# Mode of Action of *Bacillus pumilus* in Suppressing *Pseudoperonospora cubensis* (Berk and Curt) Rostow, the Pathogen of Downy Mildew of Cucumber

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## ABSTRACT

Microscopic examinations by light and scanning electron microscopy of the cucumber downy mildew lesions treated by liquid culture of *Bacillus pumilus* showed direct effects that appeared as loss in turger and osmolysis of sporangia and hyphae of *Pseudoperonospora cubensis* (Berk and Curt) Rostow. Surface activity of *B. pumilus* culture filtrate was verified on the red blood cells. The spectrum of Gas-Chromatography of the metabolites excreted by *B. pumilus* included various bands which mostly represented different antibiotic and surface-active compounds. Increasing of peroxidase and polyphenoloxidase in newly emerged leaves of treated plants confirmed induction of resistance against the downy mildew disease.

## INTRODUCTION

Downy mildew is a severe disease in cucumber plants in Egypt. Use of fungicides for disease control causes environmental disorder, in addition the causal agent, *Pseudoperonospora cubensis* (Berk and Curt) Rostow, has developed resistance to fungicides (Klinkenberg *et al.*, 1998). Many species of *Bacillus* are known to suppress several fungal plant pathogens belong to many genera such as; *Rhizoctonia, Sclerotinia, Fusarium* in addition to *Pseudoperonospora cubensis* (Fiddman and Rossall, 1994). Bacterial antagonists assume their antagonistic effects, mainly by the production of antifungal antibiotics which seem to play a major role in the biological control of plant pathogens (Leifert *et al.*, 1995). Many of these antifungal substances have been characterized and identified as peptide antibiotics (Katz and Demain, 1977). The antifungal peptides produced by *Bacillus* species included mycobacillins (SenGupta *et al.*, 1971), iturins (Isogai *et al.*, 1982) and bacillomycins (Peypoux *et al.*, 1981). El-Gremi *et al.*, (2012) stated that *P. cubensis* on cucumber plants was suppressed and controlled mostly by a local isolate of *Bacillus* sp.

The present study was designed to verify the mechanism of action of the most effective isolate identified as *B. pumilus*.

## MATERIALS AND METHODS

### *Pseudoperonospora cubensis* inoculum and plant inoculation

Infected cucumber plants that showed ideal downy mildew symptoms caused by *P. cubensis*, were collected from different locations at Kafr Elsheikh and Elgharbiya governorates, Egypt. The infected plants were transferred to the laboratory and maintained in humid chambers (~ 90 % RH) at 20°C and with full overnight darkness for sporulation. The sporangia were then washed off by shaking the infected leaves gently in distilled water for few minutes. Spore suspension was adjusted to $5 \times 10^4$ sporangia/ml, using a hemocytometer. Inoculation with *P. cubensis* was artificially performed onto two weeks aged cucumber seedlings, having fully expanded true leaves, according to Samoucha and Cohen (1984). Sporangial suspension was sprayed onto the upper and lower surfaces of the leaves. Each plant received 10-20 ml spore suspension. The infected plants were irrigated whenever needed and fertilized as recommended with calculated doses of the mineral elements.

### Preparation of *Bacillus pumilus* metabolites

To estimate maximum production of the antibiotic metabolites, the method described by Awais *et al.* (2007) was followed. Flasks of 500 ml capacity, containing 100 ml nutrient broth medium, were inoculated, each with 1 ml from 3 days old nutrient broth culture. The cultures were incubated at 30°C and 150 rpm for 7 days. The production of antibiotic metabolites was determined daily by determination of the disease inhibition after treating the infected plants. The liquid culture showed maximum disease inhibition was used for obtaining free cell supernatant. Cells were precipitated at 10000 rpm centrifugation for 15 min.

### Nature of mode of action

The prepared cell free supernatant was used to treat the cucumber plants therapeutically (for direct effect) as well as prophylactically (for indirect effect).

## a- Direct effect

Prepared *B. pumilus* filtrate was sprayed on infected plants showed the appearance of proliferated downy growth of *P. cubensis*. Plants sprayed with tap water only served as check (control). On the 4th day after treatment, specimens of lesions were sampled and prepared for microscopic examination. Comparing with the check, any abnormality or deleterious effects on the tested pathogen *P. cubensis* were observed, using light and scanning microscope. For light microscope, epidermic smears from leaves bearing downy mildew lesions were mounted on slides in lactophenol. For scanning electron microscope (SEM), pieces of cucumber leaves bearing lesions of downy mildew were processed according to the technique of Harley and Fergusen (1990). Pieces (~4 mm$^2$) were fixed in 3% glutaraldehyde and 0.2 M phosphate buffer (pH 7.2) for 24 h at 4°C, followed by exposure to Osmium tetraoxide (1% OsO$_4$) for one hour at room temperature. The samples were dehydrated by pathing through ascending concentrations of acetone, then dried till the critical point and finally, they were sputter coated with gold. The examination and photographing were done through a Jeol Scanning Electron Microscope (JSM-T.330 A) of the Central Laboratory, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Since an osmolysis was microscopically observed in *P. cubensis* treated with *B. pumilus* culture filtrate, surface activity of the filtrate was verified on the red blood cell membrane as recorded in the method of Lawrence (1950). A suspension of chicken red blood cells was prepared, using 0.8 saline solutions in heparinized tubes. Portions of the *B. pumilus* culture filtrate were added to the cell suspension (3-100% v/v). Other tubes, contained blood cell suspension, were amended by portions of saline solution and served as check. Tubes were left stable for 20 min and precipitation of the red cells and clearing of suspension were compared. The treated and the untreated blood cells were examined under light microscope for features of lysis.

## b- Indirect effect (induced plant resistance)

In this experiment, infected 30 days old plants were treated with the free cell culture filtrate. Plants treated with tap water served as control. After 10 days, new healthy emerged uninfected leaves from the treated plants were sampled and tested as indicators of induced resistance (the activity of peroxidase and polyphynoloxidase). According to Maxwell and Bateman (1967), plant tissues were cut into small portions and rapidly ground with 0.1 M Sodium phosphate buffer at pH 7.1 (2ml buffer /g of fresh tissue) in a cooled mortar. These triturated tissues were filtered through four layers of cheese-

cloth and the filtrates were centrifuged at 3000 rpm for 20 min at 6°C. The supernatant fluids were used to estimate the activity of enzymes.

**Peroxidase activity**: As described by Allam and Hollis (1972), activity of peroxidase enzyme was determined colorimetrically by measuring the oxidation of pyrogallol to purprgallin in the presence of H$_2$O$_2$. The reaction mixture contained 0.5 ml of 0.1 *M* Potassium phosphate buffer solution at pH = 7.0, 0.3 ml sample extract, 0.3 ml of 0.05 M pyrogallol, and 0.1ml of 1.0% H$_2$O$_2$, then completed with distilled water up to 3.0 ml. The activity was expressed as absorbance change per minute at 425 nm.

**Polyphenoloxidase activity**: The activity of Polyphenoloxidase was measured by the colorimetric methods of Maxwell and Bateman (1967). The reaction mixture contained 1.0 ml sample extract, 1.0 ml of 0.2 Sodium phosphate buffer at pH = 7.0 and 1.0 ml of 10$^{-3}$ *M* catechol and then completed the final volume to 6.0 ml with distilled water. The reaction mixture was incubated for 30 minutes at 30°C. The activity of Polyphenoloxidase was expressed as optical density at 495 nm.

## Chemical characterization of *B. pumilus* metabolites
### a- Extraction of metabolites

The cell free supernatant was adjusted to pH 2.5 with 6 *M* HCL and the precipitate was collected by centrifugation at 15000 rpm for 20 min (Mckeen *et al.*, 1986). Precipitates were extracted twice with 80% methanol, pooled and dried. Stock solutions of 50 mg/ ml were made by dissolving the powders in 80% methanol, and stored at 4°C.

### b- GC-MS analysis

The method of hydrolysis-methanolysis was reported by Aveldano and Horrocks (1983). It was used to obtain the methyl esters without dehydration of the ß-hydroxy fatty acids and to avoid the formation of the mixture of α- and ß-monounsaturated fatty acids. Volatile compounds in the extracts were analyzed by gas chromatography and mass spectrometry. The GC-MS analysis for derivatizing extract from bacterial filtrate using BSTFA [N,O-bis-(trimethylsilyl trifluoroacetamide) + TMCS (trimethyl chloro-silane)], as derivative kit, was performed using Agilent 6890 N gas chromatography instrument coupled with an Agilent MS-5975 inert XL mass selective detector and an Agilent auto sampler 7683-B injector (Agilent Technologies, Little Fall, NY, USA) with capillary column HP-5MS (5% phenyl methylsiloxane) with dimension of 30 m x 0.25 mm id x 0.25 µm film

thickness (Agilent Technologies, Palo Alto, CA, USA). Initial oven temperature was 40°C, held for 2 min, ramped at 6°C min$^{-1}$ to 180°C and ramped at 10°C min$^{-1}$ to 250°C and held for 3 min. The ions were detected in the range of 30-350 m/z. Mass-spectra of the unknown compounds were compared by Chem-Station 6890 Scale Mode software with two libraries (NIST and Wily), which provided best information about the identification of active compound in the polar extract (Liu et al., 2008).

### Statistical analysis

A complete randomized design was used for the laboratory and glasshouse experiments. Each experimental design had its replications. Data were transformed before subjection to analysis of variance, using IRRl Stat Computer Program and zero values were replaced by minimum values before transforming the data. Means were compared using LSD, the method of Steel and Torrie (1980) and multiple range tests according to Duncan (1955).

## RESULTS AND DISCUSSION

### Nature of mode of action
#### 1- Direct effect
The light and Scanning Electron Microscope (SEM) examinations (Fig. 1) illustrate loss of turgor in sporangia and collapse of sporangiophores of P. cubensis, as a result of treatment with B. pumilus. Osmolysis features denoted to a potential surface activity of substances excreted by B. pumilus. This potential action was proven by treatment of the red blood cells with the culture filtrate of B. pumilus. In (Fig. 2C), untreated cells are intact and well suspended containing their own hemoglobin, whereas in (Fig. 2T) the treated cells liberated their hemoglobin and precipitate as debris at the bottom of the tube. Light microscopy of the two tubes contents (Fig. 3) shows that the untreated cell blood remained intact (Fig. 3A), whereas membranes of red cells were down, collected as debris at the bottom in the test tube (Fig. 3B).

#### 2- Indirect effect
The activity of peroxidase and polyphynol-oxidase, as indicators of induced resistance, were estimated in the upper new emerged leaves from previously treated cucumber plants. The recorded levels of peroxidase were significantly higher in leaves sampled from plants treated with B. pumilus filtrate. Estimated peroxidase activity (optical density) reached 1.366 O.D, compared with that in untreated leaves (0.757 O.D). In addition, polyphynoloxidase gave significantly high level in leaves treated with B. pumilus filtrate (1.202 O.D), compared to the untreated leaves (0.473 O.D).

### Chemical characterization of B. pumilus metabolites:
GC-MS analysis of the methanol extracted metabolites of B. pumilus (Fig., 4 and Table, 1) represented peak numbers and areas of compounds. Totally, thirty-three ones were determined, mostly included fatty acids, known as Hexadecanoic, n-Hexadecanoic, Octadecanoic, 8-Octadecanoic, 9-Octadecanoic, Pentadecanoic, Hepta-decanoic, dodecatrienoic, Nonanoic and Decanoic acids. Other organic compounds such as; amines, amides, organic acids, esters, heterocyclic compounds, alcohols and ketones were also included. Mass-spectrum was obtained and confirmation of compounds was done by a comparison of the retention time and mass-spectrum with those in the Wiley 7n.1, NIST98.1 and Pest.1 libraries.

Downy mildew is a severe disease in cucumber plants in Egypt therefore; there is an urgent need to find an alternative mean to control the disease. El-Gremi et al., 2012 directed their studies to produce cucumber under greenhouse cultivations using bio-control agents, as alternative to synthetic fungicides. The liquid culture of B. pumilus showed antagonistic activity against the pathogen so; antagonistic effects due to excreted metabolites were hypothesized.

In the present study, direct effect of B. pumilus filtrate on the infective propagules (sporangia and zoospores) of the pathogen (P. cubensis) was verified and microscopic examination of the treated symptom lesions showed that sporangia suffered from osmolysis.

Surface activity of the used filtrate was proven on the red blood cells. The spectrum of gas-chromatography of the excreted metabolites included various bands which represent different known antibiotic components (fatty acids) having surface activities. In addition, cyclotetrasiloxane octamethyl, which is used as a surfactant in certain pesticide products, was also found to be produced by B. pumilus (Kenny, 2011)

Generally, numerous members of Bacillus species are known as producers of lipopeptides belong to the surfactin, iturin, and fengycin families (Zuber et al., 1993). Fengycin is an antifungal lipopeptide complex produced by Bacillus subtilis F-29-3 (Vanittanakom and Loeffler, 1986). It consists of two main components, fengycin A and fengycin B., the lipid moiety of both analogs is more variable, as fatty acids. They have been identified as anteiso-pentadecanoic acid (ai- C15), iso- hexadecanoic acid (i- C16), n-hexadecanoic acid (n-C16), and there is an evidence for further saturated and unsaturated residues up to C18.

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Fig. (1): Scanning Electron Microscopy (SEM) of *Pseudoperonospora cubensis* in lesion on infected cucumber leaf. (A: untreated lesion showing normal growth of sporangia, B: lesion treated with *B. pumilus* culture filtrate showing collapse and collapse osmolysis in sporangia).
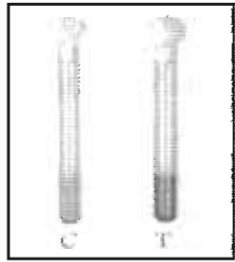


Fig. (2): Effect of *B. pumilus* culture filtrate on red blood cells. (C: intact normal suspended untreated cells, T: impaired treated cells with released hemoglobin).
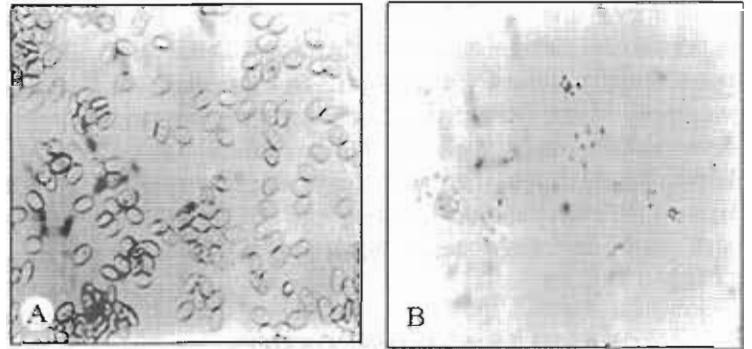


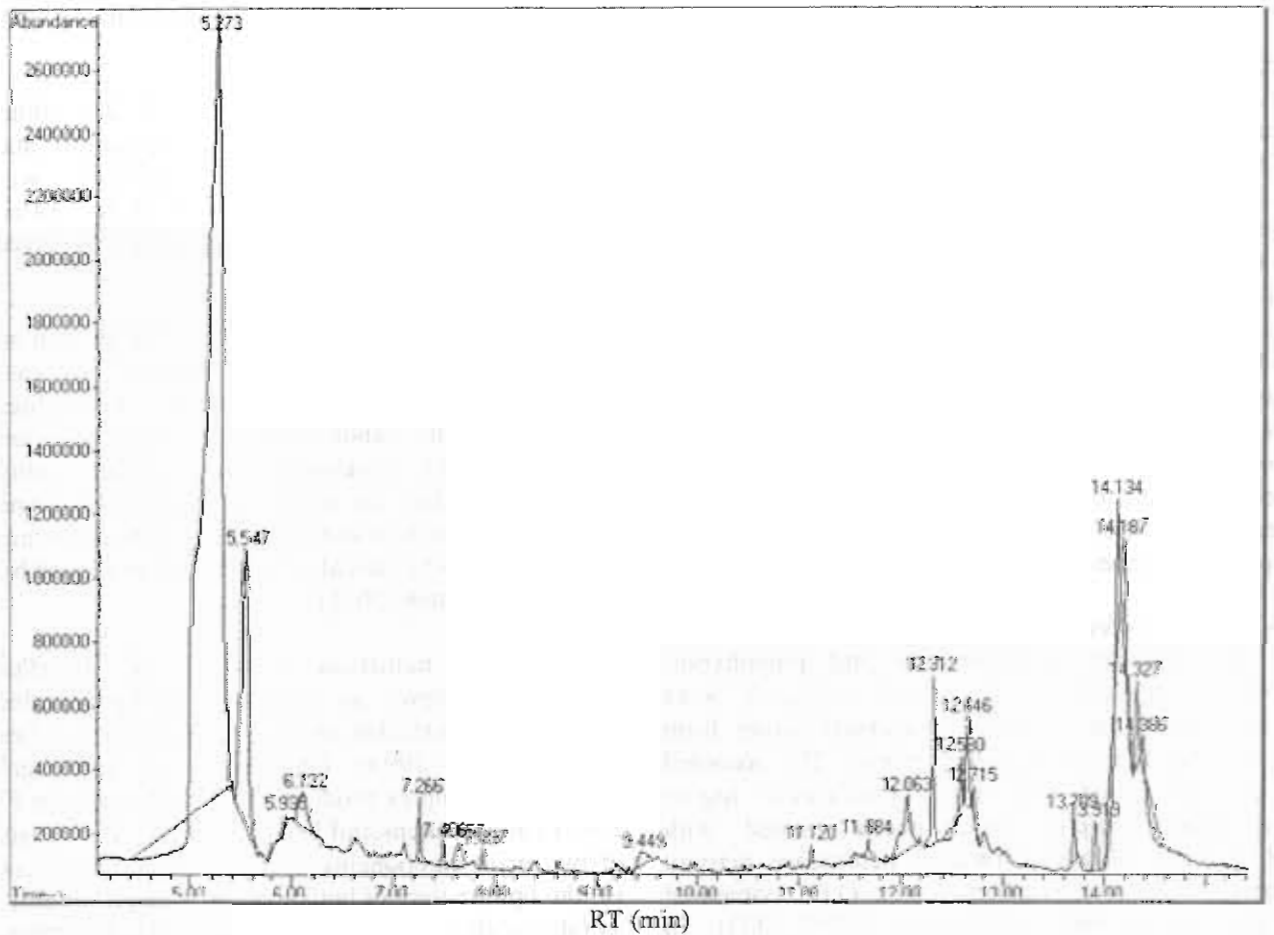Fig. (3): Light microscopy of untreated (A) and treated (B) red blood cells.



Fig. (4): GC-MS analysis of metabolites excreted by *Bacillus pumilus*.

Table (1): Potential compounds screened in GC-MS chromatography of *Bacillus pumilus* metabolites

| Peak | RT (min) | Compound | Molecular formula |
|---|---|---|---|
| 1 | 5.935 | 2,6,10-dodecatrienoic acid,3,7,11-trimethyl-,methyl ester | $C_{16}H_{26}O_2$ |
| 2 | 5.998 | Acetic acid, trifluoro | $CF_3CO_2H$ |
| 3 | 6.124 | Acetic acid, trifluoro | $CF_3CO_2H$ |
| 4 | 6.610 | Acetic acid, trifluoro | $CF_3CO_2H$ |
| 5 | 7.119 | Acetic acid, trifluoro | $CF_3CO_2H$ |
| 6 | 7.268 | Cyclotetrasiloxane, octamethyl or NUC silicone vs 7207 | $C_8H_{24}O_4SI_4$ |
| 7 | 7.486 | Pentasiloxane, dodecamethyl | $C_{12}H_{36}O_4SI_5$ |
| 8 | 7.657 | Acetic acid, trifluoro | $CF_3CO_2H$ |
| 9 | 7.898 | Nonanoic acid, methyl ester | $C_{12}H_{22}O_2S_2$ |
| 10 | 8.401 | Acetic acid, triflers | $CF3CO2H$ |
| 11 | 8.624 | Decanoic acid , methyl ester | $C_{13}H_{26}O_2$ |
| 12 | 9.179 | But-2- enamide, n,n-dimethyl | $C_8H_{16}Ne\ O$ |
| 13 | 9.448 | DL-Proline , 5-oxo-, methyl ester or L-Glutamic acid | $C_6H_9NO_3$ |
| 14 | 9.574 | Pyrrolidine, 2-ethyl- 1-methyl | $C_4H_9N$ |
| 15 | 9.609 | Piperidine, 2-propyl | $C_9H_{19}N$ |
| 16 | 11.119 | Tetradecanoic acid , methyl ester | $C_{15}H_{30}O_2$ |
| 17 | 11.565 | 1,1,2,3-Tetramethylcyclohexane | $C_{12}H_{19}CL$ |
| 18 | 11.686 | Pentadecanoic acid , methyl ester | $C_{16}H_{32}O_2$ |
| 19 | 11.869 | Tetra propylene | $C_{12}H_{16}O_2$ |
| 20 | 12.063 | Biphenyl (Dodemorph) | $C_{18}H_{35}NO$ |
| 21 | 12.183 | Pyrrolo[1,2,-a]piperazine-3,6-dione | $C_{15}H_{13}NO_2$ |
| 22 | 12.309 | Hexadecanoic acid, methyl ester or Palmitic acid , methyl ester | $C_{17}H_{34}O_2$ |
| 23 | 12.447 | n-Hexadecanoic acid or Pentadecanecarboxylic acid or Coconut oil fatty acids | $C_{17}H_{34}O_2$ |
| 24 | 12.590 | n-Hexadecanoic acid | $C_{17}H_{34}O_2$ |
| 25 | 12.647 | n-Hexadecanoic acid | $C_{17}H_{34}O_2$ |
| 26 | 12.716 | Dibutyl phthalate | $C16H22O4$ |
| 27 | 12.819 | 2-Hydrxy-3,5,5-trimethyl-cyclohex-2-enone | $C_{15}H_{22}O_2$ |
| 28 | 13.711 | 8-Octadecenoic acid , methyl ester | - |
| 29 | 13.917 | Heptadecanoic acid , 16-methyl- , methyl ester | $C_{19}H_{38}O_2$ |
| 30 | 14.135 | 9-Octadecenoic acid or trans-.delta. 9-Octadecenoic acid | $C_{18}H_{34}O_2$ |
| 31 | 14.186 | Heptadecene-(8)-carbonic acid –(1) or Oleic acid or 9-Octadecenoic acid | $C_{17}H_{36}$ $C_{18}H_{34}O_2$ |
| 32 | 14.329 | Octadecenoic acid or steric acid or Vanicol or Neo-Fat 18 or Oleic acid | $C_{27}H_{56}O_8$ $C_{18}H_{34}O_2$ |
| 33 | 14.386 | Octadecenoic acid or steric acid or Vanicol | $C_{18}H_{34}O_2$ |

In the present study, components of fatty acids had been detected. Although application of bioagents at late stages of plant infection may be suitable for curative purposes and to decrease the potential secondary inoculum, the internal pathogen remained far away from the antifungal activities of the antagonist (Sadoma, 1995). An additional prophylactic action, which was revealed on the newly emerged plant leaves, was referred to probable host induced resistance and this was proven as a fact since the activities of both peroxidase and poly phenoloxidase raised in the newly emerged untreated leaves. Most of the reviewed indicators for induced resistance in plants are the peroxidase and polyphenoloxidase enzymes (Li *et al.*, 1991). Protection resulted from induced systemic resistance (ISR), elicited by *Bacillus* spp. has been reported against leaf-spotting fungal and bacterial pathogens (Choudhary and Johri, 2009).

Obtained data revealed a significant increase in both enzymes; peroxidase and polyphenoloxidase in specimens sampled from plants pretreated with the tested bioagent, compared with the untreated plants. El-Kafrawy and Al-Ashaal (2008) reported that the oxidative enzymes (peroxidase and poly phenoloxidase) increased in the least susceptible cucumber cultivars to downy mildew than in the susceptible ones. Lignin biosynthesis was mediated by the peroxidase- $H_2O_2$ system. Cell wall-bound peroxidase was probably involved in the generation of hydrogen peroxide, which in return was necessary for lignification (Edreva, 1989). Lignification has been suggested as a mechanism for disease resistance (Dean and Kuc, 1987). In addition, peroxidase generating hydrogen peroxide may function as an antifungal agent in disease resistance. Hydrogen peroxide inhibited pathogens directly and or it might generate other active free radicals that

were antimicrobial antibiotic and cytotoxic activities against pathogens (Peter, 1989). Polyphynol oxidase was also involved in the lignification of plant cells that contributed to the formation of defense barriers against pathogens (Mohammadi and Kazemi 2002). Polyphynoloxidase activity correlated with resistance to *P. cubensis* in cucumber (Li *et al.*, 1991). Indeed, increased polyphynoloxidase activities were correlated with defense against pathogens in several plants, including cucumber, wheat, potato, cotton and rice (Mohammadi and Kazemi, 2002).

Finally, *B. pumilus* excreted metabolites had been defined as surface active materials which directly inhibited the pathogen and on the other hand, indirectly enhanced host plants stand against the pathogen (*P. cubensis*) through increasing plant enzymes, responsible for resistance. Such biocontrol agent is promising for control practices against downy mildew on cucumber (Peng and Kuc 1992). On the other hand, polyphenoloxidase had an important role in physiological functions in plant growth and development and in plant defense against pests and pathogens (Li and Steffens 2002). The active quinines produced by polyphynoloxidase may possess direct.

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