ГРИБЫ — ВОЗБУДИТЕЛИ БОЛЕЗНЕЙ РАСТЕНИЙ

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PECTOLYTIC ENZYMES PRODUCED BY *ALTERNARIA ALTERNATA* IN CULTURE AND DURING COLONIZATION OF COMMON WHEAT KERNELS

ОЗЕР Н. ПЕКТОЛИТИЧЕСКИЕ ФЕРМЕНТЫ, ПРОДУЦИРУЕМЫЕ *ALTERNARIA ALTERNATA* В КУЛЬТУРЕ И ПРИ ЗАРАЖЕНИИ ЗЕРЕН ПШЕНИЦЫ

Alternaria alternata (Fr.) Keissler is the fungus most commonly associated with black point disease in common wheat (Triticum aestivum L.). The disease is characterized by a brown to black discoloration at embryo end, the surrounding area and crease (Machacek, Greaney, 1938; Greaney, Wallace, 1943; Kilpatrick, 1968; Bhowmink, 1969; Conner, Thomas, 1985; Fernandez et al., 1994). Studies of host resistance, isolate virulence, environmental influence and the importance of kernel growth stages are restricted to the symptomatological observation or isolation of pathogen from ripe wheat kernels in the field or greenhouse (Machacek, Greaney, 1938; Adlakha, Joshi, 1974; Conner, Davidson, 1985; Conner, Thomas, 1985; Conner, 1987; Conner, 1989; Conner et al., 1990). In order to study the host-parasite relationship, it was also necessary to determine the ability of the pathogen for pectolytic enzyme production during kernel colonization.

Pectolytic enzymes such as pectin lyase (PNL) and polygalacturonase (PG) may have important roles in the infection process (Bateman, Basham, 1976; Collmer, Keen, 1986). PNL and PG have been shown to be produced in vitro by *A. alternata* isolated from rotting fruits of tomato, apple and lemon, only quantitatively (Mehta et al., 1974, 1975; Tak et al., 1985; Mahmoud, Omar, 1994; Omar, Mahmoud, 1994; El Shaieb, Malibari, 1995). Their role during the pathogenesis has been investigated only on tomato fruits (Mehta et al., 1974; Omar, Mahmoud, 1994). Differences in peroxidase from resistant and susceptible wheat cultivars to black point caused by *A. alternata* were observed using the isoelectric focusing (Williamson, 1997). To my knowledge, no information is available on the involvement of pectolytic enzymes in the development of *A. alternata* on wheat kernels.

The present study describes the capacity of *A. alternata* obtained from black pointed kernels to produce pectolytic enzymes and isoenzymes in culture and during the colonization of the common wheat kernels at different growth stages.

Material and methods

Seven *A. alternata* isolates, A1, A15, A18, A35, A46, A52, A60, obtained from embryo tissues of common wheat kernels with symptoms of black point in 2000 and 2001 were used in this study. They were cultured on potato carrot agar (PCA) containing white potato (20 g L^{-1}), carrot (20 g L^{-1}) and agar (20 g L^{-1}) at 21 °C for 10 days (Simmons, 1994). For the enzyme preparation, isolates were surface-cultured in Czapek's liquid medium (pH 5.0)

containing 10 g L^{-1} of citrus pectin (Sigma) as sole carbon source. The inoculum was one agar disc (6 mm in diam.) cut from the edge of cultures on PCA was used for inoculation. The cultures were statically grown for 7 days at 21 °C in 250 ml Erlenmeyer flasks containing 50 ml medium.

Healthy common wheat kernels (cv. Flamura 85) were collected from the experimental area of the Faculty of Agriculture in Tekirdap at five different growth stages including anthesis (Growth Stage, G. S. 10.5.2, Feekes scales; Large 1954), flowering complete (G. S. 10.5.4), milk (G. S. 11.1), mid-dough (G. S. 11.2) and ripe (G. S. 11.4). The kernel samples were surface-sterilized by immersion in 1 % sodium hypochlorite solution for 3 min and rinsed three times with sterile distilled water and dried on absorbent paper. Then, they were placed on sterile filter paper (blotter method) moistened with sterile distilled water in petri dishes. One isolate of *A. alternata*, producing the highest amount PNL and PG, was selected for inoculation of kernels. Inoculation followed the procedure described by Statler et al.(1975) with some modification. One drop of spore suspension, consisting of 1×10^5 conidia mL^{-1} in a 0.1 % aqueous solution of Tween 20, was placed on to each kernel. Eight drops of 25 % lactic acid were added to 1000 ml of the conidia suspension to retard bacterial growth. Kernels were incubated in Petri dishes enclosed in aluminum foil for 5 days at 21 °C. As a control, an aqueous spore-free solution of Tween 20 was used. Black point incidence was determined based on the percentage of kernels with symptoms of black point (a dark, brown to black discoloration). To check for the absence of contaminants, some infected kernels were aseptically placed on PCA in sterile Petri dishes, but only *A. alternata* grew under these conditions.

The experimental unit was a Petri dish containing ten kernels and there were twenty-five replicate Petri dishes of each growth stage arranged in a completely randomized design.

Seven days-old cultures of *A. alternata* isolates were collected from liquid media and the mycelium was removed by filtration in a Buchner funnel.

The enzyme preparations from the kernels were obtained by grinding infected tissues in an ice-cooled mortar in 0.05 M Tris-HCl buffer pH 7.8 (g tissue/mL^{-1}), containing 0.1 M KCl, 5 g L^{-1} cysteine and 10 g L^{-1} insoluble polyvinylpolypyrrolidone (Sigma). The slurry was then strained through three layers of cheesecloth.

Filtrates from liquid culture and inoculated kernels were centrifuged at 15 000 g for 20 min at 4 °C. The supernatant was dialyzed against several changes of distilled water at 4 °C and filter-sterilized through a 0.22 mkm membrane (Millipore). The same procedures were applied to uninoculated kernels.

All the activities were first assessed to determine pH and temperature conditions required for optimal enzyme activity. Activities were quantified under the pre-established optimal conditions.

PNL activity was assayed spectrophotometrically by measuring the absorbance at 235 nm. An increase in absorbance of 1.73 indicated the formation of 1 mkmol of unsaturated uronide (Zucker, Hankin, 1970). One unit of enzyme activity catalyzed the formation of 1 mkmol of unsaturated uronide min^{-1} from 2.5 g L^{-1} (w/v) citrus pectin in Tris HCl buffer (0.1 M, pH 8.5) at 35 °C.

PG activity was determined by measuring the increase of reducing-end groups over time. Reducing groups were measured by a modified method of Nelson (1944), using D-galacturonic acid (Sigma) as a standard. One unit of PG activity produced 1 mkmol of reducing group min^{-1} from 2.5 g L^{-1} polygalacturonic acid (Sigma) in sodium acetate buffer (0.1 M, pH 4.5), at 35 °C. All the enzyme assays were repeated three times.

Isoenzyme separation by isoelectric focusing (IEF) was realized horizontally on Mini IEF cell apparatus (Bio Rad) by using 0.4 mm thick polyacrylamide gels containing 5 % (v/v) ampholyte (Sigma) covering the pH range of 3.5—10.0. The gels were run at 200, 450, 600 and 950 V for 15, 30, 20 and 25 minutes, respectively. After IEF, the gels were overlaid with ultrathin (0.4 mm) agarose gels for PNL and PG isoenzyme detection, prepared as described by Ried and Collmer (1985). For PNL isoenzyme detection, the 10 g L^{-1} agarose (Sigma) gel contained 1 g L^{-1} pectin buffered at pH 8.5 with 50 mM Tris-HCl; for PG detection the 10 g L^{-1} agarose gel, contained 1 g L^{-1} polygalacturonic acid in 50 mM sodium acetate buffer, pH 4.5.

IEF polyacrylamide gels overlaid with ultrathin agarose gel were incubated at 100 % humidity for 60 min at 35 °C. Activity bands were visualized by staining the agarose overlay for 30 min in 0.5 g L^{-1} ruthenium red (Sigma), followed by rinsing in distilled water. PNLs and PGs appeared as white bands. The isoelectric point (pI) values of pectolytic isoenzymes were estimated from a regression equation of standard protein versus the distance migrated.

Data on disease incidence, PNL and PG activity were analyzed using variance and means were separated according to Duncan Multiple Range Test (P = 0.05).

Results and discussion

PNL and PG production by seven *A. alternata* isolates grown in a medium containing pectin as sole carbon source are shown in Table 1. All isolates produced PNL and PG. However, there were significant differences among the isolates, A18 and A52 had the highest ability to produce PNL in culture. PG production from the isolate A52 was also significantly higher than from other isolates, therefore it was chosen to inoculate the kernels.

The optimum temperatures and pH were 35 °C and 8.5 for PNL production and, 35 °C and 4.5 respectively, for PG production.

Black point symptoms were observed in the kernels inoculated with *A. alternata* at all growth stages but with significant differences in disease incidence (Table 2).

Black point incidence was significantly higher in the kernels at the stages of anthesis and complete flowering. However the inoculated kernels at the stage of flowering complete resulted in the highest level of black point.

PG production was observed in the extracts from the infected kernel at all growth stages tested (Table 2). However, PNL activity was not found in any of the extracts. PG activity was significantly higher during the colonization of the kernels by *A. alternata* at the stages of anthesis and flowering complete, especially in the latter stage. The activity decreased in the kernels of the following growth stages.

No PG or PNL activity was detected in the extracts from control kernel samples.

The dialysed extracts from culture filtrates and infected wheat kernels were subjected to thin layer polyacrylamide gel IEF and evaluated for PNL and PG isoenzymes. Two faint PNL bands with pI 9.3 and 8.9 were observed in the culture filtrates of the isolates A18, A46 and A52 (data not shown). Five PG isoenzyme bands focusing at pI 4.5, 5.2, 5.8, 7.5 and 8.4 were detected from the extracts of the isolates (Fig. 1).

All isolates produced the acidic pI 4.5, 5.2 and 5.8 bands. However, the pI 5.8, acidic PG band appeared very faint in the extract from the isolates A15, A18 and A46. In extracts from

Table 1

**Pectin lyase and polygalacturonase activity produced
by the *Alternaria alternata* isolates on liquid medium**

Isolates	Pectin lyase activity	Polygalacturonase activity
A1	1.43 b	1.11 bc
A15	0.86 bcd	1.53 bc
A18	2.78 a	1.83 b
A35	0.66 cd	1.71 b
A46	1.21 bc	0.88 c
A52	2.73 a	3.04 a
A60	0.57 d	1.53 bc

N o t e. Values presented are the means of three replicates. Means within a column followed by the same letter are not significantly different (P = 0.05).

Polygalacturonase activity from kernels inoculated with *Alternaria alternata* isolate A52 and disease incidence on kernel samples

Growth stage	Disease incidence, %*	Polygalacturonase activity**
Anthesis	48.00 a***	1.52 b
Flovering complete	55.21 a	1.89 a
Milk	22.53 b	0.17 c
Mid-dough	10.81 c	0.11 c
Ripe	10.22 c	0.09 c

* Disease incidence is expressed as percent black pointed kernels after inoculation with *A. alternata.* Values presented are the means of twenty-five replicates.
** Activity in reducing units per gram weight of extracted black pointed kernels after inoculation with *A. alternata.* Values presented are the means of three replicates.
*** Means within a column followed by the same letter are not significantly different (P = 0.05).

the isolates A1, A15, A18, A35, A52 and A60, alkaline PG bands at pI 7.5 and 8.4 were found, pI 7.5 being less distinct in A1, A18 and A52. The same isoenzyme patterns were not detected in extracts of the isolate A46.

Five PG isoenzymes at pI 4.5, 5.2, 8.1, 8.4 and 8.6 were detected from the kernels inoculated with the isolate A52 at the stages of anthesis and complete flowering (Fig. 2).

Two PG bands at pI 5.8 and 6.5 were also observed in the latter stage. The kernels inoculated with the pathogen at the stages of milk and mid-dough exhibited one PG bands at pI 8.6. The pathogen produced a PG band at only pI 7.5 in the kernels at the ripe stage. In accordance with the results obtained from quantitative PNL assays, no PNL isoenzymes were found after IEF in wheat kernels at all growth stages tested.
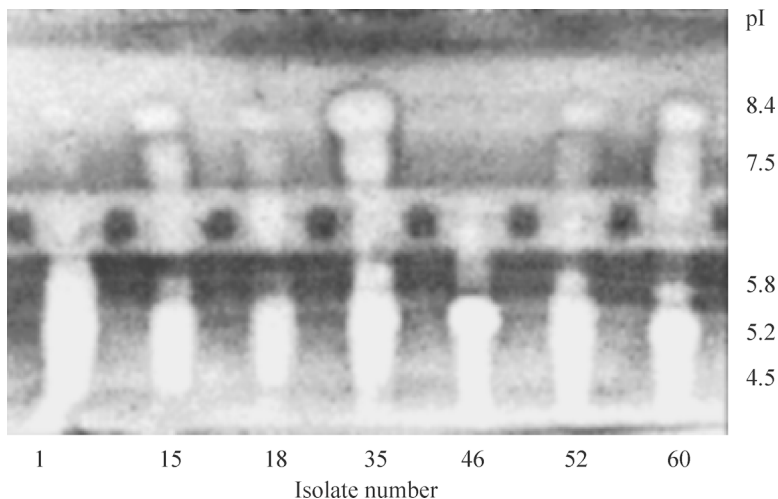

Fig. 1. Polygalacturonase isoenzyme pattern from culture filtrates of seven *Alternaria alternata* isolates grown in liquid medium supplemented with pectin as carbon source. Position and pI values of the polygalacturonase bands are indicated on the right axis.
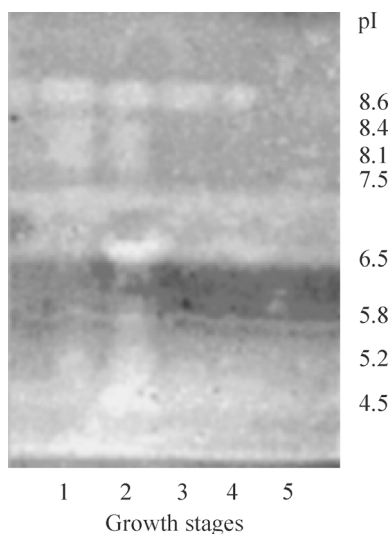
Fig. 2. Polygalacturonase isoenzyme pattern from inoculated wheat kernels with *Alternaria alternata* isolate A52 at five different growth stages, (1) anthesis, (2) complete flowering, (3) milk, (4) mid-dough, (5) ripe. Position and pI values of the polygalacturonase bands are indicated on the right axis.

PG and PNL production have previously been detected in crude extracts of *A. alternata* isolated from tomato, apple and lemon (Mehta et al., 1974, 1975; Tak et al., 1985; Mahmoud, Omar, 1994; Omar, Mahmoud, 1994; El Shaieb, Malibari, 1995). In the current report, it was shown that PG and PNL are expressed in the *A. alternata* isolates obtained from naturally infected common wheat kernels with black point disease. When isolate A52, with high PG and PNL production in liquid culture, was inoculated to wheat kernels at different growth stages, only PG was found. This result is not consistent with the finding of Mehta et al. (1974) and Omar and Mahmoud (1994) who recorded the role of both PG and PNL in tomato fruits infected with *A. alternata*. Pectolytic activity detected in inoculated wheat kernel appeared to be of fungal origin, since neither PG nor PNL activity was recorded in control tissues.

Three isolates produced very faint bands of PNL isoenzymes. The situation of PNL isoenzymes from the *A. alternata* isolates tested in present study might be due to the experimental conditions chosen for the growth of the isolates on culture medium. However, the absence of PNL and multiple PNL isoenzyme patterns from infected kernels indicates that this enzyme is not a cell wall-depolymerising factor during host tissue colonization.

A. alternata isolates in liquid culture had five PG izoenzymes, that were either acidic or alkaline. Acidic PG forms (pI 4.5, 5.2 and 5.8) were detected in extracts from all isolates. Isolates A1, A15, A18, A35, A52 and A60 expressed also basic PGs at pI 7.5 and 8.4. Isolate A52, selected for inoculation tests, produced all of them during fungal invasion of inoculated kernels at different growth stages.

The stage, at which plants were infected by *A. alternata* in the field, significantly influences the incidence of black point. Machacek and Greaney (1938) speculated that the infection occurred either at anthesis or during late stages of kernel development. Adlakha and Joshi (1974) reported high rates of infection at anthesis. Conner (1987, 1989) stated that black point incidence increased as the amount of irrigation and precipitation received during the milk, mealy or mid-dough stages increased. However, several reports noted that high black point incidences on ripe kernels could occur in susceptible common wheat cultivars artificially inoculated with *A. alternata* at anthesis (Conner, Davidson, 1985; Conner, Thomas, 1985; Conner et al., 1990) or mid-dough stage (Conner, Thomas, 1985). On the basis of these results, in vitro production of symptoms and pectolytic enzymes by inoculation of

detached kernels at different growth stages with spores of *A. alternata* may be warranted in order to clarify the role of the growth stages of the kernels for black point infection.

Pectolytic enzymes are supposed to be important virulence factors (Collmer, Keen, 1986; Alghisi, Favaron, 1995; Mendgen et al., 1996). In the present study, isolate A52 exhibited the highest PG activity and multiple PG isoenzyme patterns, and had the highest ability to infect wheat kernels at the stage of anthesis and complete flowering, especially at the latter stage. PGs at pI 4.5, 5.2, 5.8 and 8.4 from liquid culture were present in extracts from inoculated kernels at the stage of complete flowering. This result suggests that these PGs may be determinants for the colonization of wheat kernel. Three new PG isoenzymes at pI 6.5, 8.1 and 8.6 were also detected in the extracts from the kernels at this stage. However the pathogen did not produce PG isoenzymes at pI 5.8 and 6.5 in the kernels at anthesis stage.

My results demonstrated different degrees of susceptibility among the growth stages of wheat kernel, «complete flowering» being the most susceptible and «ripe» the most resistant. From the present study it also appears that some PG isoforms produced during invasion of different kernel tissues may be a pre-requisite for the infection of *A. alternata.*

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Р Е З Ю М Е

Проведена оценка активности пектинлиазы (PNL) и полигалактуроназы (PG) из культуральных фильтратов семи изолятов *Alternaria alternata*, полученных из семян мягкой пшеницы (Triticum aestivum), которые поражены черным зародышем. В результате изоэлектрического фокусирования были определены их изоэнзимы. Изоляты различались по активности ферментов. У трех изолятов наблюдались два слабых бэнда PNL. Все изоляты образовывали PG изоэнзимные паттерны с бэндами, различающимися по интенсивности и значениям pI. Здоровые зерна были отобраны в стадиях раннего цветения, полного цветения, молочной, молочно-восковой, восковой и полной спелости и затем инокулированы изолятами с высокой активностью ферментов. Определена частота встречаемости зерен с черным зародышем, а также PNL и PG активность. Ни в одной из стадий не было обнаружено активности PNL и ее изоферментов. Однако изоляты *A. alternata* различались по вирулентности, качественной и количественной продукции PG в зернах в различных стадиях роста. Полученные результаты показали, что PG может определять вирулентность в течение колонизации зерен.

Ключевые слова: *Alternaria alternata*, Triticum aestivum, пектинлиаза, полигалактуроназа.

SUMMARY

The culture filtrates of seven *Alternaria alternata* isolates obtained from the black pointed kernels of common wheat (Triticum aestivum) were assayed for pectin lyase (PNL) and polygalacturonase (PG) activity. After isoelectric focusing the patterns of their isoenzymes were defined. PNL and PG activity were detected in all isolates and the differences were also found in the activities of the isolates. Two faint PNL bands were observed in three isolates. All isolates produced PG isoenzyme patterns with bands differing in intensity and in pI values. Healthy wheat kernels were collected at the stages of anthesis, complete flowering, milk, mid-dough and ripe separately, and they were inoculated with an isolate being able to high enzyme activity. Infected kernels were sampled for the black point incidence and assayed for PNL and PG activity. No PNL activity and isoenzymes were found in inoculated kernels in any stage. However the isolates of *A. alternata* differed in virulence and in quantitative and qualitative PG production in the kernels at different growth stages. The results suggest that particular PGs from *A. alternata* may contribute to virulence during kernel colonization.

Key words: *Alternaria alternata*, Triticum aestivum, pectolytic enzymes.