Full Length Research Paper

Carbon sources and pH effect on pectinolytic activity production by *Ascochyta rabiei* isolated from chickpea (*Cicer arietinum L.*) in West Algeria

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In Algeria, *Ascochyta* blight is a major limiting factor in chickpea production. We did not record any difference between the isolates while basing ourselves on the morphological and cultural characters, but the pectinolytic activity could differentiate the ten isolates of *Ascochyta rabiei*. Activity of pectin methyl esterase was baseless in the presence of glucose, but variable in the presence of the citrus pectin and polygalacturonic acid. We recorded the weak production ranging between 0.19 and 0.55 µeg/ml/min; however the strong activity was between 2.97 and 5.88 µeg/ml/min in the presence of citrus pectin and polygalacturonic acid, respectively. On the other hand, the polygalacturonase activity was present in culture filtrates using three sources of carbon. Weak production was recorded in the presence of glucose (0.28 to 2.63 µmole/min), however the strong activity was recorded in the presence of 16.29 µmole/min of polygalacturonic acid. The maximal production of polygalacturonase was 11.7 µmole/min in the presence of the polygalacturonic acid and 0.9 µmole/min in the presence of glucose after six days of incubation at pH 5. The polygalacturonase activity was between 0.3 and 0.62 µmole/min after 4 days of incubation at pH 8. However, optimum production was obtained at pH 5.5. It should be noted that there is an important difference in the production of polygalacturonase with simple sugars glucose and pectin, indicating that the production of the enzyme is also stimulated by the presence of inducers. The exo-polygalacturonase is present in all carbon sources, suggesting that this enzyme could probably be of a constitutive nature in *Ascochyta rabiei*.

Key words: *Ascochyta rabiei*, pectinolytic activities, pectin méthylesterase, polygalacturonase.

INTRODUCTION

Ascochyta blight (*Ascochyta rabiei*) is a devastating disease of chickpea (*Cicer arietinurn*) and is considered a major limiting factor in chickpea production in the Mediterranean region. Management of *Ascochyta* blight requires an integrated approach, which often includes the use of certified disease-free seed, fungicide seed treatments, cultivar resistance and foliar fungicides. *A. rabiei* can infect the embryo or cotyledon of the seed and form mycelium and pycnidia on or just below the seed coat. Inoculum of *A. rabiei* may also reside on the surface of the seed (Dey and Singh, 1994; Kaiser and Kusmenoglu, 1997). Chickpea is the world's third most important pulse crop after bean (*Phaseolus vulgaris*) and peas (*Pisum sativum*). *Ascochyta* blight caused by *A. rabiei* (Pass.) is a

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serious foliar disease; the infection results to severe yield losses in Asian and African countries and all areas of chickpea production in the world (Singh et al., 1997; Ilarslan and Dolar, 2002; Gan et al., 2006). The complex chemical composition and physical structure of plant cell walls make them potentially pathogenic microorganisms and difficult to penetrate and degrade. Various polymers, including cellulose and protein, are embedded in a matrix of highly branched polysaccharides (Dean and Timber-lake, 1989). Many phytopathogenic fungi are known to produce range of cell wall degrading enzymes that macerate plant cell walls, including pectolytic enzymes such as pectin methyl esterase (PME), polygalacturonase (PG), pectin lyase (PNL) and pectate lyase (PL). These enzymes contribute in the process of infection and deve-lopment of disease in plants which is caused by phyto-pathogenic fungi (Chilosi and Margo, 1998; Douaiher et al., 2007). Pectin is a natural product which can be found in the cell wall of all higher plants. The role of pectinolytic enzymes in the pathogenesis and virulence of phyto-pathogenic microorganisms has been widely discussed. It has been suggested that differences in pectinase production in strains of pathogen may account for differences in the host range or in the virulence and in the extent of the damage and symptoms caused to the host (Comparini et al., 2000). Several studies, concerning the correlation between the *in vitro* enzymatic activities and the pathogenic power, showed that most aggressive isolates are the ones which produce most hydrolytics enzymes. The pathogen is frequently seed-borne, surviving for extended periods internally or externally on chickpea seed (Maden, 1983). This indicates that fungal metabolism becomes oriented towards synthesis and secretion of a whole arsenal of enzymes that are able to digest almost the complete plant cell wall. Pectinase is one of the proteins found after fungal growth on plant cell wall. Extensive knowledge of this very diverse exopro-teome is an important step towards the full understanding of fungal/plants interactions (Martínez-Trujillo et al., 2009). Plant pathogenicity and spoilage of fruits and vegetables by rotting are some other major manifesta-tions of pectinolytic enzymes (Singh et al., 2005). There is little published information on the factors that influence the efficiency of transmission of *A. rabiei* from infected seeds. In general, disease development is influenced by the plant, pathogen, environment and human activity (Agrios, 1997; Sharma et al., 2010). The main objective of this study was to determine the effect of different fac-tors such as carbon source, pH and operating parameter such as incubation time which could affect the production of PG from *A. rabiei* in culture filtrate.

MATERIALS AND METHODS

Fungal isolates and maintenance

A. rabiei isolates used in this study were collected from five different West regions of Algeria in 2006 and 2008. To isolate the fungus from diseased plants, stems showing typical *Ascochyta* blight symptoms were cut into 1 cm segments, surface-disinfested in 6% sodium hypochlorite for 3 min, rinsed in sterile distilled water for 3 min (Chen et al., 2004). Stem pieces were placed on culture medium CSMDA: 40 g chickpea seed meal, 20 g Dextrose (Sigma), 20 g Agar (Sigma), 1 L distilled water. Single conidia of *A. rabiei* were germinated and grown on CSMDA medium at 21°C with a 12 h alternating light-photoperiod (Khan et al., 1999; Ilarslan and Dolar, 2002; Bayraktar et al., 2007). After 6 days of incubation, the presence of *A. rabiei* was confirmed by microscopic observation of conidia at 100x magnification and pure cultures were obtained by single spore isolation. These isolates were used in the experiments and preserved for long-term storage as conidia and mycelia on sterile filter paper at -20°C (Wise et al., 2009).

Effect of different substrates on the mycelia growth

The isolates of *A. rabiei* were cultivated on medium CSMDA con-taining 1% of different carbon source such as glucose, citrus pectin and polygalacturonic acid. After 10 days of incubation at 22°C, the evaluation of the radial growth of the isolates was carried out by the measurement of two perpendicular diameters of the colonies. To evaluate the mycelia growth, we measured the diameter of each colony after 10 days of incubation, we took the average of two per-pendicular diameters of each colony, and then subtracted diameter of the plug of inoculums.

Sample preparation

To obtain extracellular enzymes, five 5 mm plugs of mycelia from a 10 day old culture were transferred to 50 ml of growth medium CSMD in 250 ml Erlenmeyer flasks and incubated with agitation at 125 rpm at 25°C for 10 days. Culture filtrate was collected on filter paper by filtration. This constitutes the crude enzymatic extract which will be analyzed for pectinolytic activities.

Pectin methyl esterase activity

For PME assay, the pectin-solution pH was adjusted to 7.0 with 0.02 N NaOH and the reaction mixture contained 25 ml of the crude enzyme, 5 ml of 0.2 M sodium oxalate and 25 ml of substrate. The reaction mixture was incubated at 30°C. During the course of the reaction, the pH of the reaction mixture was maintained at 7.0 with 0.02 N NaOH. The amount of 0.02 N NaOH added was recorded every 15 min. Enzyme activity was expressed as microequivalents of ester hydrolyzed per minute per ml (Abu-Bakr et al., 2003).

Polygalacturonase activity

Polygalacturonase activity was measured by titration of reducing groups with the iodine-thiosulphate method (Zaitlin and Coltrin, 1963; Sakai et al., 1982). The reaction mixture (15 ml) contained 12 ml of polygalacturonic acid (0.5%) in 0.025 M citrate-phosphate buffer (pH 4.0) with 0.02% sodium azide and 3 ml of the enzyme solution. After 2 h of incubation at 30°C, 5 ml of reaction mixture was withdrawn, allowed to react with 5 ml of O.1 N iodine solution and the residual iodine was titrated with 0.05 N $Na_2S_2O_3$ solutions. The difference in titer values between the control and the treated samples is expressed as enzyme activity. The solution was acidified with 10 ml of (1 N) HCl, and the remaining iodine was titrated with 0.1 N sodium thiosulfate solution. A blank titration without pectin was carried out at the same conditions, and the difference in titer gave the amount of 0.1 N (diiode) (I_2) solution reduced by 10 ml of pectin solution (Jermyn and Tomkins, 1950). Enzyme activity was expressed (one unit will liberate 1.0 µmole/min of galacturonic acid

from polygalacturonic acid per minute at pH 4.0 and 25°C)

Effect of pH on polygalacturonic activity

The CSMD liquid cultures supplied with 1% citrus pectin, glucose and polygalacturonic acid was used for the production of PG and PME. After autoclaving, the medium-pH was adjusted to pH 5 and 8 using either sterile 0.1 N NaOH or 0.1 N HCl. These mediums were inoculated by three pieces 5 mm diameter of marginal fresh colony, and incubated at 22°C for 10 days on a rotary shaker at 100 rpm. At 2, 4, 6, 8 and 10 days incubation, liquid from three flasks was collected and the mycelium was removed by filtration and weighed, then the culture filtrates were centrifuged at 8000 g for 20 min at 4°C and then the supernatants were dialyzed against distilled water at 4°C. Protein concentrations were determined by the method of Bradford, with bovine serum albumin used as the standard.

RESULTS AND DISCUSSION

Pectinolytic enzymes or pectinases are a heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in plants. Pectinolytic enzymes are widely distributed in higher plants and microorganisms. They are of prime importance for plants as they help in cell wall extension and softening of some plant tissues during maturation and storage. They also aid in maintaining ecological balance by causing decomposition and recycling of waste plant materials.

Effect of different substrates on the mycelia growth

Growth and production of pectinase by *A. rabiei* with different carbon sources are presented in Table 1. The radial growth of the isolates can be divided according to the carbon source, for a growth of 2.8 ± 0.6 to 3.5 ± 0.5 cm with glucose, 2.5 ± 0.8 to 3.3 ± 0.4 cm with citrus pectin and 1.8 ± 0.5 to 2.9 ± 0.2 cm with polygalacturonic acid. It appears that the diameter growth cannot be used reliably as a criterion to differentiate between *A. rabiei* isolates. A viability is also observed on the pigmentation of pycnidia, it varies from brown to black, but it is not always uniform for the same isolate. The attribution of a type of color is not clear in this case. Only a few isolateshad pycnidia uniform color (brown or black), but the majority had intermediate colors. As regards the alternating zones, pycnidia was lower among our isolates

The PME activity was measured in a quantitative manner according to the classical technique of reducing power. The activity with different carbon sources was variable. No activity was observed in the presence of glucose in all the isolates of *A. rabiei*. However, in the presence of citrus pectin, the lowest activity of 0.6 µeg/ml/min was obtained for isolate A30, the maximum value of 2.97 µeg/ml/min was observed for isolate A3 in the presence of citrus pectin and 5.88 µeg/ml/min in polygalacturonic acid. Among ten isolates, only six produced PME enzymes and the rate value varied from 0.6 to 5.88 µeg/ml/min. Four strains (A3, A26, A27 and

A30) produced PME in citrus pectin and five strains (A3, A11, A25, A26 and A31) in the presence of polygalacturonic acid.

The PG activity was found in all strains in the presence of all carbon sources, it was observed to be between 0.28 and 0.86 µmole/min for isolate A3. The activity of A30 is low; it is between 0.19 and 0.72 µmole/min in the presence of citrus pectin and polygalacturonic acid. However, it is 1.47 µmole/min in the presence of glucose. The activity of the strain A26 is higher with citrus pectin and glucose then polygalacturonic acid (Table 1). As compared to the low quantity and poor diversity of the proteins secreted after growth on glucose, this exoproteome displayed a great richness in very diverse cell wall degrading enzymes and reveals the probable high fungus reactivity towards environmental variations. The induction of protein secretion by growth on hop cell wall suggested that the fungus secretes proteins for degradation of the cell wall.

Effect of pH on polygalacturonic activity

According to results of the extracellular PME and PG activities, isolate A12 was selected for further optimal PG activity. When we compared the activity of PG among all the isolates, we noticed that the three isolates (A6, A27 and A12) recorded high activity (16.69, 8.37 and 7.94 µmole/min) of PG in the presence of polygalacturonic acid, against other isolates in the presence of citrus pectin activity (3.16, 1.46 and 0.23 µmole/min) among isolates A12, A27 and A6 and finally it was 2.41, 1.42 and 0.28 µmole/min among isolates A27, A12 and A6 in the presence of glucose, for this reason, we chose the isolate A12 for this test. Under laboratory conditions, when isolate A12 was grown in medium containing different carbon sources, it produced both PG at different pH, irrespective of the medium containing glucose, apple pectin and polygalacturonic acid (1%, w/v). The curve line PG was characterized by an increase in activity, reaching maximum value at 4th and 6th day, followed by a visible decrease. The exo-PG activity is expressed in the different media between pH 5 and 8 with the polygalacturonic acid and the pectin and it is not detectable on glucose (Figures 1 and 2). It has been shown that the exo-PG activity, the proteins concentration and fungal biomass production were proportional in the culture conditions and in the various carbon sources used. The maximal production of PG was 11.7 µmole/min in the presence of the polygalacturonic acid (3.47 µmole/min) with the citrus pectin and 0.63 µmole/min in presence glucose. During the growth, the pH of the culture was strongly changed in all cultural filtrate containing apple pectin, glucose and polygalacturonic acid. Isolate A12 was able to grow and produce PG in a broad pH range (5 and 8). However, optimum production was obtained at pH 5.
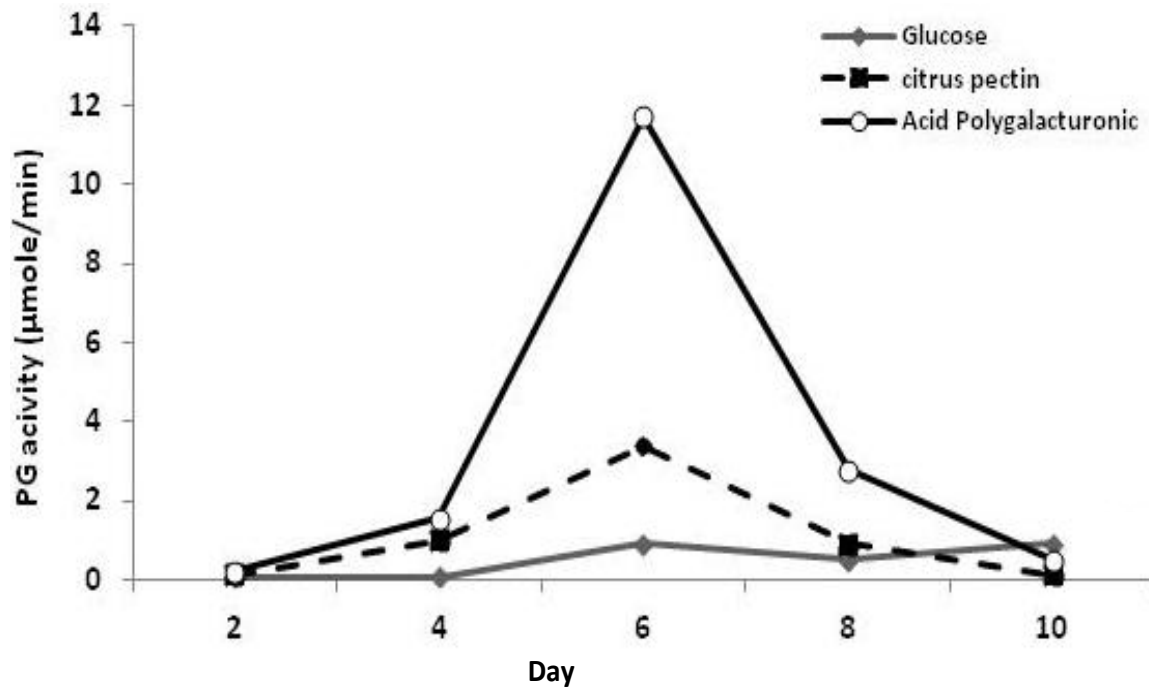
Figure 1. Influence of various carbon sources, pH 5 and incubation time on the polygalacturonase activity
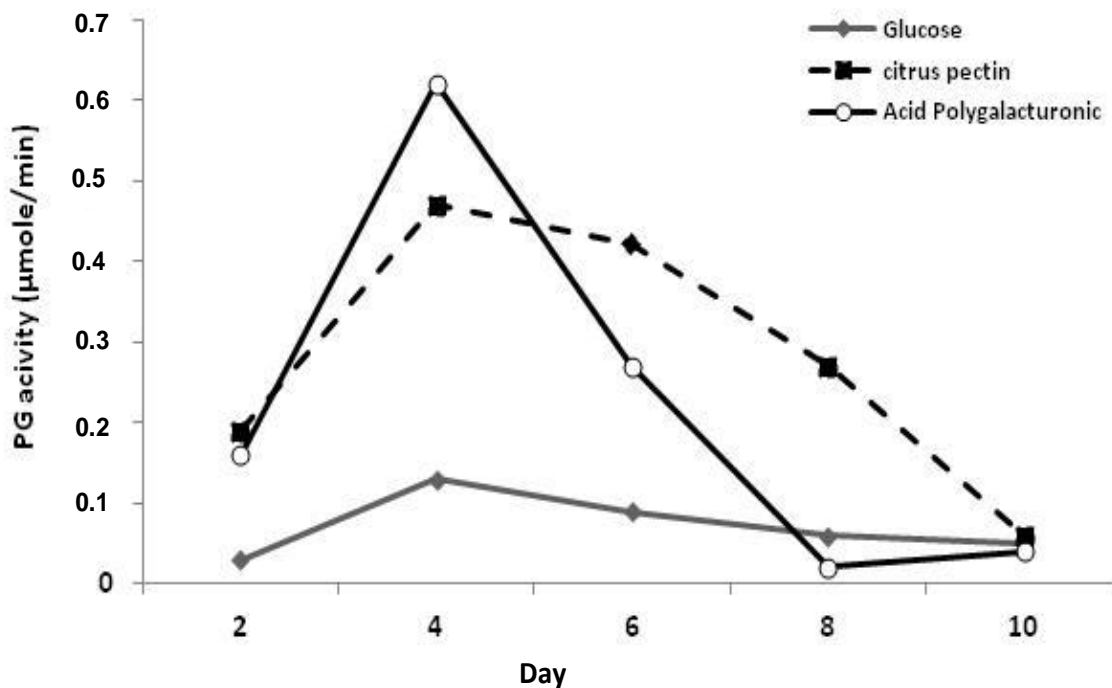

Figure 2. Influence of various carbon sources, pH 8 and incubation time on the polygalacturonase activity

Effect of pH on polygalacturonic activity

Several works could differentiate between fungi on the basis of the enzymatic activities produced *in vitro* and *in* *vivo* and various factors and suggest a key function of these enzymes in pathogenicity (Malvessi et al., 2004; Maccheroni et al., 2004). The variations in the activity of the pectic enzymes according to morphology and the cul-

tural aspect were announced in many other pathogens of the plants. Marchi et al. (2001) reported that the polygalacturonase and polymethylgalacturonase were detected in the culture filtrates of thirteen isolates of *Phaeomoniella chlamydospora*, and they did not record any difference between the isolates based on the morphological and cultural characters, but the pectinolytic activity could differentiate between these isolates. The study of the production of enzymes by fungi phytopathogens in the culture filtrates showed that they produced enzymes such as xylanase, cellulase and pectinase and the level of production is more important at the more aggressive isolates as compared to the other isolates which are slightly aggressive (Lalaoui et al., 2000; Douaiher et al., 2007), which could correlate between the production of these enzymes *in vitro* (filtrate of culture) and their role in pathogenesis. The works of Yoshida et al. (2003) showed the presence of three pectic enzymes (pectate lyase, polygalacturonase and pectin lyase) in the culture filtrate of *Rhizopus oryzae* cultivated in a liquid medium pectin and separately macerated roots. On the other hand, El-Hendawy et al. (2002) did not detect the activities of pectin methyl esterase and polygalacturonase in the culture filtrates, but they were present in extracts of roots of carrot and pepper fruits infected by *Erwinia*. In addition, the culture filtrates and extracts of infected vegetable tissues contained the activity of pectin lyase. Enzymatic activities of PME and PG were found in the filtrates of ten isolates. They are stronger and are detected more precociously in the filtrates. But these results are not enough to show the implication of these enzymes in the pathogenicity. It would be desirable to show the role of these enzymes in the pathogenic capacity of *Ascochyta* by seeking them rather directly on the level of the interactions of chick-pea/*Ascochyta*. It has been reported that microbial pectinases account for 25% of the global food enzymes sales. Almost all the comercial preparations of pectinases are produced from fungal sources. *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes (Singh et al., 2005; Martínez-Trujillo et al., 2009). Therefore, development of effective disease manag, identification and characterization of fungal species has been based on morphological characters such as size and shape of conidia and aspersoria, teleomorph state and cultural characters such as colony color, growth rate and texture. These criteria alone have not always been adequate due to overlap in morphological characters; and phenotypic variation among related species and under different environmental conditions. To overcome the inadequacies of these traditional techniques, polymerase chain reaction amplification of specific gene sequences was employed to characterize and analyze the taxonomic complexity of various genera (Taylor and Ford, 2007; White and Chen, 2007). PG was produced practically on

all carbon sources. PG productivity was measured also in media containing glucose as the only carbon source. This fact suggests that PG could be constitutive in *A. rabiei*. It should be noted that there is an important difference in the production of PG with simple sugars glucose and pectin, indicating that the production of the enzyme is also stimulated by the presence of inducers. A remarkable fact is that PG is present in all carbon sources, suggesting that this enzyme could probably be of a constitutive nature.

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