Biological control of *Pythium aphanidermatum* causal agent of root and/or collar rots of papaya (*Carica papaya*) by *Pseudomonas fluorescens*

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Abstract

*In vitro* and *in vivo* tests were conducted to evaluate *P. fluorescens* antagonistic properties against *P. aphanidermatum*. Four discs of 5 mm diameter taken from *P. fluorescens* cultures were placed on PDA media, 3 cm apart from central disc (10 mm diameter) covered with fungus. Antagonism was verified by observing the presence (+) or absence (-) of an inhibition zone between the antagonist and the pathogen using a light microscope and on culture media. For the *in vivo* experiments, four treatments were made: plants treated + bacteria + pathogen, untreated + bacteria + pathogen, plants inoculated with the pathogen and healthy control. *In vitro* test showed antifungal activities of *P. fluorescens* against *P. aphanidermatum*. Mycelia in the inhibition zone were altered completely when observed under the microscope and could not grow on new culture media. Application of bacteria reduced disease incidence to 18%.

Keywords: antagonist, bacteria, fungicidal, fungus.

1. Introduction

Root rot of papaya (*Carica papaya* L) caused by some species of *Pythium* spp. especially by *Pythium aphanidermatum* responsible of most yield losses in some zones where papaya is cultivated. Morton *et al.* [1] reported that species of *Pythium* caused substantial losses of papaya in Africa and Asia. The disease, have forced some producers in India to leave their orchards [1]. This disease caused the loss of the whole papaya tree (death) or sometimes his stunting, where the characteristics symptoms were appeared. The disease caused wilt of the plant which dies in the following days. The observation of the plant roots show destruction of root and/or collar tissue [2]. The problem becomes particularly worrying in the raining season. In addition, treatments by curative ways after establishment of the disease are ineffective.

There was no resistant variety of papaya of root rot caused by *P. aphanidermatum*. In addition, chemical treatments are more and more contoured and induce pathogens resistance [3, 4]. Indeed, in most developed countries and particularly in Côte d’Ivoire, there was the problem of excessive utilization of chemical fungicides. Koffi *et al.* [5] showed that on five chemical fungicides used in papaya farms to control root rot caused by *P. aphanidermatum*, two of them has not target the family of Oomycetes. In addition, researches on biologic antagonists utilizations are not be making in Sub-Saharan Africa and particularly in Côte d’Ivoire. Though biologic antagonists, especially bacteria, has been used successfully to control some plants diseases. Some bacteria such as *Bacillus* spp., *Pseudomonas* spp. and *Streptomyces* spp. have been widely used to control seedling blight of maize and root rot of pepper [6-9]. In Morocco, Snissi *et al.* [10] showed the efficacy of two strains of *Pseudomonas* spp. against fusariose disease of tomato.

In this case, this study suggested to test the being (existence) of antifungal activity of *Pseudomonas fluorescens* against *Pythium aphanidermatum* responsible of papaya root rot. By *in vitro* test the existence of antifungal properties of *Pseudomonas fluorescens* against *Pythium aphanidermatum* were checked. After that, *in vivo* test to evaluate the capacity of *P. fluorescens* to protect papaya against root rot has been done.
2. Materials and methods

2.1 Materials

- Papaya plants
  Papaya plants are belonging to the variety called « golden » obtained from seed of SCB (Société d’Etude et de Développement de la Culture Bananière). Seeds were sown in pots (diameter 11 cm, height 13.5 cm and volume 1 l), each of them contained 350 g of soil and 75 g of compost sterilized by autoclaving (pressure 1 bar) during 30 min. Forty days after the emergence, papaya seedling were transferred in pots containing treated soil for the tests.

- The pathogen *P. aphanidermatum*
  The strain of *P. aphanidermatum* used in this test coming from the "Laboratoire de Biologie et d’Amélioration de la Production végétale" of "Université Nangui Abrogoua". It was isolated on papaya and was conserved on Potato Dextrose Agar (PDA). For the experiments, the strain was inoculated to young plants of papaya and isolated once again from diseased plants.

- Biocontrol agent
  The bacteria *Pseudomonas fluorescens* was used like biological antagonist, and coming from the "Laboratoire de Biologie Cellulaire et Moléculaire de l’Université de Neuchâtel (Switzerland)". The strain was maintained in culture on Nutrient Agar (NA).

2.2 Methods

- Production of inocula
  Inoculum of *P. aphanidermatum* which to be used for plants infection was prepared according the method of sand mixing with corn powder (CMS: corn meal sand) of Haware and Nene [11]. The strain of *P. aphanidermatum* maintained on PDA was inoculated at CMS in the bottle of 5 cm of diameter with 5 inocula of 2 cm² by bottle. Cultures were incubated for 14 days in the darkness, at 28° C ± 1 (Fig 1). Discs of 5 mm of diameter were taken on NA containing *Pseudomonas fluorescens* and transferred in the liquid media Nutrient Broth (NB) prepared in the bottle of 100 mL. Inoculum of *P. fluorescens* was prepared 48 h before soil treatment at 28° C ± 1.

![Fig 1: Inoculum of *Pythium aphanidermatum* making using Haware et Nene [11] method.](image)

- In vitro test
  For measure the antagonism degree of *P. fluorescens* against *P. aphanidermatum*, a disk of 10 mm of diameter of *P. aphanidermatum* was placed in the center of the Petri dish of 9 cm contained PDA medium. Four disks of 5 mm of diameter of *P. fluorescens* were placed in the diagonal position at 6 cm of *P. aphanidermatum* in the same Petri dish. Ten Petri dishes were prepared and incubated at 28° C ± 1 for three days. The antagonism activity was verified by two ways: (i) for direct observation in Petri dish by the presence (+) or absence (-) of inhibition zones between the pathogen and he antagonist and (ii) by the observation in the optic microscope of the interaction zones.

- Microscopics observations
  Microscopic comparison was made between the control culture of the pathogen and the pathogen in the interaction zones. The observations were made under optic microscope (X 400).

- Tests in vivo
  Forty days after their emergence, papaya plants were transplanted from germination pots to experimental pots containing 400 g of humus soil. The plants are inside the greenhouse which isolates them to the soil at ambient temperature (27-30° C) and the daylight. The experiment includes the following 5 treatments:
  - Inoculated soil with *P. aphanidermatum* + *P. fluorescens* 10 ml + treated plants;
  - Inoculated soil with *P. aphanidermatum* + *P. fluorescens* 10 ml + untreated plants;
  - Inoculated soil with *P. aphanidermatum* + *P. fluorescens* 20 ml + treated plants;
  - Inoculated soil with *P. aphanidermatum* + *P. fluorescens* 20 ml + untreated plants;
  - Inoculated soil with *P. aphanidermatum* + untreated plants (inoculated control or positive control); Soil without inoculum + non-inoculated plants (healthy control or negative control).
  Inoculations of *P. aphanidermatum* and *P. fluorescens* were made one week before transplanting. About thirteen g (12.84 g) of CMS containing *P. aphanidermatum* (fungal inoculum) was used per pot, an inoculum unit corresponding to one gram of fungal culture on CMS [12]. Five days and 7 days after inoculation of *P. aphanidermatum*, the same pots containing the same pathogen were treated (soil) with 10 mL and 20 mL of bacterial solution (bacterial inoculum concentration varied from 3.10⁸ to 9.10⁸ cel/mL). Ten papaya plants were used for each treatment. The experiment was replicated three times. It was noticed that some plants in nursery have been treated with bacterial solution two weeks ago before they transplantation. One batch was treated with 20 mL of bacterial solution, and the other batch with 40 mL in two treatment of 10 and 20 mL in the meantime of three days.

- Measured parameter
  Symptoms evaluation was made during a period of 12 weeks after seedlings transplantation. Symptoms evaluation scale with four points was used (0-3) method of Vakalounakis and Fragkiadakis [13]. Disease incidence (%) was calculated using the formula of Song et al. [14]:

\[
\text{Disease incidence} (\%) = \left( \frac{\text{Number of infected plants}}{\text{Total number of plants}} \right) \times 100
\]

- Statistics analysis
  Analysis of variance with two criteria of classification (ANOVA 2) was conducted to determine the effect of treatment (factor A) and applied doses (factor B) on the incidence of the disease. In the case of difference between the means, they were compared using Newman Keuls tests at 5%. The software used was Statistica 6.0.

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3. Results and Discussion

3.1 Results

- **In vitro action of antibiotic components of* P. fluorescens* on mycelium development of* P. aphanidermatum**

Figure 2 shows the growth of microorganism. The fungal pathogen *P. aphanidermatum* grows normally on PDA (Fig. 2a), while *P. fluorescens* grows actively on NA (Fig. 2b). In the Petri dishes, where it was been inoculated simultaneously with *P. aphanidermatum* in the center and *P. fluorescens* in four corners of the dish, *P. aphanidermatum* was inhibited. The inhibition zones showed clearly that antimicrobial compounds with fungicide properties have been produced by the bacterium (Fig. 2c). Microscopic observations of interaction zones between *P. fluorescens* and *P. aphanidermatum* showed in obvious manner the antagonist effect of the bacterium. Microparasitic action of *P. fluorescens* leads of the denaturation of lipidic and proteinic components of *P. aphanidermatum* mycelium. This action ends by the lysis of parasite hyphes (Fig. 3).

The antifungal compounds released by the bacteria have fungicide effect on the pathogen. His action is also as well as on the mycelium and on the spores. No mycelium development and germination of spore were observed from the portion of inhibition zone cultivated on new PDA for two weeks (Fig. 4). These results permitted to understand in one part the antimicrobial secretions of *P. fluorescens* were antioomycete. In other part, these molecules have fungicide action against *P. aphanidermatum*.

![Fig 2](image)

**Fig 2:** (a-c) Pathogen culture and antagonist bacterium; (a) Pur culture of *Pythium aphanidermatum* on PDA (b) Culture of *Pseudomonas fluorescens* on NA (c) Inhibition of mycelium growth of *P. aphanidermatum* by *P. fluorescens* in the interaction zone.

![Fig 3](image)

**Fig 3:** (a-c) Effect of antioomycete substances of *P. fluorescens* on mycelia tissue of *P. aphanidermatum*. (a) Microscopics of normal structure of hyphae of *P. aphanidermatum* alone on PDA. (b) Disorganization (inside interaction zone) of mycelium structure 3 to 5 days after confrontation with *P. fluorescens*. (c) lysis and destruction of *P. aphanidermatum* mycelium 10 days after confrontation with *P. fluorescens*. 
Fig 4: Obvious fact of the fungicide effect of released substances inside interaction zone by *P. fluorescens* with absence of *P. aphanidermatum* growth.

> **In vivo evaluation of antifungal activity of *P. fluorescens* against *P. aphanidermatum***

The results of the *in vivo* effect of *P. fluorescens* on *P. aphanidermatum* show that antagonist bacterium reduce significantly the disease incidence of *P. aphanidermatum*. Disease incidence in the pots with inoculated soil with the pathogen without bacterial treatment was assess at 76% while, the soil in pots treated with *P. fluorescens* have incidence of 18.6% (Fig. 5). Nevertheless, this reduction was high compared with the healthy control where there was no bacterial treatment and non-pathogen inoculation. Statistic result show significative difference between incidence of positive or healthy plants control (0%) and plants where the soil was inoculated with *P. aphanidermatum* and control with *P. fluorescens* (18.6%).

Bacterial treatment of the inoculated soil by the pathogen is sufficient to guarantee the suppressive effect of this on. Indeed, there was no statistic difference between disease incidence of the treatment Pt+Py+Ps (treated plants with *P. aphanidermatum* in the nursery + + *P. aphanidermatum* inoculated to soil + *P. fluorescens* inoculated to soil) and this treatment Pnt+Py+Ps (untreated plant + *P. aphanidermatum* inoculated to soil + *P. fluorescens* inoculated to soil). Incidence was reduce at 17.5% for the treatment Pt+Py+Ps while it is to 19.65% for the treatment Pnt+Py+Ps (Fig 5).

The two volumes 20 mL and 40 mL of bacterial solution (3.10^8 à 9.10^8 cel/mL) used for the bacterial treatment of the inoculated soil (by the pathogen) have the both reduce at the same level disease incidence. Indeed, statistical analysis show that there was no significative difference between disease incidence of treatment V1=20 mL (18.98%) and treatment V2= 40 mL (18.16%). These two bacterial concentrations have the same inhibitor effect against the pathogen. Volume V1 was not too small to reduce antagonist action and volume V2 was not too high to reduce disease incidence (Fig. 6).

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**Fig 5:** Effect of treatments on rot incidence due to *P. aphanidermatum* (bands with the same letter are not significantly different from another at Newman and Keuls tests ($\alpha=5\%$))

Pt+Py+Ps: treated plants +*P. aphanidermatum* inoculated to the soil + *P. fluorescens*

Pnt+Py+Ps: untreated +*P. aphanidermatum* inoculated to the soil + *P. fluorescens*

Ti: Positive control (Inoculated soil by *P. aphanidermatum* without bacterial treatment)

**Fig 6:** Effect of bacterial concentration on disease incidence (bands with the same letter are not significantly different from another at Newman and Keuls tests($\alpha=5\%$)).
3.2 Discussion

The in vitro action of *P. fluorescens* against *P. aphanidermatum* obvious by the inhibition zones shows fungal activity of the bacterium. Most authors showed antimicrobial activity of bacteria, particularly *Pseudomonas* species on fungal pathogens. So, Walker et al. [15] showed that the antifungal metabolite of *Pseudomonas antimicrobica* inhibited efficiently germination and elongation of germinal tube of *Botrytis cinerea*. Ezzyani et al. [6] also showed the establishment of inhibition zone due to in vitro antifungal activity of *Streptomyces rochei* on *Phytophthora capsici*.

Microscopic observations of mycelium tissue inside inhibition zone showed the absence of spore and the denaturation of the mycelium. The culture of a portion of this zone on the new zone showed the absence of spore and the denaturation of the bioprotection. Indeed, it was isolated more secondary depending that *P. fluorescens* produced antibiotics responsible of the bioprotection. Indeed, it was isolated more secondary substances produce by *P. fluorescens* which were fungicides antioomycete properties. Howell and Stipanovic [16] held this protection against seedling blight due to *Pythium ultimum* of cotton seeds at two antibiotics: pyoluteorine and pyrrolnitrine, produce by *P. fluorescens*. Kraus and Loper [17] allowed this protection to the oomycine produce by the same bacterium. Cucumber protection against rot caused by *P. ultimum* is due according to Maurhofer et al. [18] to pyoluteorine.

In vivo tests confirm activity of antioomycete composites released by the bacterium. Indeed, soil bacterial treatments reduced disease incidence from 76 % to 18.6 %. These results showed the potential biocontrol of bacterium *P. fluorescens*. Similar effects of bioprotection of *P. fluorescens* were mentioned by Weller [19]. He used this bacterium against the fungi *Gaemannomyces graminis* var. *triticis* of wheat. Field tests permitted to increase the yield from 10 to 15 %. Similarly, Becker and Cook [20] showed the suppressive effect of *P. fluorescens* inside the soil naturally infected by species of *Pythium*. They observed a protection of wheat plants by the bacterium against *Pythium* rots.

Bacterial treatment reduce significantly disease incidence but not guarantee integral protection of the plants. This protection is susceptible to biogeochemical variations inside the soil. The tests showed that losses recorded (incidence 18 %) on soil treated with the bacterium were observed 10 to 12 weeks after plants emergence. Protection assured by *P. fluorescens* remained efficient so long that his population is so abundant in the rhizosphere. As soon as that the soil factors were unfavorable to him (bacterium), the protection becomes inefficient. This result is in consonance to Ryder and Jones [21] they showed that the success of utilization of an antagonist depends of his invasion of the infection. This assess is confirm by Harman [22] who report that failure of establishment in first time of the antagonist will lead to the failure of biological suppression. For Lindeman [23], it is important to consider origins of edaphic conditions of the antagonist for his successful utilization.

4. References