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Extracellular Enzymes of *Fusarium graminearum* Isolates

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ABSTRACT

Fusarium graminearum *isolates from three different agroecological regions in Argentina were examined according to the production of different extracellular enzyme activities of potential biotechnological interest: pectinases (PGase: polygalacturonase and PMGase: polymethylgalacturonase), cellulase (CMCase: carboxymethylcellulase) and hemicellulase (xylanase). The isolates were grown in minimum salt medium supplemented with 0.25% glucose, 0.125% citric pectin and 0.125% oat bran as carbon sources and/or enzyme inducers. PGase activity was detected early (after two days of incubation) in all the cultures; it was found to be the highest for all the isolates. PMGase was high only for those isolates of the II region. CMCase and endoxylanase activities were particularly found at late stages (after four and seven days of incubation, respectively) and the maximum values were lower than pectinase activities.*

Key words: Extracellular enzymes, *Fusarium graminearum*, pectinases, cellulases, hemicellulases

INTRODUCTION

Nowadays, enzymes have become an important tool to obtain various industrial products. Enzymes of different origin and activity are utilized in food, chemical, textile and pharmaceutical industries. Various species of filamentous fungi are the main organisms that produce industrial enzymes (Alkorta et al., 1998). Phytopathogenic fungi can produce highly specific enzymes utilized for degrading the cell walls, either in plant infected tissues (*in vivo*) or under culture conditions (*in vitro*). These fungi are of interest for the search of new enzyme activities due to their potential application in bioconversion processes (Riou et al., 1992).

Fusarium graminearum is an important phytopathogen of crop plants in Argentina. It is the causal agent of the Fusarium head-blight, a destructive disease of wheat, which under favorable environmental conditions can lead to serious economical losses in the agro-feeding industry. Its damage changes according to the geographical location of the infection, altering the quality parameters of grains, their weight, carbohydrate and protein composition.

Little is known about the mechanisms involved in the virulence degree of this phytopathogen in cereals. However, it could arise from qualitative and quantitative differences in the production of mycotoxins and enzymes that degrade the plant cell wall (Jenczmionka and Schafer, 2005). Gilbert et al. (2001) reported the production of several mycotoxins and ergosterol in *F. graminearum* isolates with different degree of damage on cereals. In *Fusarium* spp. different enzymes have been detected; they can degrade polysaccharides on the cell wall in corn grains (Marín et al., 1998) whereas xylanases and cellulases have been found on wheat leaves (Klechkovskaya et al., 1998). In addition, ß-glycosidase and xylanase activities were found to be incremented in rye grains

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(Schwarz et al., 2001). Cereal grains showed cavities and furrows in endosperm of starch granules, evidencing damage caused by amylases (Jackowiak et al., 2002) as well as protein degradation resulting from the presence of proteinases (Nightingale et al., 1999; Pekkarinen et al., 2000).
In biotechnological processes, i.e., bioconversion of plant biomass, it is convenient to use enzyme mixtures with complementary activities. Thus, new enzymatic pools must be analyzed. In this study, the production of different enzyme activities obtained by different local isolates of *F. graminearum* were characterized taking into account their potential biotechnological application.

MATERIALS AND METHODS

Biological material and inocula
Isolates of *F. graminearum* from different agroecological regions (II, IV and V; three isolates per region) in Buenos Aires province, Argentina, were utilized. These regions show the greatest production of wheat in Argentina. Isolates were kept in tubes with 2% potato-agar under a layer of mineral oil at 4ºC. Inocula were prepared from 10-mm plugs cut out from the margin of a 5-day-old colony growing on Petri dishes (2% potato-agar) at 26ºC.

Culture medium
Different isolates were cultured in Czapek-Dox medium, modified by Reyes and Byrde (Martínez et al., 1991), supplemented with yeast extract (0.1%). Glucose (0.25%), citric pectin (0.125%) and commercial oat bran (0.125%) were used as carbon source and/or enzyme inducers.

Culture conditions – Enzyme crude extract
Cultures were performed in 100-mL Erlenmeyers with 20 mL culture medium. After inoculation, they were cultivated at 28ºC in darkness at 150 rpm. The supernatant was separated from the mycelium by centrifugation at 7,650 × *g* for 30 min. Supernatant was stored at -20ºC until use.

Determination of enzyme activities
Pectinases
Polygalacturonase (PGase) and polymethylgalacturonase (PMGase) activities were determined at 40ºC with 0.1% polygalacturonic acid and citrus pectin (Sigma) as substrates, respectively in acetate buffer (50 mM, pH 5.0) using galacturonic acid as standard.

Hemicellulases
Xylanase activity was measured at 50ºC with 0.2% oat spelt xylan (Sigma) as substrate in acetate buffer (50 mM, pH 5.0) using xylose as standard.

Cellulases
Endo-1,4-β-glucanase activity (CMCellulase) was determined at 50ºC with 1% carboxymethylcellulose (Sigma) as substrate in acetate buffer (50 mM, pH 5.0) using glucose as standard.
All enzyme activities were determined by measuring the formation of reducing groups by the Somogyi-Nelson method (Somogyi, 1952). One enzymatic unit was defined as the amount of enzyme necessary to release 1 μmol of reducing sugars per minute under the above mentioned reaction conditions.

RESULTS AND DISCUSSION

The wheat producing regions in Argentina were grouped according to the grade of damage attributable to *F. graminearum* in: slight (IV and V regions), intermediate (II region) and serious (I and III regions). In this work, isolates from IV, V and II regions were examined according to the *in vitro* production of different enzyme activities. In general terms, the substrates utilized for inducing these enzymatic activities showed no specificity, perhaps due to the fact that they were not pure in most cases, with no clearly defined composition. Nevertheless, the combined use of this type of substrates was adequate from a qualitative and quantitatively point of view to enhance the production of plant cell wall degrading enzymes (Alconada and Martínez, 1994; 1996).
All the isolates of *F. graminearum* grew in a homogeneous and disperse filamentous form. They produced different extracellular enzyme activities which were responsible for the degradation of plant cell-wall polysaccharides.

Pectinase activities were the first to be detected on the 2nd day of culture in all the isolates. PGase activity was detected at different incubation times, which were very high from the beginning,

reaching a maximum of 5,400 mU/mL on 7th day of culture (IV and V regions) (Fig. 1 A). Despite the fact that the biological function of PGase activity is controversial concerning the plant-pathogen interaction, it is considered as an important parameter in these interactions. In *F. solani* and *F. oxysporum*, a strict correlation was found between its production and degree of damage observed (Lang and Dornenburg, 2000). PMGase activity was smaller than PGase activity, reaching its maximum value on 7th day (1,530 mU/mlL in isolates of II region (Fig. 1 B). Xylanase activity was detected on 7th day of culture, evidencing maximal values of 880 and 840 mU/mL in isolates from the II and V regions, respectively (Fig. 1 C). CMCase activity was detected on 4th day of incubation, showing maximum values similar to those found in the different groups ranging between 7th and 9th day of incubation (320-330 mU/mL (Fig. 1 D).

Pectolytic enzymes were the first to appear and the major ones, mainly the PGase activity, while cellulase and xylanase activities appeared later and in a lesser magnitude. The *in vitro* enzyme production of the *F. graminearum* isolates could suggest that although similar enzymes were detected, their sequential and differential production profile might be related to differences in the aggressiveness of the isolates. Mechanisms involved in enzymatic activity induction in diverse microorganisms are regulated by certain factors, which may vary from one organism to another one. Thus, the existence of catabolic repression produced by those products resulting from the hydrolysis should be taken into account in the above mentioned systems (Sengrupta and Ghosh, 1991).

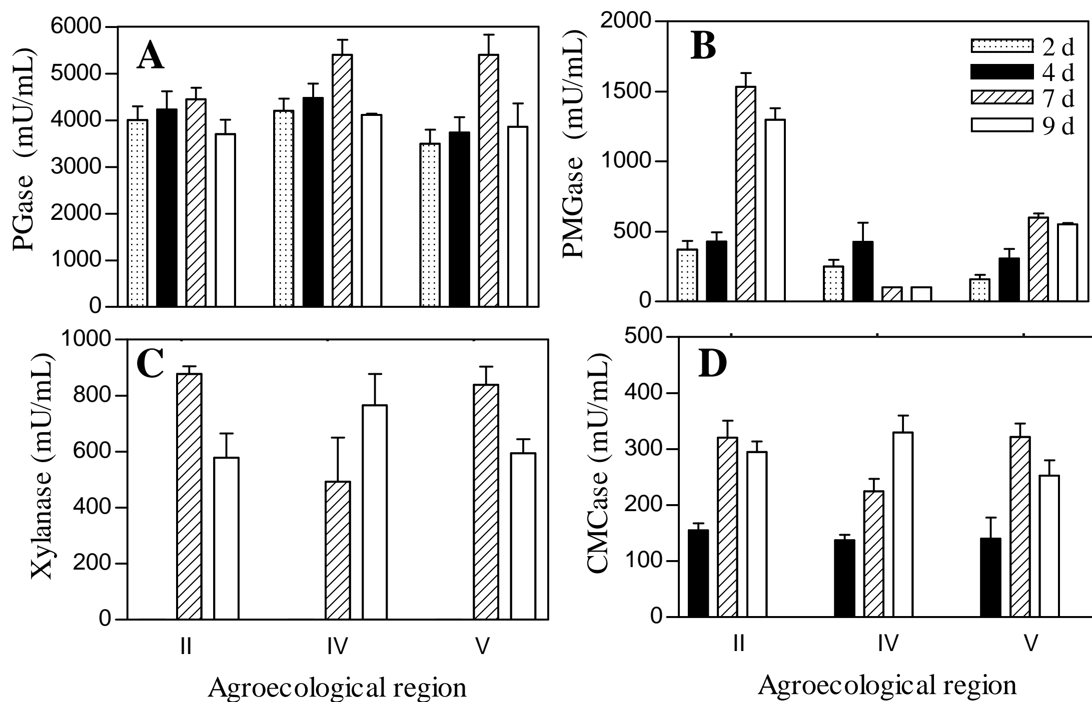

Figure 1 - Time course for the production of PGase (A), PMGase (B), xylanase (C), and CMCase (D) activities of *F. graminearum* isolates from different agroecological regions in Argentina. Values are means of 8 replicates +/- SD. See text for experimental details.

Due to the fact that the application of enzymatic systems containing pectinase, cellulase and hemicellulase activities is essential for the biomass conversion, the isolates under study constitute a tentative good enzyme source for this purpose. Pectic enzymes are among the most utilized commercially, with different biotechnological purposes (Alkorta et al., 1998; Kashyap et al., 2001). It must be noted that fungal PGases are mainly used in juice clarification (Lang and Dornenburg, 2000).

CONCLUSIONS

Different isolates of *F. graminearum* produced *in vitro* various extracellular enzyme activities related to the degradation of the main components of plant cell wall. Virulence of the isolates could be related to the capacity of producing the extracellular enzymes detected, involved in the fungal infection onto the host plant. The sequential production *in vitro* of the enzymes could be related to their production *in vivo* and to the tissue organization in the spike of the host plant. In this sense, pectinase activity was produced before than hemicellulases and cellulases, indicating that pectic enzymes were needed to increase the accessibility of cell-wall components for degradation by other enzymes, cell lysis and plant tissue maceration. It could be assumed that the pool of extracellular enzymes produced by *F. graminearum* could be a potential biocatalyst to be used for the bioconversion of plant materials.

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