

ENZYMATIC ACTIVITIES OF FUNGI ISOLATED FROM LETTUCE LEAVES

Al-Homeidan, Hoda H.

Botany Department, College of Education-Riyadh University for Girls
P.O. Box 27104, Riyadh 11417, Kingdom of Saudi Arabia

ABSTRACT

Six fungal species, namely: *Alternaria alternata*, *Botrytis cinerea*, *Fusarium rousm*, *F. tricinctum*, *Stemphylium botryosum* and *Thielaviopsis* sp. were isolated from lettuce leaves collected from Riyadh markets during summer months in 2005. The fungal isolates were examined for their ability to produce five extracellular enzymes, namely: amylase, lipase, protease, cellulase and phenoloxidase, on solid media. They were also examined for production of one endoenzyme which is phosphatase. The results showed that all isolates were able to produce amylase though with variable magnitudes; the highest amylase activity was displayed by *F. rousm* followed by *F. tricinctum*. Concerning lipase activity *Thielaviopsis* sp. and *F. rousm* showed the highest potential; *A. alternata* and *F. tricinctum* came next, whereas *B. cinerea* and *S. botryosum* failed to produce lipase. Regarding protease enzyme, only two isolates (*A. alternata* and *F. rousm*) were able to produce the enzyme. The present study showed also that *A. alternata* followed by *B. cinerea*, *F. rousm* and *S. botryosum* were able to produce cellulase, whereas the other two fungal species did not display any cellulase activity. On the other hand, all isolates, except *B. cinerea* do not produce phenoloxidase. Meanwhile, the largest colonial diameter on agar media was yielded by *F. rousm* followed by *S. botryosum*, *A. alternata*, *F. tricinctum*, and *B. cinerea*, respectively. This investigation also showed that the cell-free extracts of all the fungal isolates tested appeared to catalyze the phosphate (adenosine 5-monophosphate, AMP) hydrolysis; and the following sequence was displayed for acid phosphatase activity of the experimental fungal isolates: *A. alternata*, *B. cinerea*, *S. botryosum*, *Thielaviopsis* sp., *F. tricinctum* and *F. rousm*, in descending order, respectively.

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

INTRODUCTION

Alternaria spp. causes leaf spot disease. These leaf lesions often exhibit a zonate appearance (Pernezy and Raid, 2006). *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 that they are able to kill host cells during the infection process (Kars and Vankan). The grey mold disease caused by the fungus *B. cinerea*, can invade the seedling stage through maturity as well as causes a post-harvest decay (Van Kan, 2005). *Fusarium* spp were frequently isolated from lettuce leaves (Mercier and Reeleder, 1978; Garibaldi *et al*, 2002 & 2004). *Stemphylium botryosum* was reported as leaf spot pathogen on numerous crops including lettuce (Netzer *et al*, 1985 (Padhi and Snyder, 1954; Clancy and Brophy, 1989; Koike *et al*, 2001). Black root rot of lettuce is caused by the fungus *Thielaviopsis basicola* (Sala *et al*, 2003), although triple red lettuce cultivar is resistant to *Thielaviopsis* (Sala and Costa, 2005).

The disease can occur at any stage of plant growth, from seedlings in containers to plants ready for market (Gilardi et al, 2004). Enzymatic activity in a large number of phytopathogenic fungi has been reported by many investigators (Gabr and Mansour, 1982; Rodeia and Goncalves, 1986; Lunyong *et al*, 2002; Urairuj *et al*, 2003).

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 endoenzyme (acid 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 them in 2% of sodium hypochlorite for 30 seconds. All tissues were dried on sterilized filter papers and then placed on Czapek Dox medium with 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 potatoes 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 inoculums for the following experiments (Bahkali, 1999).

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 plugs 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 (Gabr and Mansour, 1982).

Lipase: Lipase activity was determined by growing fungi on a medium containing lipid (Tween 20) as the primary source of carbon. The medium contained the following (g/l) : peptone, 1.0; yeast extract, 0.10; agar 15.0; the medium was sterilized by autoclaving at 5 psi for 20 minutes. Tween 20 was sterilized separately and added to the medium at a concentration of 10 ml/l. 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 (Betro *et al*, 1997).

Protease: Protease activity was assayed using casein hydrolysis medium, which contained 1% skimmed milk. After incubation at 30°C , the diameter of the clear zone was measured (Lumyong *et al*, 2002).

Cellulase : Cellulolytic activity was tested by using cellulose medium composed of: 1.0 g NH₄ H₂ PO₄, 0.2 g KCL, 0.2 g MgSO₄.7H₂O, 0.2 g CaCl₂, 250 ml of 4% ball milled cellulose, 18 g agar, and 750 ml distilled water (Padhi and Snyder, 1954). The medium was sterilized by autoclaving at 5 psi for 20 minutes and poured into 9 cm Petri dishes (15-20 ml / 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 basal medium, resulting in an opaque medium. Cellulase activity was then visualized by the formation of cleared zones around fungal growth (Mansour *et al*, 1985; Pointing, 1999).

Phenoloxidase: 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 (Rodeia and Goncalves,1986).

Acid phosphatase: The fungi were grown on Czapek Dox liquid medium for 10 days at 27 °C. Thereafter, the mycelial mats were harvested by filtration, , rinsed with distilled water, blotted on filter paper, and then homogenized with acid-washed sand (sea sand was washed with sulfochromic solution, neutralized and washed several times with distilled 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-monophosphate (AMP) and 0.5 ml of the last cell free extracts, 160 μ Na₂CO₃-NaHCO₃ buffer at pH 9.2, were incubated at 40 °C for 30 min. Liberated phosphate was measured spectrophotometrically at 530 nm. One unit of phosphatase activity was defined as the amount of the enzyme which released 1μ moles orthophosphate. Protein determination was carried out following the Lowry method using albumin as the standard (Lowry *et al*, 1951)

Statistical analysis

Data obtained were statistically analyzed by F test, Least Significant differences (LSD) which was determined at 0.05 level of probability (Snedecor and Cochran, 1980)

RESULTS AND DISCUSSION

Six fungal species namely: *A. alternata*, *B. cinerea*, *F. rousm*, *F. tricinctum*, *S. botryosum* and *Thielaviopsis* spp. Were isolated from lettuce leaves collected from Riyadh markets during summer 2005. All of these fungi have been reported to be among the phytopathogenic fungi. The biomass growth of the fungal isolates tested and their ability to produce exoenzymes 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. tricinctum*, *Thielaviopsis* spp., *A. alternata*, *B. cinerea* and *F. rousm* respectively. The highest activity of amylase enzyme was revealed as: *F. rousm*, *F. tricinctum*, followed by *A. alternata* and *Thielaviopsis* spp, *B. cinerea* and finally, *S. botryosum*. Amylase activity of *F. rousm* twice *F. tricinctum*, 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		Lipase		Protease		Cellulase		Phenoloxide	
	CD	AZ	CD	AZ	CD	AZ	CD	AZ	CD	AZ
<i>A. alternata</i>	38	3	40	8	35	7	16	55	-	-
<i>B. cinerea</i>	35	2	10	0	0	58	25	15	+	+
<i>F. rousm</i>	78	10	30	11	31	6	65	15	-	-
<i>F. tricnictum</i>	40	5	40	8	34	0	0	0	-	-
<i>S. botryosum</i>	47	1	10	0	30	0	60	5	-	-
<i>Thielaviopsis</i> 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 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 which produced lipase enzyme were *F. rousm*, *A. alternata* and *F. tricnictum* then *Thielaviopsis* spp. 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 *Thielaviopsis* spp. F-test showed high significant differences between myceliel growth and lipase production. Fungal lipase enzyme plays a role in the infection of plants, Berto 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 ((Berto *et al*, 1999)

Protease: The results in Table (1) shows good growth of *A. alternata*, *F. tricnictum*, *F. rousm*, and both *S. botryosum* and *Thielaviopsis* spp 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 results presented that the production 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* spp were unable to grow on 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* spp. growth and cellulase production Table (1). F-test revealed a significant

difference (significance level of 5%) between mycelial growth and the cellulase production. Dahm showed that the fungi which produce cellulase enzyme might become pathogenic to their hosts (Dahm, 1987). On the other hand, low cellulolytic activity may be preferable to the host to achieve limited penetration (Cao and Crawford, 1993).

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 (Doumas *et al*, 1986) All the lettuce fungi which were tested produced acid phosphatase. *A. alternata* produced 1.40 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. tricinctum*, and 0.22 milliunit of *F. rousm* respectively Fig (1). These results correspond with other investigations (Redlak *et al*, 2001). The production of phosphatase may contribute to the increased phosphate uptake in fungi (Dahm, 1987).

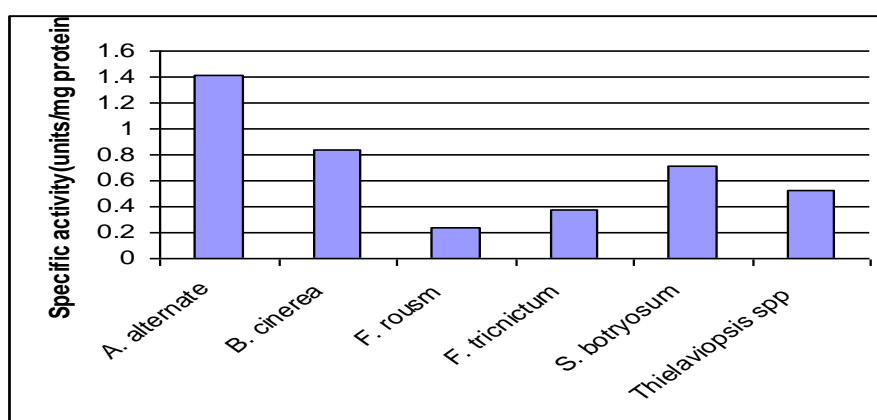


Fig. (1) : Acid phosphatase activity of fungal spp. Isolated from lettuce leaves.

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 (Kars and Va Kan,2004). 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 fungal pathogens. The mycotoxins are known to be carcinogenic, causing several serious ailments in humans and animals (Narayanasamy, 2005).

REFERENCES

Bahkali, A.H. (1999) Cell wall degrading enzymes produced by the phytopathogenic fungus *Verticillium tricorpus*. Saudi J. Bio. Sci. 6, 2: 133-145.

- Berto, P. ; Commenil, P. ; Belingheri, L. and Dehorter, B. (1999) Occurrence of a lipase in spores of *Alternaria brassicicola* with a crucial role in the infection of cauliflower leaves. F E M S Microbiol. 108, 183-189.
- Berto, P. ; Belingheri, L. and Dehorter, B. (1997) Production and Purification of novel extracellular lipase from *Alternaria brassicicola*. Biotechnol. 19, 533-536.
- Cao, W. and Crawford, D. L. (1993) Carbon nutrition and hydrolytic and cellulolytic activities in the ectomycorrhizal fungus *Pisolithus tinctorius*. Can. J. Microbiol. 39, 529-535 .
- Clancy, K. J. , and Brophy, G.(1989) Evaluation of six fungicides for control of *stemphylium* leaf spot or lettuce. Ann. App. Boil. 114 46-47.
- Dahm, H. (1987) Cellulolytic and Pectolytic activity of Nonmycorrhizal fungi associated with roots of forest trees. Acta Microbiologia Polonica. 36, 4 317-324.
- Doumas, P. ; Berjand, C. ; Callejand, M. ; Coupe, M. ; Espian, C. and Anzac, J. (1986) phosphatase extracellulaires et nutrition phosphate chez les champignons ectomycorhiziens et les plantes hotes Physiol Veg. 24, 173-184.
- Gabr, M.A and Mansour , F.A.(1982) . Studies on amylase production by some Streptomyces species from Egyptian soil.Proc.Egypt.Bot.SOC.,3 (Mansour Conf.) : 59-82
- Garibaldi, A. ; Gilardi G. and Gullino, M. L.(2002) First report of *Fusarium oxysporum* on lettuce in Europe. Plant Disease. 86, 1052.
- Garibaldi, A. ; Gilardi G. and Gullino, M. L.(2004) First report of *Fusarium oxysporum* causing vascular wilt of lambs lettuce (*valerianella olitoria*) in Italy. Plant Disease. 88, pp 83.
- Gilardi, G. ; Omodei, M. and Garibaldi, A. (2004) Attacks of *Thielaviopsis basicola* on lamb's lettuce in northern Italy. IF 2, 84-50
- Kars, I. and van Kan, J.A.L. (2004) Extracellular enzymes and metabolites involved in pathogenesis of *Botrytis*. In "*Botrytis* : Biology, pathology and control" Kluwer Academic Publishers, the Netherlands. 99-118
- Koike, S. T. ; Henderson, Diana M. and Butler E. E. (2001) leaf spot disease of spinach in California caused by *Stemphylium botryosum*. Plant Disease. 85, 2 126-130.
- Lowry, O. H. ; Rosenbrough, N. ; Farr, A.and Randall R. J. (1951) Protein measurement with the follin phenol reagent. J. Biol. Chem. 193, 265-275.
- Lumyong, S.; Lumyong, P.; Mckenzie, E. H. C.and Hyde, D. K. (2002).Enzymatic activity of endophytic fungi of six native seedling species from Doi Suthep-Pui national park, Thailand. Can. J. Microbiol. 48, 1109-1112.
- Mansour, F.A., El- Esawy,A.A and El Sayed, S.A. (1985). Studies on the cellulolytic activities of some Streptomyces isolated from Egyptian soil. Proc. 2nd Conf. Agric. Bot. Sci. Mansoura, Egypt, pp.112-142.
- Mercier,J. and Reeleder, R. D. (1987) Effects of the pesticides maneb and carbaryl on the phylloplane microflora of lettuce. Canadian- Journal of Microbiology. 33, 3 212- 216.
- Narayanasamy, P. (2005).Postharvest Pathogens and Disease Management. Wiley – Interscience. pp59
- Netzer, D. ; Globerson, D. ; Weintal C. H. and Elyassi R. (1985) Sources and inheritance of resistance to *stemphylium* leaf spot of lettuce. Euphytica. 34, 2 393-396
- Padhi, B. and Snyder, W. C. (1954) *Stemphylium* leaf spot of lettuce. Phytppathology. 44 175 – 180.

- Paterson, R. R. M. and bridge P. D. (1993) Biochemical techniques for filamentous fungi. International Mycological Institute. Bakeham Lane Egham Surrey. 22-23.
- Pernezny, K. and Raid, R (2006) *Alternaria* Leaf Spot (*Alternaria sonchi*, *Alternaria* spp.) Specific Common Diseases. Florida Plant Disease Management Guide: Lettuce and Endive
- Pointing, S.B. (1999).Qualitative methods for determination of lignocellulolytic enzyme production by tropical fungi. Fungal Diversity. 2, 17-33.
- Redlak, K. ; Dahm, H. ; Ciesielska, A. and Strzelczyk, E. (2001) Enzymatic activity of ectendomycorrhizal fungi. Biol. Fertil. Soils. 33, 83-90.
- Rodeia, N. T. and Goncalves, A. M. (1986) Polyporaceae- cellulasic and phenoloxidasic activities. Bol. Soc. Brot. 2, 59 43-57.
- Sala, F.C.; Costa, C.P.; Teixeira-Yanez, L.D.D. and Blat, S.F. (2003) First report of root rot incited by *Thielaviopsis basicola* on lambs lettuce (*Valerianella oleria*) in Europe. Hortic. Bras.21.
- Sala, F. C. and Costa, P. D. (2005) PiraRoxa: Triple red lettuce cultivar. Hortic. Bras. 23, 1 158-159.
- Snedecor , W. and Cochran , G.(1980).Statistical methods.7th edn.The Iowa State University Press , Ames , Iowa .
- Van Kan, J.A.L. (2005) Infection strategies of *Botrytis cinerea*. Acta Horticulturae. 669 77-90.
- Urairuj, C. ; Khanongnuch, C. and Lumyong, S. (2003) Ligninolytic enzymes from tropical endophytic Xylariaceae. Fungal Diversity.13, 209-219.

النشاطات الإنزيمية للفطريات المعزولة من أوراق الخس

هدى بنت حمد الحميدان

قسم النبات - جامعة الرياض للنبات - كلية التربية -

ص . ب . ٢٧١٠٤ الرياض ١١٤١٧ المملكة العربية السعودية

يهدف هذا البحث نحو دراسة الانشطة الانزيميه لسته انواع من الفطريات (هي ألترناريا ألترناتا، تريكنيكتوم، ستيمفيليوم بوتريوزوم، تم عزلها من اوراق نبات الخس اثناء التسويق خلال اشهر الصيف بمدينه الرياض

اخذت قدره هذه الفطريات علي انتاج خمس انزيمات خارجيه هي(الاميليز، الليبيز، البروتيز، السيلوليز و الفينول اوكسيديز) بالاضافه الي انزيم داخلي هو إنزيم الفوسفاتيز الحامضي)

و قد أظهرت النتائج ان جميع الفطريات موضوع الدراسة تبدي قدرات متفاوتة علي هذه الانزيمات. فبالنسبه لانزيم الاميليز كان فطر فيوزاريوم روزم هو اكثر الفطريات قدره علي انتاج هذا الانزيم يليه في ذلك فطر فيوزاريوم تريكنيكتيوم، في حين اظهر فطر ثيلا فيوبسس، فيوزاريوم روزم قدره عاليه علي إنتاج انزيم الليبيز، يلي ذلك فطر الترناثيا الترناثا و فيوزريوم تريكنيكتيوم بينما لم يستطع أي من بوتريثس سينيري أو ستيمفيليوم بوتريوزوم إنتاج الليبيز وبالنسبة لانزيم البروتيز فقد أظهرت النتائج ان هناك معزولتين فقط هما(فيوزاريوم زوم و الترناثيا الترناثا) ينتجان هذا الإنزيم. الإنزيم السيلولوز فقد اظهر كل من الترناثيا الترناثا، بوتريثس سينيري، فيوزريوم روزم و ستيمفيليوم بوتريوزوم نشاطا ملحوظا علي تحلل السيلولوز، في حين لم تستطع أي من فيوزاريوم تريكنيكتوم و ثيلافيوبسس إنتاج إنزيم السيلوليز. أما فيما يتعلق بإنزيم الفينول اوكسيديز فقد وجد أن هناك فطر واحد فقط (هو بوتريثس سينيري) ينتج هذا الإنزيم .

و من جهة أخرى وجد ان اكبر فطر للمستعمرة الفطرية النامية على الأوساط الغذائية المختلفة كان فطر فيوزاريوم روزم، ثم ستيمفيليوم بوتريوزام، فيوزاريوم تريكنيكتوم، بوتريثس سينيري علي التوالي . وبالنسبة لقدرة المعزولات موضوع الدراسة على إنتاج إنزيم الفوسفاتيز الحامضي فقد أوضحت النتائج أن المستخلص الخالي من الخلايا لجميع الفطريات المختبرة يحفز التحلل المائي للفوسفات من الأدينوسين أحادي الفوسفات (AMP) ، ويمكن ترتيب المعزولات تبعا لنشاطها الأنزيمي ترتيبا تنازليا على النحو التالي: ألترناريا ألترناتا ، بوتريثس سينيري ، ستيمفيليوم بوتريوزام ، ثيلافيوبسس ، فيوزاريوم تريكنيكتوم ثم فيوزاريوم روزم.