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Evaluation of relationship between β-1, 3- glucanase production and biocontrol capacity of *trichoderma* isolates

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ABSTRACT

Seven fungi were isolated from five plant hosts collected from 15 governorates. The mean percentage of fungal recovery from the governorates showed that *Trichoderma* spp. (35.44%) *Fusarium* spp.(28.45%),and *Rhizoctonia solani*(16.00%) were the most dominant fungi, other fungi occurred at frequencies ranged from 1.33 to 7.79 % . Fifteen isolates of *Trichoderma* spp. were screened for their biocontrol activity against soil- borne fungal pathogens under greenhouse conditions by using eight pathosystems. The tested isolates showed variable levels of antagonism within each pathosystem. When the same isolates were *in vitro* screened for β-1,3-glucanase,prodution20% of the isolates were high producers, 46.67% were medium producers, and 33.33% were low producers. Regression analysis was used to study the effect of β-1,3- glucanase activity (independent variable) on percentage of surviving seedlings (dependent variable)in each pathosystem.In almost all pathosystems.the in vitro efficiency of *Trichoderma* isolates in producing for β-1,3-glucanase was not significantly correlated with the percentage of surviving seedlings, which was used as a parameter for evaluating the antagonistic activity of *Trichoderma* isolates under greenhouse conditions. This finding may indicate that the *in vitro* β-1,3- glucanase activity of *Trichoderma* isolates is of no practical value because it cannot be used as a criterion to predict Their *in vivo* performance .Grouping the isolates by cluster analysis,based on their antagonistic patterns, was not related to their β-1,3-glucanase activity .these results may suggest that β-1,3-glucanase may not be involved in the biocontrol process of the tested isolates. Similary, grouping the isolates by cluster analysis, based on their RAPD banding patterns, was not related to their β-1,3-glucanase activity, this result indicates that RAPD banding patterns were unable to differentiate among the isolates based on their β-1,3- glucanase activity.

INTRODUCTION

Trichoderma Pers. is a genus of hyphomycetes. Its species are among the most commonly encountered soil fungi (Roiger *et al.,* 1991). *Trichoderma* has been shown to act as a mycoparasite against a range of economically important aerial and soilborne plant pathogens. Several modes of action have been proposed to explain the suppression of plant pathogens by *Trichoderma;* these mides of action include production of antibiotics, competition for key nutrients, production of cell wall degrading enzymes, stimulation of plant defense mechanisms ,and combination of these possibilities(Vazques-Garciduenas *et al*.,1998).

The initial interaction between *Trichoderma* and its host is characterized by the chemotrophic growth of hyphae of the mycoparasite towards the host(Chet and Elad, 1983)). When the mycoparasite reaches the

host, its hyphae often coil around it or are attached by hook-like structure (Elad *et al.,* 1983a). Following these interactions, the mycoparasite penetrates the host mycelium, apparently, by partially degrading its cell wall. Susceptible host mycelia show rapid vacuolation, collapse, and disintegration (Elad *et al.,* 1983b; Benhamou and Chet, 1993).

Cell walls of most phytopathogenic fungi contain chitin as structural backbone arranged in regularly ordered layers and β-1,3- glucan as a filling material arranged in an amophic manner (Gajera *et al*.,2012).however, oomycetes,such as *Pythium* are exceptional in that their cell walls contain

β-(1,3)-(1,6)-glucans and cellulose instead of chitin as their major structural components(Viterbo *et al*.,2002).

The importance of β-1,3- glucanase as a key enzyme responsible for lysis and degradation of fungal cell wall has been reported (Cook and Baker,1983). This enzyme has been shown to be produced by *Trichoderma* spp. and may be an important factor in their biological control activity (EL-Katatny *et al*.,2000).

β-1,3- Gucanase hydrolyzes the O-glycosidic linkages of β-glucan chains by two mechanisms. Exo- β-1,3- glucanase (EC3.2.1.58)hydrolyzes a substrate by sequentially clearing glucose residues from the nonreducing end, and endo- β-1,3- glucanase(EC3.2.1.39)cleave β. linkages at random sites along the polysaccharide chain, releasing short oligosaccharides (Vazques-Garciduenas *et al*.,1998; Noronha and Ulhoa,2000; Marco and Felix,2007). Degradation of β-glucan by fungi is often accomplished by the synergistic action of both endo- and exo- β-glucanase ; in fact, in most cases multiple β-glucanase rather than a single enzyme have been found (Vazques-Garciduenas *et al.,*1998).The level of β-1, 3- glucanase activity secreted by *Trichoderma* was found to be proportional to the amount of glucan present in the inducer. *Trichoderma* ptoduces at least seven extracellular β-1- 3- glucanase upon induction with laminarin, a soluble β-1, 3- glucan.*(*Vazques-Garciduenas *et al.,*1998).

The main objective of this study was to assess the role of β-1,3-glucanase in the biocontrol activity of *Trichoderma* spp by using eight pathosystems. prevalence of *Trichoderma* spp was also evaluated compared with prevalence of some major root- colonizing fungi.

MATERIALS AND METHODS

Isolation, identification, and quantification of *Trichoderma* spp. and other fungi from roots of five hosts (tomato, cotton, cucumber, peanut, and onion).

Diseased plants at the seedling stage of growth through maturity were collected at random from 15 governorates (Gharbiya, Daqahliya, Minufiya, Ismailiya, Damietta, Sharqiya, Kafr El-Sheikh, Beheira, Qualyubiya, Giza, Fayoum, Beni-Swaif, Minya, Assiut, and Sohag) during 2012 and 2013. Each sample included from 20 to 30 seedings affected with a variety of damping-off symptoms or rotted roots of 10 to 15 adult plants. The seedlings and roots collected at each field were stored at 4^{o}C until fungal isolation was

performed. Seedlings and roots of mature plants were washed thoroughly under running tap water for 24 hr to remove any adhering soil. Small pieces (approximately 0.5cm long) of necrotic root tissues were surface sterilized with 10% Clorox solution for 2 minutes, and washed several times with sterilized water. The surface sterilized pieces were then blotted dry between sterilized filter papers and plated (5 pieces/plate) onto potato-dextrose agar (PDA) medium amended with streptomycin sulfate or penicillin G. and rose Bengal (100-200 mg/L each) as bactericides. The plates were incubated at 26±3^{o}C for 3-7 days. The developing colonies were identified according to Gilman (1966) or Barnett and Hunter (1979). Colonies of each fungus were expressed as percentage of the total developing colonies.

Evaluation of biocontrol capacity of *Trichoderma* isolates by using different pathosystems.

Substrate for growth of each selected isolate of *Trichoderma* spp. was prepared in 500-ml glass bottles, each bottle contained 50 g of sorghum grains and 40 ml of tap water. Contents of bottles were autoclaved for 30 minutes. Isolate inoculum, taken from one-week- old culture on PDA, was aseptically introduced into the bottle and allowed to colonize sorghum for 3 weeks. The same method was used for preparing inocula of the pathogenic fungi used in the pathosystems (Table 1). The present study was carried out by using autoclaved clay loam soil. Batches of soil were infested separately with inoculum of each *Trichoderma* isolate at a rate of 50 g/kg of soil. For each *Trichoderma* isolate, eight batches of infested soil were placed on greenhouse benches and separately infested with the pathogenic fungi (Table 1) at rates of 1, 5, 5, and 1 g/kg of soil for *Rhizoctonia solani, Sclerotium rolfsii, S. cepivorum*, *and Pythium* sp., respectively. After thoroughly mixing, infested soil was dispensed in 25-cm -diameter clay pots and these were planted with 10 seeds of each host or onion transplants (Table 1) per pot. There were five pots (replicates) for each treatment. In the control treatments, *Trichoderma* isolates were not added to the pathogen-infested soil. The greenhouse temperature ranged from 16.5±2.5 to 30.5±3.5^{o}C during the experiment. Percentages of surviving seedlings were recorded 45 days after planting.

Table 1. Pathosystems (Ps) used in evaluating antagonistic activity of *Trichoderma* isolates under greenhouse conditions.

Pathosystem (P)	Host	Pathogen
P_1	Cotton, cv. Giza 89	*Rhizoctonia solani*
P_2	Bean, cv. Bolista	*Rhizoctonia solani*
P_3	Cucumber, cv. Bazindra	*Rhizoctonia solani*
P_4	Sesame, cv. Giza 32	*Rhizoctonia solani*
P_5	Sesame, cv. Giza 32	*Sclerotium rolfsii*
P_6	Sesame, cv. Giza 32	*Pythium* sp.
P_7	Cucumber, cv. Bazindra	*Pythium* sp.
P_8	Onion, cv. Giza 60	*Sclerotium cepivorum*

Screening *Trichoderma* isolates for β-1,3- glucanase activity.

β-1,3- glucanase activity of *Trichoderma* isolates was determined according to the method of El-Katatny *et al*. (2000). In this method, spore suspension inoculum of each *Trichoderma* isolates (1.0 x 10^6 spores/ml of culture broth) was used to inoculate two 100- ml flasks containing 20 ml of unbuffered mineral synthetic medium supplemented with dried mycelium of *sclerotium rolfsii* as the sole carbon source (5 g/l). The cultures were grown at 30^{o}C for 5 days without shaking. Culture filtrates were centrifuged at 4^{o}C for 10 min at 5000 x g and the clear supernatants were stored at- 20^{o}C until assayed.

β-1,3- glucanase activity was assayed using a colorimetric method. by incubating 500 µL of 5.0%(w/v)laminarin in 50 mM acetate buffer (pH= 4.8)with 200 µLenzyme solution at 45 ^{o}C for 30 min and determination of the reducing sugar with dinitrosalicylate reagent. The amount of reducing sugar released was calculated from a standar curve for glucose, and the activity of β-1و3- glucanase was expressed in nkat (nmol/s)

Evaluation of genetic diversity of *Trichoderma* isolates by using random amplified polymorphic DNA (RAPD) analysis.

DNA isolation and RAPD analysis.

Trichoderm isolates were grown for 22 days at 25-30^{o}C on liquid Czapec medium. The mycelium was harvested by filration through cheesecloth, washed with distilled water several times. DNA was isolated from 500 mg of fresh mycelium of each isolate using Qiagen kit for DNA extraction. The exracted DNA was dissolved in 100 µLof elution buffer. The concentration and purity of the obtained DNA was determined by using "Gene Quanta" system, Pharmacia Biotech. The purity of the DNA for all samples was between 90-97%. Concentration was adjusted to 6 ng/µl for all samples using TE buffer (pH 8.0).

Polymerase chain reaction (PCR) mixture was prepared with PCR bead tablets (manufactured by Amessham Pharmacia Biotech.), which contained all the necessary reagents except the DNA template and the 10-mer primer. The kits of Amessham Pharmacia Biotech also incude the following primer: RAPD analysis primer 4:6-d (AAGAGCCCGT)-3

Thirty ng from each DNA exracted sample and 5 µl of the 10-mer random primer (15 ng/ml) were added to a PCR bead tablets. The total volume was completed to 25 µl using sterile distilled water. The amplification protocol was carried out as follows using PCR unit 11 Biometra: Denaturing at 95^{o}C for 5 min, 45 cycles each consisted of the following steps: Denaturing at 95^{o}C for 1 min, annealing at 36^{o}C for 1 min, extension at 72^{o}C for 2 min. Final extension was carried out at 72^{o}C for 5 min and hold at 4^{o}C. Five µl 6x tracking buffer (manufactured by Qiagen was added to 25 µl of the amplification product.

Amplification product analysis

The amplified DNA (15 µl) for all samples was electrophoresed using the electrophoretic unit WIDE mini-sub-cell GT (Bio-Rad) on 1% agarose containing

0.5 μg/ml ethidium bromide at 75 constant voltage, and determined with UV transilluminator.

Gel analysis

Gel was scanned for molecular weight (bP) and amount (%) of bands by the gel documentation system AAB (Advanced American Biotechnology, Fullerton CA, 92631). The different molecular weights of bands were determined against DNA standard (G317 A. Promega Inc., USA) with molecular weights 1000, 750, 500, 300, 150, and 50 bp.

Statistical analysis of the data

Analysis of variance (ANOVA) of the data was performed with MSTAT-C statistical package. Least significant difference (LSD) was used to compare between frequency means of the isolated fungi. Correlation, regression, and cluster analyses were performed with the software package SPSS 6.0.

RESULTS

Seven fungi were isolated from 15 governorates (Table 2). The mean percentage of fungal recovery from governorates showed that *Trichoderma* spp (35.445%), *Fusarium* spp (28.45%),and *Rhizoctonia solani* (16.00%).were the dominant fungi . Other fungi occurred at frequencies ranged from 1.33 to 7.79%.

Table 2. Frequency (%) of *Trichoderma* spp. and other fungi isolated from 15 governorates..

Fungus	Isolation frequency (%)
Trichoderma spp.	35.44[a]
Rhizoctonia solani	16.00
Fusarium spp.	28.45
Pythium spp.	7.79
Macrophomina phaseolina	7.03
Sclerotium cepivorum	3.98
Sclerotium rolfsii	1.33

[a]Mean of 15e replicates (governorates).

LSD (p≤ 0.05)= 9.11

Trichoderma isolates showed variable levels of antagonism within each pathosystem (Table3).For example , isolate T89 was highly effective as a biocontrol agent in pathosystem 2 (P2) as it increased the surviving plants by 153.33% on the other hand, isolate T65 was ineffective. Another example was isolate T84, which increased survival by 100% in P3 while isolate T102 was ineffective.

Fifteen isolates of *Trichoderma* were screened for β-1,3 -glucanase activity (Table 4) these isolates could be categorized into three groups: high producers(T36,T51,and T89),medium producers (T1,T2,T3,T4,T9,T74,and T88),and low producers (T65,T67,T84,T102 , and T111),In other words,20% of the isolates were high producers ,46.67% were medium producer, and 33.33% were low producers.

Table 3. Evaluation of antagonistic capacity of 15 isolates of *Trichoderma* spp. by using different pathosystems under greenhouse conditions.

Trichoderma isolate (T)	Surviving seedlings (%)							
	Pathosystem (P)							
	P_1	P_2	P_3	P_4	P_5	P_6	P_7	P_8
T_1	60	75	95	55	25	60	100	50
T_2	60	70	45	80	20	73	93	42
T_3	60	65	85	85	50	73	87	83
T_4	70	70	75	70	20	53	67	58
T_9	60	75	60	60	5	67	100	25
T_{36}	65	50	75	70	25	73	87	100
T_{51}	70	65	40	70	20	43	100	50
T_{65}	30	30	40	50	25	67	100	58
T_{67}	35	60	45	25	15	67	93	25
T_{79}	50	40	70	70	5	67	100	75
T_{84}	50	60	90	90	20	87	80	58
T_{88}	55	60	65	80	35	60	100	58
T_{89}	30	76	60	45	30	60	100	67
T_{102}	25	15	45	40	40	80	93	50
T_{111}	40	50	100	75	15	80	60	67
Control[a]	15	30	45	30	20	53	53	17

[a]**Autoclaved soil was infested only with the pathogen of each pathosystem.**

Table 4. Screening of 15 isolates of *Trichoderma* spp for β-1,3-glucanase activity.

	β-1- 3 glucanase activity.	
Isolate no.	**(nkat/ml)**	**Category**
T1	2.80	Medium
T2	2.90	Medium
T3	2.80	Medium
T4	2.50	Medium
T9	2.90	Medium
T36	3.7	High
T51	4.50	High
T65	0.40	Low
T67	0.40	Low
T79	2.90	Medium
T84	1.80	Low
T88	2.40	Medium
T89	6.30	High
T102	0.14	Low
T111	0.90	Low

Regression analysis was used to study the effect of β-1,3- glucanase activity (independent variable)on percentage of surviving seedlings (dependent variable) in eight pathosystems (Table 5). The two variables were positively correlated in P2 ($r=0.62, p\leq0.05$)while they were negatively correlated in P6(r=

0.50,p ≤0.10)T he correlation between the two variables was nonsignificant in the other pathosystems.

5-

A phenogram based on dissimilarity distance (DD) generated from cluster analysis of antagonism patterns of *Trichoderma* isolates is presented in Fig1. The smaller the DD,the more closely the isolates were related in their antagonism patterns. Six groups of *Trichoderma* isolates (isolates T84,T111; isolates T3,T79, T36;isolates T2,T9,T51,T88;isolates T1,T4; isolates T65,T102, and isolates T67,T89, respectively)were identified by cluster analysis. Grouping the isolates by cluster analysis was not related to their β-1,3-glucanase activity. For example, isolates T79 and T2 showed the same level of β-1,3- glucanase activity; hower, the were placed in two remotely related groups.T he high β-1,3- glucanase producer T51 and the medium producer T88 were placed in The same group. The low producer T67 and the high producer T89 were also placed in the same group.

Fig.1. Phenogram based on average linkage cluster analysis of bicontrol capacity 15 isolates of *Trichoderma* spp. by using eight pathosystems. Biocintrol capacity of the isolates was evaluated based on the surviving seedlings (%). β-1,3-Glucanase.activity was expressed as nkat/ml.

Aphenogram based on similarity level (SL)generated from cluster ananlysis of RAPD banding patterns of *Trichoderma* isolates is presented in Fig 2. The greater the SL,the more closely the isolates were related in their

RAPD banding patterns.Three groups of *Trichoderma* isolates were identified by custer analysis at SL_s 78.60, 82.17, and 75.33%. Isolate T4 showed an unique DNA profile quite different from those of the remaining isolates. Although 71.43%of The medium producer isolates were placed in the third group, grouping the isolates by cluster analysis was not related to their β-1,3-glucanase activity because isolates of each of the first and second group showed variable levels of β-1,3- glucanase activity.

Fig.2. RAPD banding patterns of *Trichoderma* spp. isolates obtain by a random decamer primer and electrophoresed on agarose gel. β-1,3- Glucanase activity was expressed as nkat/ml.

DISCUSSION

The present study demonstrated that *Trichoderma* spp were the most dominant fungi compared with the other root- colonizing fungi. This result is in agreement with the previous reports, which indicated that *Trichoderma* spp. were among the most commonly encountered soil fungi (Roiger *et* al.,1991).

The isolation of *Trichoderma* spp. from widely separated locations in 15 governorates may suggest that *Trichoderma* spp. are well adapted to colonize plant roots under a wide range of environmental conditions (edaphic factors, crop rotations, irrigation systems,temperature, and so on).In the present study, *Trichoderma* spp were recovered from five plant hosts belonging to five different genera. This may indicate that host specificity in *Trichoderma* spp. was lacking on colonizing plant roots.

Trichoderma isolates showed variable levels of antagonism within each pathosystem. This variability could be attributed to the differential effects of host cultivar and /or pathogen isolate on the performance of *Trichoderma* isolates. That is,a single isolate of a pathogen can be highly

sensitive to the application of a single isolate of *Trichoderma* but may exhibit minimal sensitivity to the application of another *Trichoderma* isolate (Asran *et al* 2005).similarly, a host cultivar can be highly responsive to the application of a *Trichoderma* isolate but may show minimal response to the applicaton of another isolate of *Trichoderma* (Asran,2007).

Production of extracellular β-1,3- glucanases increase significantly when *Trichoderma* spp.are grown in a medium supplemented with either autoclaved mycelium or host fungal cell walls. These abservations, together with the fact that β-1,3- glucan is among the main structural components of most fungal cell walls, are the basis for the suggestion that β-1,3- glucanases produced by *Trichoderma* spp. play an important role in the destruction of phytopathogenic fungi (Va`zques- Garciduenas *et al* .,1998).

Surprisingly, in most pathosystems of the present study, the *in vitro* efficiency of *Trichoderma* isolates in producing β-1,3- glucanase was not significantly correlated with the percentage of surviving seedlings,which was used as a parameter for evaluating the antagonistic efficiency of *Trichoderma* isolates. This result may indicate that the *in vitro* β-1,3 -glucanase activity of *Trichoderma* isolates is of no practical value because it cannot be used as criterion to predict their *in vivo* performance under greenhouse conditions.

We suggest some possible explanations for the lack of correlation between the *in vitro* β-1,3- glucanase production by *Trichoderma* isolates and there *in vivo* antagonism.It seems reasonable to assume that the biocontrol activity of *Trichoderma* isolates cannot be simply explained by the production of β-1,3- glucanase . This is because other modes of action (Vazques- Garciduenas *et al* .,1998).were as important as β-1,3- glucanase or even more important in the biocontrol process of the tested group of *Trichoderma* isolates. The optimal activity of fungal β-1- 3 glucanase is usually in the range of pH4.0 -6.0(Noronha and Ulhoa,2000) the values of pH of the Egyptian soil range from 7.92- 9.15 (Aly and Kandil ,1999).Therefore, this range is unfavorable for β-1,3- glucanase activity by *Trichoderma* isolates applied into the soil. Recognition is an important factor in the mycoparasitic activity of *Trichoderma* isolates(Herrera-Estrella and Chet,1999). Therefor, the lack of biocontrol activity under greenhouse conditions may be due to the failure of *Trichoderma* isolates to recognize their host (the pathogen).

The peat cultivation and dual cultures showed that β-1,3- glucanase activity was induced in *Trichoderma* by the presence of *Pythium* . Further, purified endo β-1,3- glucanase from *Trichoderma* was inhibitory to the germination and growth of encysted *Pythium* zoospores (Thrane *et al*.,1997). Surprisingly, the *in vitro* production of β-1,3- glucanase and the *in vivo* antagonism of *Trichoderma* was negatively correlated ($r = -0.502, p \leq 0.10$) in the *Pythium* – sesame pathosystem. It is difficult to account for this result, considering the aforementioned reports, and we do not have an immediate biological explanation for it.

Grouping *Trichoderma* isolates by cluster analysis, based on their antagonistic patterns, was not related to their β-1,3- glucanase activity. This finding is an additional evidence that β-1,3 -glucanase may not be involved in the biocontrol process of the tested isolated. Similarly, grouping the isolates

by cluster analysis, based on their RAPD banding pattern, was not related to their β-1,3- glucanase activity. This result indicates the RAPD banding patterns were unable to differentiate among the isolates based on their β-1,3-glucanase activity.

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تقييم العلاقة بين إنتاج إنزيم البيتا- 1 ، 3 – جلوكانيز والقدرة على المقاومة الحيوية لعزلات فطر التريكوديرم

على عبد الهادي علي ، عبد الفتاح عبد الحميد الوكيل ، أمل عبد المنجي عسران ، عبد الودود زكي عبد الله عاشور و محمود توفيق محمود منصور

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عزلت سبعة فطريات من خمسة عوائل نباتية جمعت من 15 محافظة . أظهرت نتائج العزل ان ألانواع التابعة لجنسي الترايكوديرما و الفيوزاريوم بالاضافة إلي فطر الريزوكتونيا سولاني هي الاكثر انتشارا، حيث وصل متوشط تكرار عزلها من المحافظات إلي 35.44 و 28.45 و 16.00% علي الترتيب، أما الفطريات الاخري فقد تراوح متوسط تكرار عزلها من 1.33 إلي 7.79% . قيمت 15 عزلة لفطر الترايكوديرما، تحت ظروف الصوبة،من حيث القدرة علي المقاومة الحيوية باستعمال ثماني منظومات مرضية . أظهرت العزلات تباينا في القدرة علي التضاد داخل كل منظومة مرضية. عندما اختبرت نفس مجموعة العزلات من حيث القدرة علي إفراز انزيم البيتا 1 ، 3 – جلوكانيز ، تحت ظروف المعمل، وجد أن 20% من العزلات كانت ذات قدرة عالية علي إفراز الانزيم و 46.67% ذات قدرة متوسطة و 33.33% ذات قدرة منخفضة، إستعمل تحليل الانحدار لدراسة تأثير إفراز انزيم البيتا- 1 ، 3 – جلوكانيز (عامل مستقل) علي النسبة المئوية للبادرات السليمة (عامل تابع) وذلك لكل منظومة مرضية. في اغلب المنظومات المرضية موضع الدراسة، كانت كفاءة عزلات التريكوديرما في إفراز انزيم البيتا 1 ، 3 – جلوكانيز تحت ظروف المعمل غير مرتبطة معنويا بالنسبة المئوية للبادرات السليمة تحت ظروف الصوبة . تدل هذة النتيجة علي ان إنتاج انزيم البيتا 1 ، 3 – جلوكانيز بواسطة عزلات التريكوديرما تحت ظروف المعمل ، ليس له اهمية من الناحية التطبيقية، لعدم قدرتة عل التنبؤ بأداء هذة العزلات، تحت ظروف الصوبة. إستعمل التحليل العنقودي لتقسيم العزلات إلي مجموعات بناء علي ما بينها من تباين في إنماط القدرة علي التضاد، إلا أن هذة المجموعات لم ترتبط بقدرة العزلات علي إفراز الانزيم . تدل هذة النتيجة علي احتمال عدم مساهمة إنزيم البيتا-1 ، 3 – جلوكانيز في عملية المقاومة الحيوية لمجموعه العزلات المختبرة . طبقت تقنية التضاعف العشوائي لمناطق متابينة من الحمض النووي دنا علي العزلات، واستعمل التحليل العنقودي لتقسيم العزلات إلي مجموعات بناء" علي ما بينها من تماثل في انماط الحمض النووي ،إلاأن المجموعات المتحصل عليها لم ترتبط بقدرة

العزلات علي إفراز إلانزيم مما يدل عل أن تقنية التضاعف العشوائي لا تصلح للتفرقة بين العزلات بناء علي قدرتها علي إفراز إلانزيم.