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ENZYMATIC ACTIVITIES OF FUNGI ISOLATED FROM LETTUCE LEAVES

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ABSTRACT

Six fungal genera were isolated from lettuce leaves collected from the market during summer months in 2005. The fungal isolates were examined for their ability to produce five extracellular enzymes on solid media, they were also examined for production of one endoenzyme notably phosphatase. The results showed that all isolates were able to produce amylase through with variable magnitude; the highest activity of amylase was displayed by *Fusarium rousm* followed by *F. tricnictum*. Concerning lipase activity *Thielaviopsis* sp. followed by *F. rousm* showed the highest potential; *A. alternate* and *F. tricnictum* come next whereas *B. cinerea* and *S. botryosum* failed to produce lipase. Regarding protease enzyme only to isolates (*A. alternate* and *F. rousm*) to produce the enzyme. The present results also showed that *A. alternate* followed by *B. cinerea, F. rousm , S. botryosum* were able to produce cellulose whereas *F. tricnictum* failed to produce cellulose. On the other hand all isolates except *B. cinerea* did not produce phenoloxidase. The highest colonial diameter on medium was yielded by *S. botryosum* followed by *A. alternata, F. tricnictum, A. alternata, F. rousm* and *B. cinerea* respectively. The findings also showed that the cell-free extracts of the tested fungi catalyzed the phosphate hydrolysis adenosine 5-monophosphate (AMP) by *A. alternata, B. cinerea, S. botryosum, Thielaviopsis* sp., *F. tricnictum and F. rousm* in decreasing order respectively.

Keywords: *Alternaria alternata, Botrytis cinerea, Fusarium rousm, Fusarium tricnictum, Stemphylium botryosum, Thielaviopsis* spp., amylase, lipase, protease, cellulase, phinoloxidase and phosphatase, lettuce.

INTRODUCTION

Alternaria spp. cause s leaf spot disease. These leaf lesions often exhibit a zonate appearance [1]. *Botrytis cinerea* is able to infect a wide spectrum of host plant species whereas other *Botrytis* species are confined to a single host species. All *Botrytis* species, whether specific or not, are necrotrophs implying they are able to kill host cells during the infection process [2]. The grey mold disease caused by *B. cinerea*, can invade susceptible plants at the seedling stage through maturity as well as causes a post-harvest decay[3]. *Fusarium* spp. were frequently isolated from lettuce leaves [4, 5, 6]. *Stemphylium botryosum* was reported as a leaf spot pathogen on numerous crops including lettuce [7, 8, 9, 10]. Black root rot of lettuce is caused by the fungus *Thielaviopsis basicola* [11] although triple red lettuce cultivar is resistant to *Thielaviopsis* [12]. The disease can occur at any stage of plant growth, from seedlings in containers to plants ready for market [13]. Enzymatic activity in a large number phytopathoginc fungi has been reported by many investigators [14,15,16,17, 18].

It has been authenticated that the infection of host plants by phytopathogic fungi is mediated and/or enhanced by numerous extracellular enzymes and metabolites elaborated by the pathogenic organisms.

The aim of this work was to isolate the pathogenic fungi from lettuce leaves and to examine their ability to produce extracellular enzymes (amylase, lipase, protease, cellulase and phenoloxidase) , in addition, to an endoenzymes (phosphatase).

MATERIALS AND METHODS

Isolation of lettuce fungi:

Lettuce leaves were collected from markets in June, July and August 2005 then were rinsed thoroughly with water, cut into small pieces and, they were surface sterilized by soaking in 2% of sodium hypochlorite solution for 30 seconds. All tissues were dried on sterilized filter papers and then placed onto Czapek Dox medium containing rose Bengal (to slow down the growth of fungi) and 5 µg of chloromphenicol or streptomycin sulfate (to inhibit the bacterial growth). The plates were incubated at 27°C. Hyphal tips of colonies growing out from the leaf tissue were transferred to fresh potato dextrose agar (PDA) slants. Pure cultures were identified according to morphology and sporulation. All fungi were grown on PDA plates, incubated at 27°C for 5-7 days and used as inocula for the following experiments [18].

Identification of fungi:

The fungi were identified at the Department of Plant Pathology, Faculty of Agriculture,Ain Shams University, Egypt.

Inoculum and culture conditions:

Agar discs containing mycelial growth of each fungus were cut from Petri dish cultures with a 5 mm diameter sterile cork borer and used to inoculate the test medium.

Enzymes assays:

Amylase: Amylase activity was assayed by growing fungi on Nutrient agar plus 0. 2 % soluble starch, PH 6.0. After 3-5 days, the plates were flooded with potassium iodide (KI) solution. A yellow zone around the colony in blue medium indicated amylolytic activity [14].

Lipase: Lipase activity was determined by growing fungi on a medium containing lipid (Tween 20) as a substrate.. The medium composed of (g/l): Peptone 1.0; yeast extract .10 and agar 15.0; Tween 20, was autoclaved separately, the medium was sterilized by autoclaving at spsi for 20 min. and added to the medium as a concentration of 10ml per liter. A positive test was performed by the occurrence of precipitated fatty acid crystals around the colony or the clearing of the medium as a result of the degradation of the fatty acids [19].

Protease: Protease activity was assayed using casein hydrolysis medium, which contained 1% skimmed milk. After incubation at 27°C , the diameter of the clear zone was measured [15].

Cellulase : Cellulolytic activity was tested by using cellulose medium (1.0g $NH_4 H_2 PO_4$) 0,2 g KCL, 0.2g $MgSO_4 . 7H_2O$; 0.2g $CaCl_2$; 250 ml of 4% ball

milled cellulose, 18.0 g agar and 750 ml distilled water) [20]. the medium was sterilized by autoclaving at 5 psi for 20 minutes and poured into 9 cm Petri dishes (15-20ml/plate). The solidified plates were inoculated with the tested fungi and incubated for 14-28 days. Cellulose agar plates involved in the incorporation of native cellulose into a besal medium, resulting in an opaque medium. Cellulase activity was then visualized by the formation of cleared zones around fungal growth [21, 22].

Phenolloxidase: This enzyme was tested on malt agar medium containing guaiacol as a phenolic compound. The mycelial growth presence and extent of the colored zone - under or around it - was recorded after 1-30 days incubation [16].

Acid phosphatase: The fungi were grown on Czapek Dox medium. Mycelia obtained in liquid Czapek Dox media were harvested by filtration, after 10 days of incubation, rinsed with distilled water, blotted on filter paper. The mycelia mass were homogenized with acid-washed sand (sea sand washed with sulfochromic solution and pa neutralized with water). The homogenate was centrifuged at 4°C for 10 min at 5000g, the supernatant was retained for immediate enzyme assay. Reaction mixtures contained 1.5 ml from 20µ moles of adenosine 5-monophosphat (AMP) and 0.5 ml of the last cell free extracts, 160 µ Na2CO3-NaHCO3 buffer at PH 9.2, liberated phosphate was measured spectrophotometrically at 530 nm. One unit of phosphatase activity was defined as the activity of the enzyme which released 1µ moles orthophosphate. Extracts were allowed to incubate at 40°C for 30 min with substrate, Protein determinations were made using the Lowry method using albumin used as the standard [23].

Statistical analysis

Data obtained were statistically analyzed by F test, Least Significant Differences (LSD) which was determined at 0.05 level of probability.

RESULTS AND DISCUSSION

Six fungal species namely: *Alternaria alternate, Botrytis cinerea, Fusarium rousm, F. tricnictum, Stemphylium botryosum* and *Thielaviopsis sp.* were isolated from letture leaves in summer 2005. All of these fungi are known as plant pathogens. The mycelial growth of tested fungi and their ability to produce enzymes on solid media is shown in Table (1).

Amylase: All of the isolates could degrade starch. The highest growth of mycelium were observed by the following fungi: *S. botryosum, F. tricnictum, Thielaviopsis* spp., *A. alternata, B. cinerea and F. rousm* respectively. The highest activity of amylase enzyme was revealed as: *F. rousm, F. tricnictum,* followed by *A. alternata* and *Thielaviopsis* sp., *B. cinerea* and finally, *S. botryosum.* Amylase activity of *F. rousm* twice *F. tricnictum*, 3.3-fold higher than *A. alternata* and *Thielaviopsis* spp., **followed by** *B. cinerea* 5- fold, and *S. botryosum* 10- fold. The statistical analysis showed a significant value between myceliel growth and the amylase production.

Table (1) extracellular enzymes activity produced by fungi which were measured by the diameter of clearing zones around fungal colonies (mm).

Fungi	amylase CD	amylase AZ	Lipase CD	Lipase AZ	Protease CD	Protease AZ	Cellulase CD	Cellulase AZ	Phenoloxidase CD	Phenoloxidase AZ
A. alternata	38	3	40	8	35	7	16	55	-	-
B. cinerea	35	2	10	0	0	58	25	15	+	+
F. rousm	18	10	30	11	31	6	65	15	-	-
F. tricnictum	40	5	40	8	34	0	0	0	-	-
S. botryosum	47	1	10	0	30	0	60	5	-	-
Thielaviopsis spp	39	3	10	5	30	0	0	0	-	-
F-test	9.13141		10.96774		0.335527		1.913178			

Note: CD = Colony diameter, AZ = Activity zone, enzymatic, production ratio in the case of phenoloxidase = The reaction was small and could only be measured as + or - , *F*-test is the analysis of variance for comparing between CD and AZ for each enzyme.

Lipase: Four of the tested fungi namely, *F. rousm, A. alternata* and *F. tricnictum* then *Thielaviopsis* sp. were able to produce lipase enzyme, while *B. cinerea and S. botryosum* were unable to produce lipase on the medium containing lipid (Tween 20) Table (1). The culture medium **elicited** enzyme production in *F. rousm* 1.38-**fold higher than** lipase production in *A. alternata* and *F. tricnictum* whereas it was 2.2-fold that of *Thielaviopsis* sp. *F*-test showed high significant differences between myceliel growth and lipase production. Fungal lipase enzyme plays a role in the infection of plants, Berto [24] found that spore surface-bound lipase interacted closely with epicuticular leaf waxes for adhesion and/or penetration of the fungal propagules during the early stages of host-parasite interactions [24].

Protease: The results in Table (1) show good growth of *A. alternata, F. tricnictum, F. rousm, and both S. botryosum* and *Thielaviopsis* sp. on casein medium. On the other hand, *B. cinerea* was unable to grow on casein hydrolysis medium. Protease enzyme was produced excessively by *B. cinerea, A. alternata* and *F. rousm*, respectively while *F. tricnictum, S. botryosum* and *Thielaviopsis* spp. were unable to produce protease although growth overall was good. The present results revealed that the magnitude of protease from *B. cinerea* was 8.29 fold higher than *A. alternata* and it was 9.67 fold higher than *F. rousm*. The statistical comparison showed significant difference between myceliel growth and protease production.

Cellulase: The cellulose medium presented excessive growth of *F. rousm, S. botryosum, B. cinerea* and *A. alternata* respectively while *F. tricnictum* and *Thielaviopsis* sp. were unable to grow on this medium. Cellulase activity was highest in *A. alternata,* followed by *B. cinerea, F. rousm* and *S. Botryosum* while *F. tricnictum* and *Thielaviopsis* spp. were unable to produce cellulase. In addition, Cellulase production in *A. alternata* was 3.67 fold higher than in *B. cinerea* and *F. rousm*, and it was 11-fold higher in *S. botryosum*. Cellulose medium showed inhibitory effects on *F. tricnictum* and *Thielaviopsis* sp. growth and cellulase production (Table, 1). *F*-test revealed a significant difference (significance level of 5%) between myceliel growth and the

cellulase production. Dahm[25] showed that the fungi which produce cellulase enzyme might become pathogenic to their hosts [25]. On the other hand, low cellulolytic activity may be preferable to the host to achieve limited penetration [26].

Phenoloxidase: All of the tested fungi were unable to grow on malt agar medium containing guaiacol except *B. cinerea*, whereas *B. cinerea* was the sole fungus which grew and produced phenoloxidase Table (1).

Phosphatase consists of a group of enzymes which catalyze the hydrolysis of phosphate monoesters. Fungi may release P from insoluble organic and inorganic forms [27]. All the lettuce fungi which were tested produced acid phosphatase. *A. alternata* produced 1.4 0 milliunit of acid phosphatase, 0.85 milliunit of *B. cinerea*, 0.70 milliunit of *S. botryosum*, 0.55 milliunit of *Thielaviopsis* spp, 0.38 milliunit of *F. tricnictum*, and 0.22 milliunit of *F. rousm* respectively Fig (1). These results are in agreement with other investigations [28] . The production of phosphatase may contribute to the increased phosphate uptake in fungi [25].

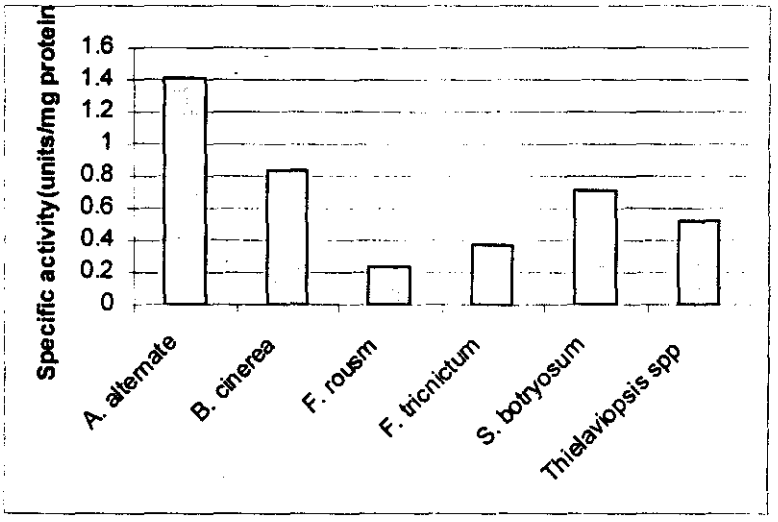

Fig (1) Acid phosphatase activity of lettuce fungi.

The infection of host plants by fungus is mediated by numerous extracellular enzyme and metabolites. Each of these compounds may play a role in different stages of the infection process [2]. The harvested products might have been infected by pathogens prior to harvest under field conditions or they might have been infected during transit and storage. The food crops are generally stored in a dry state with moisture level below 12%, to eliminate the adverse effects of mycotoxins produced by pathogenic fungi. The mycotoxins are known to be carcinogenic, causing several serious ailments in humans and animals [29].

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النشاطات الإنزيمية للفطريات المعزولة من أوراق الخس

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عزلت ستة أجناس فطرية من أوراق الخس، أثناء التسويق في أشهر صيف عام ٢٠٠٥ . اختبرت قدرة الفطريات المعزولة على إفراز خمسة إنزيمات خارجية، على مزارع صلبة، أيضا اختبر واحد من الإنزيمات الداخلية وهو الفوسفاتيز. وبينت النتائج أن النشاطات الإنزيمية الأعلى للأميليز، الليبيز، البروتيز، السيليوليز كانت في فيوزاريوم روزوم، ثم فيوزاريوم روزوم لليبيز، بوتريتس سنيري، و ألترناريا ألترناتا على التوالي، بينما لم يفرز فطري فيوزاريوم ترايسنكتوم و ثيلافيوبسس إنزيمي البروتيز والسيليوليز، ولم يفرز ستمفيليوم بوتريوزم إنزيمي الليبيز والبروتيز و بوتريتس سنيري لم يفرز الليبيز. كان أعلى نمو للغزل الفطري على البيئات لفطر ستمفيليوم بوتريوزم، ألترناريا ألترناتا و فيوزاريوم ترايسنكتوم، ألترناريا ألترناتا، فيوزاريوم روزوم و بوتريتس سنيري على التوالي. أوضحت النتائج أيضا أن المستخلص الخالي من الخلايا للفطريات المختبرة حفز التحليل المائي للفوسفات من أدينوسين ٥-أحادي الفوسفات (AMP) في ألترناريا ألترناتا، بوتريتس سنيري، ستمفيليوم بوتريوزم، ثيلافيوبسس، فيوزاريوم ترايسنكتوم و فيوزاريوم روزوم مرتبة تنازليا على التوالي.