J. Agric. Sci. Mansoura Univ., 29(2): 825 - 840, 2004

EFFECT OF 2,4-DIACETYLPHLOROGLUCINOL PRODUCED BY *Pseudomonas fluorescens* Q2-87 ON β-1,3 GLUCANASE AND CELLULASE OF *Pythium ultimum*

Youssef, S. A.[1]; K. A. H. Tartoura[1]; M. Nair [2] and J. M. Vargas[3]

1- **Botany Department, Faculty of Agriculture, Suez Canal University, Ismailia, Egypt.**
2- **Horticulture Department, Michigan State University, E. Lansing, MI 48824.**
3- **Plant Pathology Department, Michigan State University, E. Lansing, MI 48824**

ABSTRACT

In our previous study we demonstrated that the biocontrol agent, *Pseudomonas fluorescens* Q2-87 producing the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), has the ability to antagonize the phytopathogenic *Pythium* spp. by degradation of hyphal cell walls and inhibition of growth. Thin-layer chromatography (TLC) as well as high performance liquid chromatography (HPLC) techniques were used to confirm the production of 2,4-DAPG from the biocontrol agent *P. fluorescens* Q2-87. Activities of polysaccharide degrading enzymes, β-1,3 and β -1,4 glucanase (cellulase), and their relation to *Pythium ultimum* Trow cell wall degradation in relation to exogenous application of 2,4-DAPG were investigated during the first 72 h after treatment. Higher levels of β -glucanases in treated *P. ultimum* comparing to that in control were detected. Glucanase activity reached its highest peak at 12 h; however, cellulase activity reached its highest peak at 24 h after application. Native polyacrylamide gel electrophoresis also confirmed the activities of such hydrolytic enzyme in treated *Pythium* mycelia. Conclusion that bacterial cell-wall degrading enzymes origin are excluded since 2,4-DAPG isolation procedure resulted in 2,4-DAPG-free from such enzymes. The present results suggest that exogenous application of 2,4-DAPG to *Pythium* resulted in higher levels of such cell-wall degrading enzymes that participated in hyphal degradation of the oomycete *Pythium*.

Keywords: 2,4-Diacetylphloroglucinol, β -glucanase activities, *Pseudomonas fluorescens* Q2-87, *Pythium ultimum*

INTRODUCTION

Pseudomonas fluorescens has been used as a biocontrol agent against various plant pathogens because of its capability to produce one or more antibiotics (Hill *et al.* 1994; Cornin *et al.* 1997; Duffy and Défago 1999; Thomashow *et al.* 2000; Landa *et al.* 2002). Production of 2,4-DAPG is one of the antibiotics produced by several strains of *P. fluorescens*, including Q2-87 (Shanahan *et al.* 1992; Keel *et al.* 1992; Fenton *et al.*1992; Harrison *et al.* 1993; Bonsall *et al.* 1997; Dunne *et al.* 1998). *P. fluorescens* Q2-87 was isolated from the rhizosphere of wheat grown in a take-all-suppressive soil and inhibited *Gaeumannomyces graminis* var. *tritici in vivo* and *in vitro* by producing the antibiotic 2,4-DAPG (Harrison *et al.* 1993). Q2-87 strain of *P. fluorescens* producing 2,4-DAPG has the ability to antagonize the phytopathogenic *Pythium* spp. by degradation of hyphal cell wall and inhibition of mycelial growth (Youssef *et al.* 2003). Since β -1,3 glucan and cellulose (β -1,4 glucan) are two of the main structural components of

oomycete plant pathogens (Bartnicki-Garcia and Wang 1983) and cell wall-degrading enzyme activities have been described among fungi, algae and bacteria (Pitson *et al.* 1993), β -1,3 glucanase and cellulase appear to be involved in the antagonistic interaction process of *Pythium* spp. by *P. fluorescens* Q2-87. The physiological role of 2,4-DAPG produced by this strain in this process against *P. ultimum* in relation to cell wall-degrading enzymes is not studied yet.

The present investigation was carried out to achieve the following goals: (a) confirmation that whether or not Q2-87 produces the antibiotic 2,4-DAPG under the experimental condition, if so, (b) determination of β -1,3 and β -1,4 glucanase levels in *Pythium* cells, and (c) determination of the β -glucanase source that participated in hyphal cell wall degradation

MATERIALS AND METHODS

Organisms and culture conditions

Lyophilized *P. fluorescens* strain Q2-87 (NRRL, B-23374) was obtained from the Agricultural Research Service Patent Culture Collection (National Center for Agricultural Research, USDA, Peoria, Illinois). Cultures were grown in 100 ml tryptone soy broth (TSB, 30 g/ L, Difco Lab, Detroit, MI) for 24 h. Cultures were maintained at 28°C in tryptone glucose yeast medium (TGY) containing 5 g tryptone, 5 g yeast extract, 1.0 g glucose, 1.0 g K_2HPO_4 and 15 g agar in 1 liter-distilled water. The pH value was adjusted to pH 6.5. *P. ultimum* was isolated from cowpea in Georgia and kindly provided by Dr. Jeff Hoy (Louisiana State University). *Pythium* cultures were grown in solid V8 medium which consisted of 200 ml V8 juice, 3.0 g $CaCO_3$, 15.0 g agar, 820 ml distilled water. Following experiments were conducted in potato dextrose broth (PDB, 24 g/L, Difco Lab) and potato dextrose agar (PDA, 39 g/ L).

Confirmation that 2,4-DAPG produced by *P. fluorescens*

Natural product 2,4-DAPG was isolated according to a modified method of Shanahan et al. (1992). Strain Q2-87 was grown in PDB for five days in a rotary shaker (250 rpm) at 24°C. Bacterial cells were harvested after centrifugation for 10 min at 6000g and the supernatants were filtered through 0.45 and 0.22 µm nitrocellulose filter. One liter of filtrate was lyophilized and yielded 15.0 g powder. The resultant freeze-dried powder was homogenized in 90% aqueous methanol (MeOH) (ratio 1: 4, w/v) followed by centrifugation at 15000g for 30 min. The supernatant was decanted and evaporated *in vacuo* at 30°C using rotary evaporator and the remaining pellets was dried, kept at 4°C and labeled fraction A. The aqueous extract (8.0 g) was redissolved in MeOH (10 ml) and precipitated by chloroform ($CHCl_3$) to remove sugars. The precipitate was filtered off, dried under vacuum and labeled fraction B. The filtrate MeOH/$CHCl_3$ was evaporated *in vacuo* to dryness and yielded 2.5 g which labeled fraction C. Bioassay preliminary experiments indicated that fraction C only exhibited an antagonistic activity against *P. ultimum* by *in vitro* inhibition assay described below. Therefore, an appropriate sample of this fraction was redissolved in 95% MeOH; 5-10 µl was chromatographed as well as authentic 2,4-DAPG

on thin-layer chromatography (TLC) plates (Silica gel 60 F_{254}, Merck) with an chloroform: methanol (12:1 v/v) as mobile phase. The plates were viewed under UV at 254 and 360 nm and the UV visible spots were marked and further confirmed by spraying with 1% (w/v) vanillin in sulfuric acid (95%). R_f values were calculated for all bands. These bands were marked under UV light, individually scrapped from TLC plates, eluted with MeOH and subjected to *in vitro* inhibition assay.

For analytical high performance liquid chromatography (HPLC), 20 µl aliquots of MeOH extracts having antagonistic activity was analyzed by reversed-phase HPLC (0.8 x 10 cm Waters Nova-pak radial compression cartridge) eluted with a 0.1% trifluoroacetic acid-acetonitrile linear gradient (0 to 100% over 25 min, 100% for 5 min). The retention time of 2,4-DAPG was 14.5. Identification of 2,4-DAPG was confirmed by adding authentic 2,4-DAPG to a part of the sample.

In vitro antagonistic activity bioassay

Preliminary experiment indicated that the only band containing antifungal activity coincided with the UV fluorescent band at R_f 0.82. Consequently, *In vitro* antagonistic activities of the synthetic 2,4-DAPG (purchased from Toronto Research Chemicals Inc., Toronto, Canada) and the natural product produced by Q2-87 strain were assayed on PDA media according to a modified method of Vincent *et al.* (1991). Based on preliminary experiments, media was supplemented with either fraction C containing 2,4-DAPG at concentration 0.25 g/100 ml, or 5-7 µg/ml synthetic 2,4-DAPG. 5 mm mycelial plugs from the actively *P. ultimum* culture grown at 24°C for 3 days were placed in the center of the Petri dishes containing 20 ml of the amended medium. Bacterial strain Q2-87 was streaked in the middle of other Petri dishes containing PDA media as a positive control. After 24 h of pre-incubation at 24°C, mycelial plugs were placed 27 mm distant from each side of the bacterial streak. Inhibition zones were obviously seen after 48 h. Six replicates per treatment were performed.

Natural 2,4-DAPG treatment and sampling

Pythium mycelia were grown in freshly prepared PDB media for 4 days at 24°C in a rotary shaker, then the media replaced by other fresh ones supplemented with fraction C containing 2,4-DAPG (2.5 g/ L, w/v) under sterilized conditions and 2,4-DAPG-free media was used for the control. Incubation condition was identical to those for *Pythium* growth. Starvation conditions did not occur throughout the experiment because some PDA was transferred intracellular of the mycelium, thus giving growth support during the time-course study. Treated and untreated *Pythium* mycelia were collected at specified times and quickly frozen in liquid nitrogen. Samples were weighed, frozen immediately in liquid nitrogen, lyophilized followed by re-weighting and used for enzymatic assay. Levels of β -glucanases, their specific activities, and total soluble protein were without significant variations during the time-course study. Consequently, control corresponded to zero-time.

Protein extraction

Dried mycelium was ground with a morter and pestle under liquid nitrogen. The ground matter was homogenized (2 h, 4°C) in 0.05 M

phosphate buffer (1/3, w/v, pH 5.0). The homogenate was centrifuged twice at 10,000g at 4°C, the supernatant was collected and Protein concentration was determined according to Bradford (1976) using protein assay dye reagent (Bio-Rad Laboratories, Richmond, CA). Aliquots of each sample were precipitated in acetone (1/2, v/v, pH 4.5); acetone traces were vacuum dried, and the powder was resuspended in 0.05 M sodium acetate buffer (pH 5.0) and kept at –20°C. This fraction was immediately used for the determination of β-1,3 glucanase and cellulase activities.

Determination of enzymatic activities

β -1,3-glucanase (EC 3.2.1.6) activity was assayed by a modification of the method of Lima *et al.*, (1997) using Azurine-crosslinked pachyman (AZCL-pachyman; Megazyme) as the substrate. The complete reaction mixture (0.5 ml, 37°C) contained 225 µl crude enzyme preparation extracted with acetone, 25 µl AZCL-pachyman (10 mg ml^{-1}) and 50 mM sodium acetate buffer (pH 5.0). The mixtures were incubated for 20 min with moderate shaking. The reaction was stopped by adding 700 µl of Tris (20% w/v), incubated at room temperature for 3-4 min and then briefly centrifuged and the amount of blue soluble dyed fragments released from AZCL-pachyman was determined spectrophtometrically at 595 nm. Quantifiction of enzyme units was determined using a calibration curve constructed by plotting the amount of glucanase units and their optical densities and performing a linear regression. The method was linear (R>0.99) in the analyzed amount range of 0.005-.025 units of glucanase. The activities were expressed as units $h^{-1}g^{-1}$ dry weight (DW).

β-1,4-glucanase (EC 3.2.1.4) activity was assayed by a modified method of Worthington (1988) using cellulose (Sigmacell type 20, Sigma) as a substrate. The complete reaction mixture (0.5 ml, 37°C) contained 250 µl crude enzyme preparation extracted with acetone, and 250 µl 1% cellulose dissolved in 50 mM sodium acetate buffer (pH 5.0). The reaction mixtures were incubated at 37°C for 120 min followed by transferring into an ice bath for 10 min, then, centrifuged for 5 min at 2°C to clarify the supernatant. Into a cuvette, 3 ml glucose (hexokinase) determination (Sigma Stock No.16-10) was added and equilibrated to 25°C, then 0.1 ml of the supernatant was then supplemented. Immediately mix by inversion and record the maximal increase in absorbance for 5 min at 340 nm.

$$\text{Units.ml}^{-1}\text{ enzyme} = \frac{(\Delta A340\text{ test} - \Delta A340\text{ blank})(3.1)(5)(df)}{(6.22)(2)(1)(0.1)}$$

Where 3.1= final volume (in ml) of step 2, 5 = total volume (in ml) of reaction mix (step 1), df = dilution factor, 6.22 = millimolar extinction coefficient of β-NADH at 340 nm, 2 = conversion factor for 2 h to 1 h, 1 = volume (in ml) of cellulase used in step 1, 0.1 = volume (in ml) from step 1 used in step 2. The activities were expressed as units h^{-1} g^{-1} FW. All data are the means of three independent samples.

Detection of β-1,3 glucanase and β-1,4 glucanase isoforms by native PAGE

Non-denaturing PAGE [12% (w/v) acrylamide] was carried out without SDS using the buffer system according to Laemmli (1970). Detection of β-1,3 glucanase and cellulase activities during maximum activities measured

spectrophotometrically, 12 and 24 h, after application, respectively, was performed by equilibrating in 50 mM potassium acetate buffer, pH 5.0, and then incubated in 25 mM potassium acetate buffer amended with 0.5% laminarin (Sigma), pH 5.0 for 45 min at 37°C. After incubation, the gel was washed with distilled water several times and incubated with 1 M NaOH amended with 0.15% 2,3,5-triphenyl tetrazolium chloride in boiling water for 5 min. Red bands indicate β-1,3glucanase activity. (Kim and Hwang 1997). In relation to cellulase activity, the gel was equilibrated by immersion in 0.25 M Tris-maleate buffer pH 5.0 for 10 min and blotted for 3.0 h at 37ºC against substrate-containing buffered agar (0.5%, w/v) carboxymethylcellulose, CMC (Sigma, C-4888), 1.5% noble agar (Difco) in 0.1 M Tris-maleate buffer pH 0.5, as described by Bertheau *et al.* (1984). The agar gel was gently soaked for 0.5 h in 1% (w/v) Congo red, rinsed in water and 1 M NaCl, then immersed and soaked in 1 M NaCl for 20 min. After repetition of this coloring protocol, the gel was incubated in 1 M NaCl overnight. White bands indicate cellulase activity on a red background. Protein concentration was determined according to the method of Bradford (1976) using Bio-Rad protein assay. Bovine Serum Albumin (BSA) was used as the substrate. β-1,3 glucanase and cellulase activities were repeated three times with identical results.

Determination of the source of β-glucanases

Strain Q2-87 was grown in PDB supplemented with physiological concentrations of β-glucanases inducers, *i.e.* laminarin and CMC, for five days in a rotary shaker (250 rpm) at 24ºC. Bacterial cells were harvested and the supernatants were collected as described above. This supernatant was subjected for assaying β-glucanases at the same manner as previously described.

RESULTS

Confirmation that 2,4-DAPG is produced by *P. fluorescens* Q2-87

A synthetic sample of 2,4-DAPG comigrated with fraction C on TLC and these compounds formed a brilliant yellow derivative when TLC plates were visualized with diazosulfanilic acid. TLC plates developed with fraction C showed presence of three bands as detected under UV radiation at 254 and 365 nm and further visualized after spraying with diazosulfanilic acid (Fig.1). An orange-yellow spot at R_f = 0.82 corresponded to the presence of the authentic 2,4-DAPG was observed. One of these bands containing antifungal activity that was coincided with the UV fluorescent band at R_f 0.82. When this band was marked under UV light, scrapped from TLC plates, eluted with MeOH, then filtered through a 0.2 µm pore-sized filter. The filtrates were concentrated, reinjected onto the HPLC and eluted as a single peak with a retention time of 14 min (Fig. 2). Furthermore, analysis by HPLC revealed that the synthetic and natural 2,4-DAPG exhibited identical retention times. Thus, in addition to TLC, HPLC also provides a mean to identification of the DAPG produced by *P. fluorescens* Q2-87.

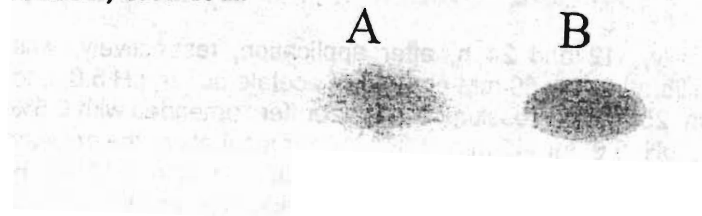

Fig. 1: TLC plate shows authentic 2,4-DAPG band (A) and fraction C bands (B). Fraction C shows a spot corresponded to the presence of the authentic 2,4-DAPG.

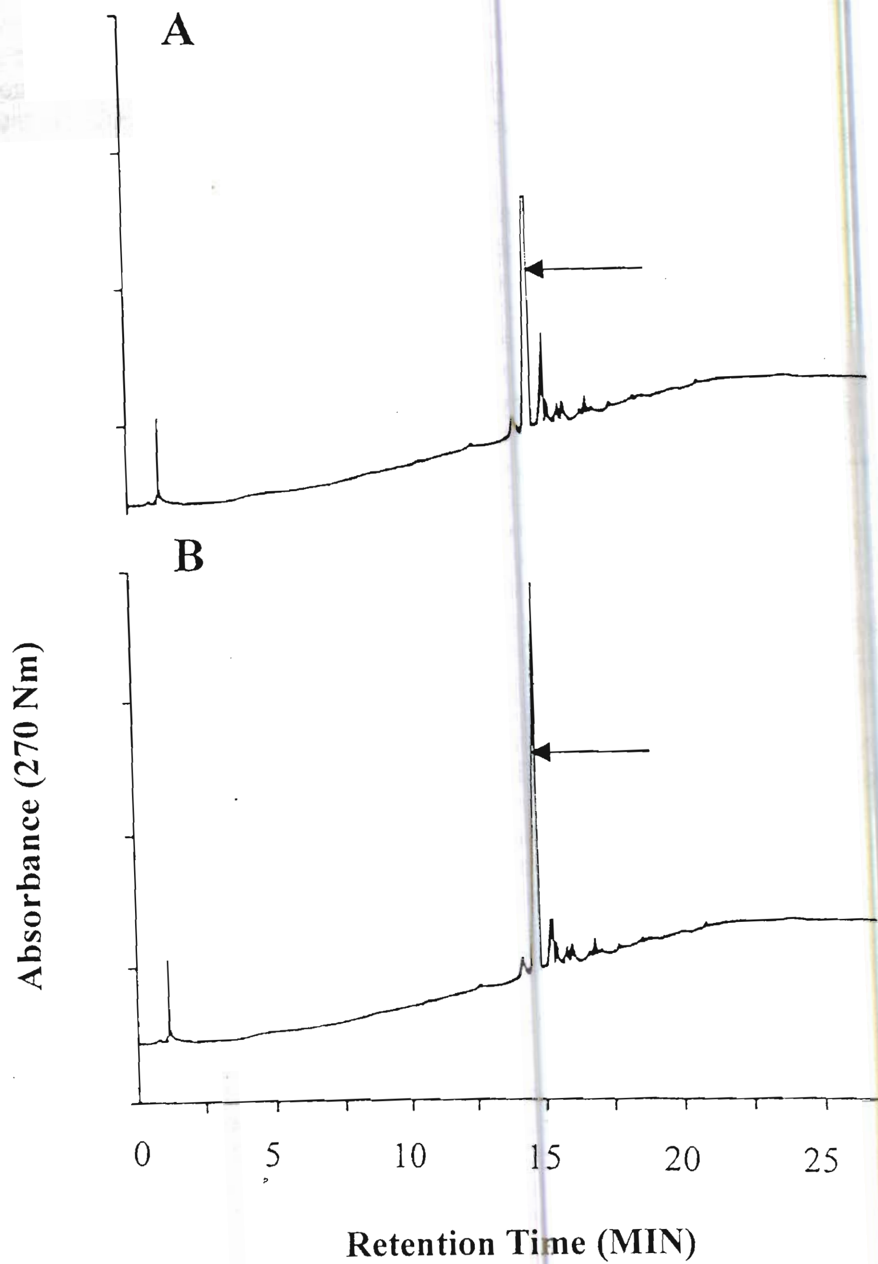

Fig. 2: HPLC chromatographic profiles of 2,4-DAPG isolated from *P. fluorescens* Q2-87. Arrow in (A) represents natural 2,4-DAPG; arrow in (B) represents natural 2,4-DAPG after adding authentic 2,4-DAPG.

Pythium inhibition bioassay

The biological activities of fraction C, authentic 2,4-DAPG, and the bacteria showed inhibitory effectiveness on *Pythium* growth (Fig. 3). Fraction C at 0.25 g/100 ml and authentic 2,4-DAPG at 50 μg/ml completely inhibited *Pythium* growth *in vitro*. Plates were streaked with *P. fluorescens* Q2-87 also inhibited mycelial growth.

Effect of natural 2,4-DAPG on β-1,3 glucanase activity

Higher levels of β-1,3 glucanase and its specific activity were found in treated *Pythium* relative to the untreated one during the time-course of study reaching the maximum level during 12 h after application (Fig. 4). Between 12 and 24 h, these activities gradually decreased. Total soluble protein level was also found to be higher in treated sample comparing to the control during the time-course of study reaching the maximum level during 6 h followed by a gradual decrease.

Effect of natural 2,4-DAPG on cellulase activity

As a response of natural 2,4-DAPG treated mycelium, cellulase activity and its specific activity levels were increased during the time-course of study and reaching the maximum levels during 24 h compared to control (Fig. 5). Total soluble proteins also increased during most of the time-course of study comparing to control and reached the maximum level during 6 h after application. Thereafter, it was gradually decreased.

Detection of β-1,3- and β-1,4- glucanase by native PAGE

Higher β-1,3- and β-1,4- glucanase isozymes were detected in 2,4-DAPG treated samples compared to untreated ones as shown in Fig. 6.

Source determination of β-1,3- and β-1,4- glucanase

β-1,3- and β-1,4- glucanase activities were not detected by means of either spectrophotometric assay or native electrophoretic technique in the 5 day-old culture supernatants of the Q2-87 strain which was either supplemented with β-1,3- and β-1,4- glucanase inducer or not. However, such enzymatic activities were present in *Pythium* mycelium treated with 2,4-DAPG (Figs. 4 and 5)

DISCUSSION

Production of antibiotics is an important mechanism proposed for the antagonistic activity of *P. fluorescens* against soil-borne plant pathogens (Shanahan *et al.* 1992; Howie and Suslow 1991). 2,4-DAPG has been shown to be the main antifungal compound produced by *P. fluorescens* strain Q2-87 (Bonsall *et al.* 1997). In the present work, production of the secondary metabolite 2,4-DAPG was confirmed by means of TLC and HPLC under the experimental conditions used in this investigation (Figs.1 and 2). The present results are in agreement with Harrison *et al.* (1993) who reported that Q2-87 produces both mono- and diacetylphloroglucinol, 2,4-DAPG is only active in exhibiting antagonistic activity against *Pythium.* However, the authors did not identify monoacetylphloroglucinol and its inhibitory effectiveness in fraction C.

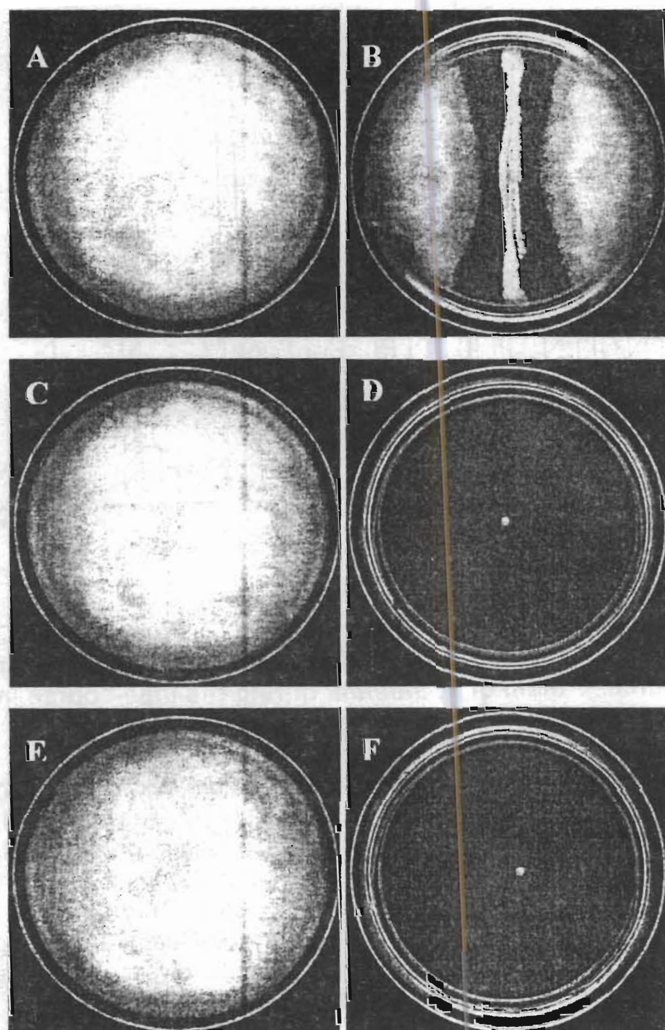

Fig. 3. Light micrographs of *in vitro* inhibition assay of *P. ultimum* on PDA. A, C, and F. Control; B, D, and F represent inhibition resulted from *P. fluorescens* Q2-87, fraction C, and synthetic 2,4-DAPG, respectively. In D and F, the *Pythium* did not grow.

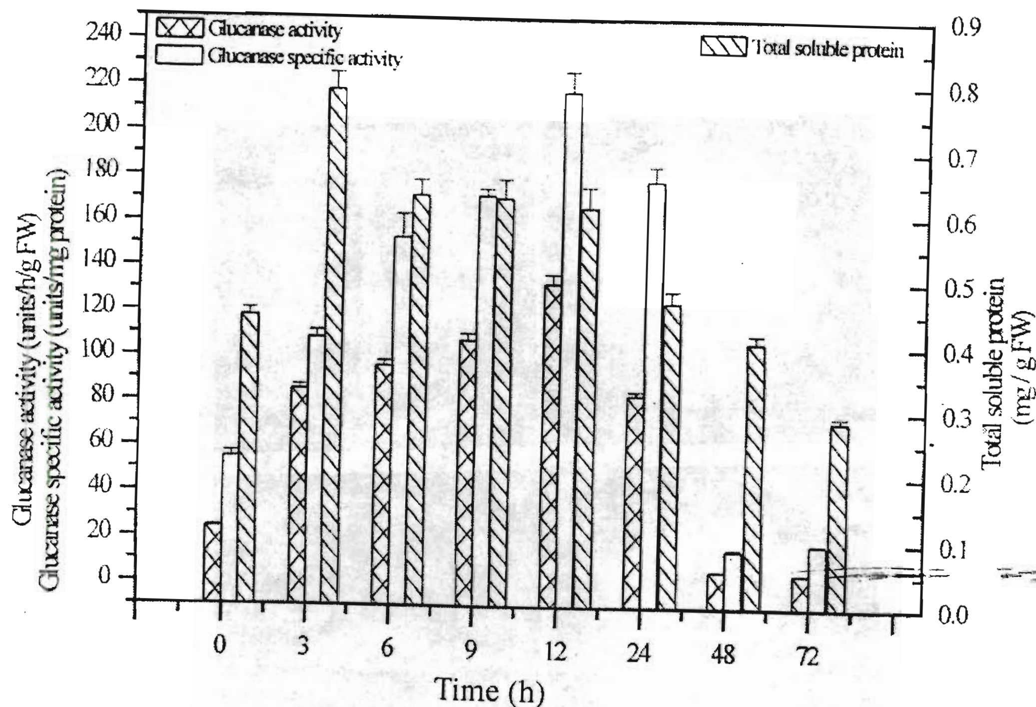

Fig. 4: Effect of 2,4-DAPG produced by *P. fluorescens* Q2-87 on glucanase activity, specific glucanase activity, and total soluble protein of *P. ultimum* during the time-course indicated. Time-zero sample represents control.

In a previous study, degradation of *Pythium* cell walls occurred after treatment with the biocontrol *P. fluorescens* Q2-87. The source of the cell-wall polysaccharide degrading enzymes that caused degradation of cell wall was unknown (Youssef *et al.* 2003). Since *Pythium* cell wall consists mainly of β-1,3- and β-1,4-linked glucans (Bartnicki-Garcia and Lippman, 1973); it requires glucanase and cellulase for lysis (Bartnicki-Garcia and Wang, 1983).

In the present work, activities of cell wall-polysaccharide-degrading enzymes, β-1, 3- and β-1,4-glucanase, showed higher activities in *Pythium* cells after application with 2,4-DAPG. The activities of both enzymes, their specific activities and total soluble proteins exceeded the control by 2-5 times during most of the time-course study as compared to control (Figs. 4, 5, and 6). The present data also indicated that untreated healthy *Pythium* having a low level of β-glucanases production. Of course, presence of these enzymes in oomycete is essential to hyphal tip growth, morphogenesis and for changes in the cell wall composition during morphogenesis, as also suggested by Bartnicki-Garcia and Wang (1983), and Pitson *et al.* (1993).

cellulasef (820x596x24b jpeg)

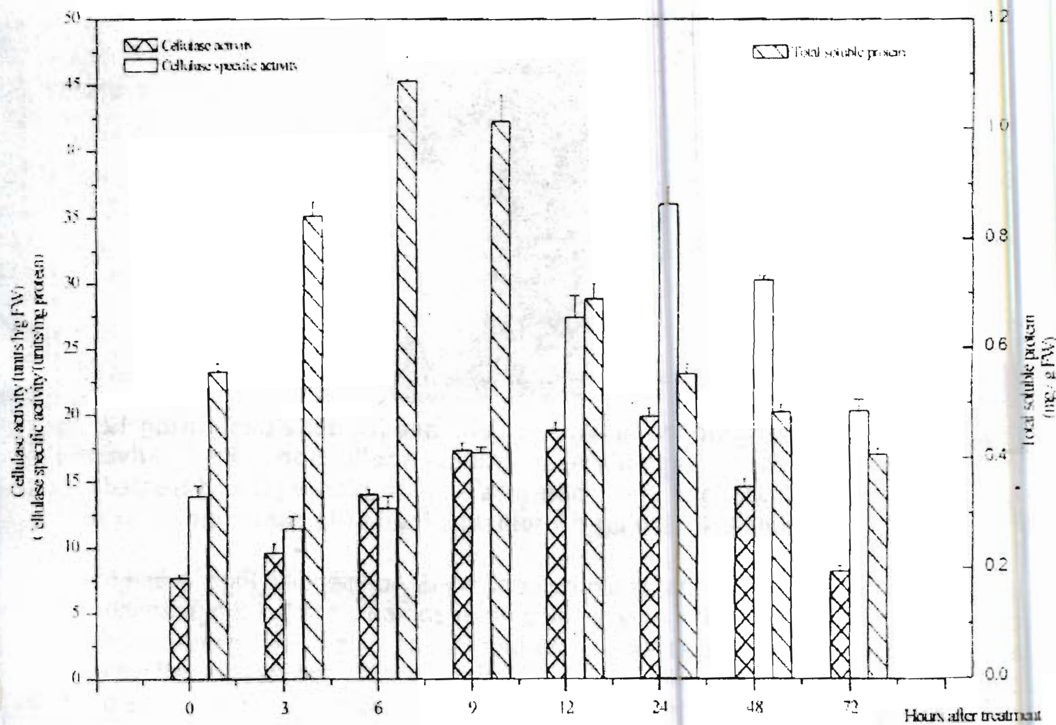

Fig. 5: Effect of 2,4-DAPG produced by *P. fluorescens* on cellulase activity, specific cellulase activity, and total soluble protein of *P. ultimum* during the time-course indicated. Time-zero sample represents control.

The loss of the *Pythium* ability to control the expression of these enzymes following 2,4-DAPG application is probably caused by disruption of the regulatory mechanism; at least, because of the plasma membranes have been reported to be a potential target for antibiotics (Lewis and Papavizas 1987). It seems likely that the production of low levels of hydrolytic enzymes, *e.g.* β-1,3 glucanases, may have allowed the release of elicitor-active molecules in the presence of 2,4-DAPG, e.g. β-1,3 glucans, from *Pythium* cell walls, which further induce synthesis of these enzymes. Alternatively, 2,4-DAPG produced by Q2-87 could be a signal molecule. Alteration of the lipid composition of the plasma membrane of *Pythium* hyphae, possibly resulting from the binding of such molecules, may have induced deregulation of membrane-bound enzymes, resulting in increasing cell wall degrading enzymes. Although significant increase of β-1,3 glucanase and cellulase activities have been observed in mycelial hyphae of *Pythium* after application, it has not been elucidated if the increased activities were due to changed enzymatic properties, increased translation, or increased gene expression.If the induction of such enzymes was found to be due to an increase in the corresponding m-RNA level, increased translation and/ or increased gene expression it might explain the response of the *Pythium* cells to 2,4-DAPG.

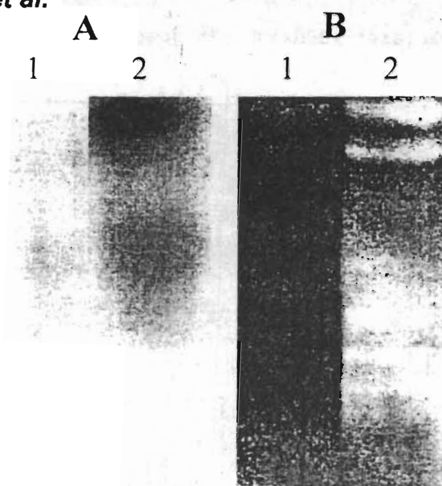

Fig. 6. Detection of glucanase (A) and cellulase (B) activities during 12 and 24 h, respectively, after application by native polyacrylamide gel electrophoresis in untreated (1) and treated (2) *P. ultimum* cells. 30 μg protein was loaded in each lane.

Thus, the latter cases would be confirmed. Additionally, Figs. 4 and 5 show that cellulase activity was peaked later compared to β-1,3 glucanase activity. This may be explained by the fact that cellulosic β-1,4 glucans are embedded in a matrix of amorphous material and successful cell-wall degradation may depend on the activity of more than one enzyme. As a result, β-1,4 glucan seems to be protected by noncellulosic β-1,3glucan, and is not ready accessible to β-1,4 glucanase as also reported by Bartnicki-Garcia and Wang (1983). Thus, it is likely that β-1,4 glucanase activity is preceded by, or coincides with, the hydrolytic activity of other enzymes, especially β-1,3- and β-1,6 glucanases

In the present study, the possibility that bacterial cell wall-degrading enzymes might be participated in inhibition and degradation of *Pythium* hyphae could be excluded for the following reasons: (a) 2,4-DAPG extraction procedure by organic solvents could eliminate any enzymatic activities that might be present in the bacterial culture, and (B) no β-glucanases activities could be detected in the supernatants of *P. fluorescens* Q2-87 even in the culture medium amended with laminarin and carboxymethylcellulose which is known to induce such enzymatic production for β-1,3 glucanase and cellulase, respectively (Winstead and McCombs 1961; Moore and Couch 1968; Janardhanan and Husain 1974). Thus, the source of these higher enzymatic activities, that caused growth inhibition and cell-wall degradation, was *Pythium* itself. However, extracellular hydrolytic enzymes by some *P.* spp., other than Q2-87 strain, are considered important determinants of the antagonistic ability of these bacteria against plant pathogens. The role of these enzymes in biocontrol is supposed to be connected to their direct action against plant pathogens (Fridlender *et al.* 1993). These authors showed that *Pseudomonas cepacia* has the ability to inhibit several plant pathogens in the greenhouse including *Pythium ultimum*, *Fusarium*

oxysporum, *Rhizoctonia solani*, and *Sclerotium rolfsii* by such cell wall-degrading enzymes. Other studies also indicated that there is significant relationship between antagonistic activity of fluorescent *Pseudomonas* and the level of β-1,3 glucanase production which suggests that β-1,3 glucanase production may be responsible for the biological suppression of phytopathogens (Sneh *et al.* 1984; Jones *et al.* 1986; Velazhahan *et al.* 1999; Meena *et al.* 2001). The modes of action of *P. fluorescens* strains differ depending on the bacterial strain, as reported by Whipps 2000.

The present results indicate that *P. fluorescens* Q2-87 was able to produce the phenolic antibiotic 2,4-DAPG under the experimental conditions used in this investigation. Growth inhibition and cell-wall degradation of *Pythium* might be attributed to 2,4-DAPG. Exogenous application of this antimicrobial compound to oomycete *Pythium* may influence the synthesis and/ or activities of cell wall-polysaccharide-degrading enzymes, β-1,3 glucanase and cellulase activities that participated in cell-wall degradation. Further, β-glucanases that involved in degradation process does not originate from bacterial cells. Further work will be required to fully elucidate the modes of biocontrol agent *P. fluorescens* Q2-87 action of a major plant pathogen, *Pythium*. The interaction of other biological compounds with applied 2,4-DAPG may elucidate this mode of action.

REFERENCES

Bartnicki-Garcia, S. and E. Lippman (1973). Fungal cell wall composition. Pages 229-252 In: Handbook of Microbiology. A. I. Laskin and H. A. Lechevaluer, eds. CRC Press, Cleveland, OH.

Bartnicki-Garcia, S. and M. Wang (1983). Biochemical aspects of morphogensis in *Phytophthora*. Pages 121-137 In: *Phytophthora*, Its Biology, Taxonomy, Ecology, and Pathology. Erwin, D. C., Bartnicki-Garcia, S., and Tsao, P.H., eds. The American Phytopathological Society, St. Paul, MN.

Bertheau, Y.; E. Madgidi-Hervan; A. Kotoujansky; C. Nguyen-The; T. Andro and A. Coleno (1984). Detection of depolymerase isoenzymes after electrophoresis or electrofocousing, or in titration curves. Anal. Biochem., 139: 383-389.

Bonsall, R. F.; D. M. Weller and L. S. Thomashow (1997). Quantification of 2,4-diacetylphloroglucinol produced by fluorescent *Pseudomonas* spp. *In vitro* and in the rhizosphere of wheat. Appl. Environ. Microbiol., 63:951-955.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254.

Cronin, D.; Y. Moenne-Loccoz; A. Fenton; C. Dunne; D. N. Dowling; and F. O'Gara (1997). Ecological interaction of a biological *Pseudomonas fluorescens* strain producing 2,4-diacetylphloroglucinol with soft rot potato pathogen *Erwinia carotovora* subsp. *atroseptica*. FEMS Microbiology Ecology, 23: 95-106.

Duffy, B. K. and G. Défago (1999). Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Appl. Environ. Microbiol., 65(6): 2429-2438.

Fenton, A. M.; P. M. Stephens; J. Crowley; M. O'Callaghan and F. O'Gara (1992). Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. Appl. Environ. Microbiol., 58:3873-3878.

Fridlender, M.; J. Inbar and I. Chet (1993). Biological control of soilborne plant pathogens by a β-1,3-glucanase-producing *Pseudomonas cepacia*. Soil Biol. *Biochem.,* 25:1211-1221.

Harrison, L. A.; L. Letendre; P. Kovacevich; E.A. Pierson and D. M. Weller (1993). Purification of an antibiotic effective against *Gaeumannomyces graminis* var. *tritici* produced by a biocontrol agent, *Pseudomonas aureofaciens*. Soil Biol. Biochem., 25:215-221.

Hill, D. S.; R. I. Stein; N. R. Torkewitz; A. M. Morse; C. R. Howell; J. P. Pachlatko; J. O. Becker and J. M. Ligon (1994). Cloning of genes involved in the synthesis of pyrrolnitrin from *Pseudomonas fluorescens* and role of pyrrolnitrin synthesis in biological control of plant disease. Appl. Environ. Microbiol., 60:78-85.

Howie, W. J. and T. V. Suslow (1991). Role of antibiotic biosynthesis in the inhibition of Pythium ultimum in the cotton spermosphere and rhizosphere by *Pseudomonas fluorescens*. Molecular Plant-Microbe Interactions, 4: 393-399.

Janardhanan, K. K. and A. Husain (1974). Production of a toxic metabolite and pectolytic enzyme by *Pythium butleri*. Mycopathol. Mycol. Appl., 52: 325-330.

Jones, J.D. G.; K. L. Grady; T. V. Suslow and J. R. Bedbrook (1986). Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens*. EMBO J., 5: 467-473.

Keel, C.; U. Schnider; M.Maurhofer; C. Voisard; J. Laville; U. Burger; P. Wirthner; D. Haas and G. Défago (1992). Suppression of root diseases by *Pseudomonas fluorescens* CHA0: Importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. Molecular Plant-Microb Interact., 5: 4-13.

Kim, Y. J., and B. K. Hwang (1997). Isolation of a basic 34 kiloDalton β-1,3 glucanase with inhibitory activity against *Phytophthroa capsici* from pepper stems. Physiological and Molecular plant pathology., 50: 103-115.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Landa, B. B.; O. V. Mavrodi; J. M. Raaijmakers; B. B. McSpadden-Gardener; L. S. Thomashow and D. M.Weller (2002). Differential ability of genotypes of 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* strains to colonize the roots of pea plants. Appl. Environ. Microbiol., 68: 3226-3237.

Lewis, J. A. and G. C. Papavizas (1987). Permeability changes in hyphae of *Rhizoctonia solani* induced by germling preparations of *Trichoderma* and *Gliocladium*. Phytopathol., 77: 699-703.

Lima, L. H. C.; C. J. Ulhoa; A. P. Fernandes and C. R. Felix (1997). Purification of a chitinase from *Trichoderma* sp. And its action on *Sclerotium rolfsii* and *Rhizoctonia solani* cell walls. Journal of Gen. Appl. Microbiol., 43:31-37.

Meena, B.; T. Marimuthu; P. Vidhyasekaran and R. Velazhahan (2001). Biological control of root rot of groundnut with antagonistic *Pseudomonas fluorescens* strains. Plant Diseases and Protection, 108: 369-381.

Moore, L. D. and H. B. Couch (1968). Influence of calcium nutrition on pectolytic and cellulolytic enzyme activity of extracts of Highland bentgrass foliage blighted by *Pythium ultimum.* Phytopathol., 58: 833-838.

Pitson, S. M.; R. J. Seviour and B. M. McDdougall (1993). Non-celluloytic fungal β-glucanases: their physiology and regulation. Enzyme Microb Technol., 15: 178-192.

Shanahan, P.; J. O'Sullivan; P. Simpson; J. D. Glennon and F. O'Gara (1992). Isolation of 2,4-diacetylphloroglucinol from a fluorescent Pseudomonad and investigation of physiological parameters influencing its production. Appl. Environ. Microbial., 58: 353-358.

Sneh, B.; Y. Dupler Elad and R. Baker (1984). Chlamydospore germination of *Fusarium oxysporum* f. sp. *Cucumerinum* as affected by fluorescent and lytic bacteria from *Fusarium*-suppressive soil. Phytopathol., 74: 1115-1124

Strunz, G. M.; R. E.Wall and Di J. Kelly (1978). Phloroglucinol derivatives from Aeromonas hydrophilla. J. Antibiot., 31: 1201-1202.

Thomashow, L. S.; O. V. Mavrodi; D. V. Mavrodi; J. M. Raaijmakers; B. B. Bonsall; R. F. McSpadden-Gardener; Y.-J. Lee; M. J. Park and D. M.. Weller (2000). 2,4-Diacetylphloroglucinol, a major determinant in the biological control of take-all disease of wheat, p. 331-335. In: De Witt, P. G. M., Bisseling, T., and Stiekema, W. J. (ed). Biology of Plant-Microbe Interactions, Vol. 2. International Society for Molecular Plant-Microbe Interactions, St. Paul, MN.

Thrane, C.; D. F. Jensen and A. Tronsmo (2000). Substrate colonization, strain competition, enzyme production in vitro, and biocontrol of *Pythium ultimum* by *Trichoderma* spp. Isolates P1 and T3. Eur. J. Plant Pathol., 106: 215-225.

Velazhahan, R.; R. Samiyappan; and P. Vidhyasekaran (1999). Relationship between antagonistic activites of *Pseudomonas fluorescens* isolates against *Rhizoctonia solani* and their production of lytic enzymes. Plant Diseases and Protection, 106: 244-250.

Vincent, M. N.; L. A. Harrison; J. M. Brackin; P. A. Kovacevich; P. Mukerji; D. M. Weller and E. A. Pierson (1991). Genetic analysis of the antifungal activity of a soilborne *Pseudomonas aureofaciens* strain. Appl. Environ. Microbiol. 57: 2928-2934.

Whipps, J. M. (2000). Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52: 487-511.

Winstead, N. N. and C. L. McCombs (1961). Pectinolytic and cellulolytic enzyme production by *Pythium aphanidermatum*. Phytopathol., 51: 270-273.

Worthington, C. E. (1988). Worthington Enzyme Manual, pp. 76-79, Worthington Biochemical Corporation, Freehold, NJ.

Youssef, S. A.; K. A. Tartoura and J. M. Vargas (2003). Antagonistic interaction between the biocontrol agent Pseudomonas fluorescens Q2-87 and plant pathogenic Pythium in relation to hyphal ultrastructural analysis. J. Agric. Sci. Mansoura Univ., 28 (12):

تأثير المضاد الحيوي الناتج من بβتيريا سيدوموناس فلوريسنس سلالة Q2-87 علي إنزيمات البيتا ١،٣ جلوβانيز والسليوليز لبيثيم ألتيمم

سحر على جمال الدين يوسف[١] - βامل أحمد حسين طرطورة[١] - موورالديهاران نـــير[٢] جوزيف فارجس[٣]

[١]قسم النبات الزراعي - جامعة قناة السويس بالأسماعيلية- مصر

[٢] قسم البساتين - جامعة ميشيجان بأمريβا

[٣]قسم أمراض النبات - جامعة ميشيجان بأمريβا

في بحث سابق للمؤلفين تم استنتاج أن الناتج الثانوي ٢،٤ داي أســيتيل فلوروجلوســينول لبβتيريا سيدوموناس فلوريسنس Q2-87 له قدرة علي تضاد أنواع بيثيــوم المســببة أمــراض للنباتات وذلβ بإحداث تغيرات فسيولوجية تؤدي إلي تحلل الجدر الخلوية وإيقاف النمو. وقد أجري هذا البحث بهدف تأβيد قدرة تلβ السلالة البβتيرية علي إنتاج ٢،٤ داي أسيتيل فلوروجلوســينول تحت الظروف المستعملة لتنميتها وأيضا لتقدير بعض الأنشطة الإنزيمية، بيتا ١،٣ جلــوβانــيز و سليوليز ، المسئولة عن تحلل بيثيوم المعامل خارجيا بهذا المضاد الحيوي المســتخلص مــن تــلβ السلالة.

أوضحت النتائج المتحصل عليها باستخدام βروماتوجرافيا الطبقة الرقيقة وجهاز التحليــل الβروماتوجرافي العالي الأداء وجود المضاد الحيوي ٢،٤ داي أسيتيل فلوروجلوسينول في بيئــة نمو البβتيريا. βما أوضحت النتائج أيضا أن هناβ مستويات عالية لإنزيمات التحلل المائي بيتــا ١،٣ جلوβانيز وسليوليز في بيثيوم المعامل بمقارنته بالβنترول وأن مصدر تلβ الإنزيمات هــو بيثيوم.

من النتائج التي تم الحصول عليها تم إقتراح أن المضاد الحيوي البβتيرى هو المســئول عن مقاومة بيثيوم حيويا بتحلل جدره الخلوية إنزيميا وإيقاف نموه الطبيعي .