion in *C. graminicola* occurred after the secretion of pectinases. The maximum of activity was detected after 15 days in all the isolates, when reducing sugars levels were at their lowest.

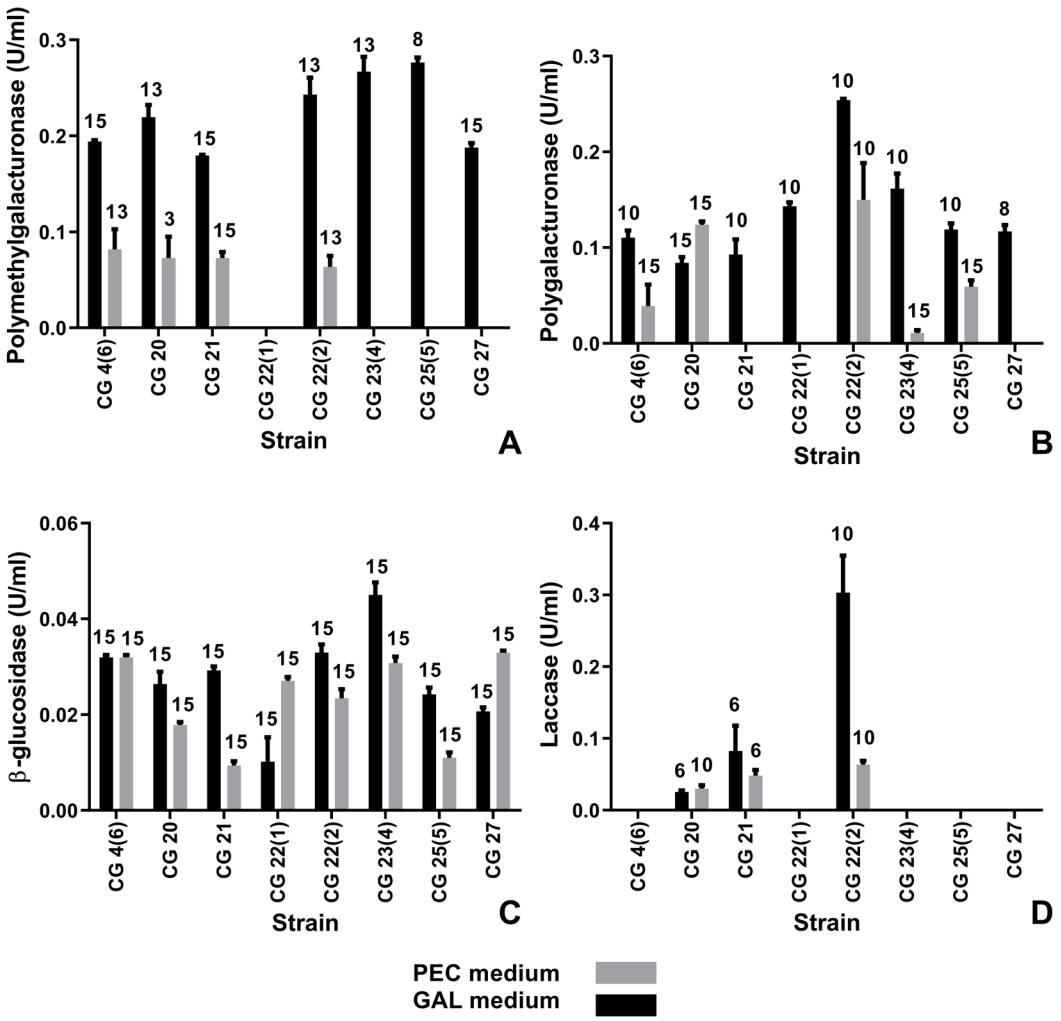

Fig. 2. Enzymatic production by *C. graminicola* isolates. **A**: Polymethylgalacturonase. **B**: Polygalacturonase. **C**: β-glucosidase. **D**: laccase, in PEC and GAL media. Numbers on top of the bars indicate the day of maximal activity, the values are the mean of three replications, error bars denote SEM.

A polyphenol oxidase characterized as laccase, was identified in the extracellular conidial mucilage of *C. graminicola* (Anderson & Nicholson, 1996) and might have a role in fungal survival and detoxification of plant phenols. Nevertheless, the roles of laccases in plant-pathogen interactions remain poorly understood (Levin *et al*., 2007), and mycelium associated laccase activity detected in this work might play a different function. Only three isolates showed laccase activity in both GAL and PEC media: CG20, CG21 and CG22(2) (Fig. 2D). The highest enzyme titres (0.063 U/ml) were obtained in GAL medium, mostly before the day of maximum growth. Similar laccase production was detected when growing *C. truncatum* in a liquid medium with pectin as carbon source, six out of ten isolates produced up to 0.044 U/ml laccase (Levin *et al*., 2007).

The results of this study show that *C. graminicola* has a high intraspecific variability when evaluating CWDEs *in vitro* production (*F*-values were respectively of 35.10, 10.64, 25.30 and 14.02 for PMG, PG, β-glucosidase and laccase, with *p*-values <0.0001), which is consistent with

the high variability observed on a genetic level when assessing ISSR markers in these same isolates (Gatica *et al*., 2014).

The pattern of *in vitro* production of CWDEs likely reflects the *in vivo* pattern of enzyme production, and their role in the pathogenic process: initial pectinolytic enzyme secretion being responsible for allowing access to other cell wall components, while subsequent cellulase activity contributing to cell lysis and tissue maceration. Similar patterns were described in other phytopathogenic fungi (Ten Have *et al*., 2002; Kikot *et al.*, 2009). Laccase activity could contribute to cell wall degradation in lignified tissues and protect against plant defence mechanisms. Additional work on the characterization of transcription and secretion of CWDEs in different phytopathogenic fungi will contribute to finding new specific targets to protect plants and accelerate the development of new agents for battling these fungi (Kubicek *et al*., 2014).

The assayed isolates of *C. graminicola* displayed notable differences in CWDEs production, not associated with a differential growth. This suggests a large intraspecific variability, which might be considered when choosing a method to deal with this pathogen. The aim of forthcoming studies will be to test the hypothesis that differences in aggressiveness among *C. graminicola* isolates causing anthracnose in maize are related with disparities in CWDE production.

Author Contributions

PN and IC designed and performed the laboratory experiments. PN and LL wrote the manuscript. All authors have read and approved the final version of the manuscript.

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