

## Enhancement of *Enterobacter Cloacae* Antagonistic Effects Against the Plant Pathogen *Fusarium Oxysporium*

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**Abstract:** The present investigation aimed to enhance and producing high efficient antagonistic mycoparasites to control the soilborne plant pathogens *F. oxysporum f. sp. sesame* and *F. oxysporum f. sp. vicia* which cause root wilt disease. Among several bacterial strains tested for their efficiency in inhibiting *F. oxy. f. sp. sesame* and *F. oxy. f. sp. vicia* growth, *P. aeruginosa* and *E. cloacae* had the highest antagonistic effects. *E. cloacae* antagonistic genes expression studies were carried out by using different carbon sources. No antagonistic effect against *F. oxy. f. sp. sesame* has been found in *E. cloacae* culture filtrate when grown with glucose as a sole carbon source. Using 1% chitin as a sole carbon source could enhance the antagonistic effects of *E. cloacae*. The highest inhibition effect was found when *E. cloacae* was grown for two days on glucose as a sole carbon source, then for five days on chitin – medium. *E. cloacae* filtrate was more efficient in inhibiting *F. oxy. f. sp. sesame* growth than in *F. oxy. f. sp. vicia* which may reflect differences between the two pathogen forma specials. Hybrids of *E. cloacae* and *P. aeruginosa* were produced by fusion of the bacterial protoplasts. About 25% of *P. aeruginosa* and 42% of *E. cloacae* cells were converted to protoplasts. The best protoplast fusion percentage was found in ten minutes after protoplasts mixing in the presence of 25% PEG 6000. The obtained fusants were very unstable which may due to the difference between both genera.

**Key words:** Antagonism, *Fusarium oxysporium*, chitinase gene expression, protoplast fusion, *Enterobacter cloacae*, *P. aeruginosa*

### INTRODUCTION

It has been estimated<sup>[2]</sup> that 31-42% of the world's crop production is lost due to disease, weeds and insects causing estimated losses of US\$ 550 milliard per annum. At the United States, it is estimated that plant disease losses, including control costs, in 2003, amounted to approximately US\$ 682.67 million resulting in a 12.64% total disease loss across all crops included<sup>[29]</sup>. This value was estimated in 2004 to be US\$ 558.92 million resulting in 10.21% total disease loss across all crops estimated<sup>[21]</sup>. Biological control of soil-borne plant pathogens by antagonistic microorganisms is a potential non-chemical means of plant disease control. Genetic modification of bacterial biocontrol agents such as *E. cloacae*, offers significant opportunities for enhancing biocontrol performance<sup>[30]</sup>. Transgenic strains of *E. cloacae* were constructed by transform them with chitinase gene<sup>[25]</sup> and used for controlling plant diseases and, also used to suppressing herbivorous insect pests<sup>[27]</sup>. The protoplast fusion can be induced by chemical and electrical fusogens<sup>[11]</sup>.

Successful production of intergeneric bacterial hybrids was done by protoplast fusion<sup>(1,22)</sup> and by mated between *E. cloacae* and *Erwinia herbicola*<sup>[28]</sup>.

This study aimed to explore some factors affecting the antagonistic efficiency of *E. cloacae* to improve its biocontrol performance against the plant pathogen *Fusarium oxysporum*. It also aimed to produce more efficient bacterial fusants in controlling the plant pathogen.

### MATERIALS AND METHODS

**Microbial Strains and Culture Conditions:** *Enterobacter cloacae* No.30 and *Fusarium oxysporum f. sp. vicia* strains were kindly obtained from Dr. A.F. Sahaab, Plant Pathology Dept., National Research Centre, Egypt. An Egyptian *Pseudomonas aeruginosa* isolate, was kindly obtained from Dr. A. Gaballa, Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt. *Fusarium oxysporum f. sp. sesame* strain was kindly obtained from Dr. S. Zedan Plant Pathology Dept., National Research

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Centre, Egypt. Luria -Bertani medium (LB)<sup>[8]</sup> was used for bacterial growth. King's agar complete medium<sup>[15]</sup> was used as a selective medium for *Pseudomonas* strains. Minimal medium for *Pseudomonas*<sup>[3]</sup> was used for growth. Potato Dextrose agar (PDA) or Broth (PDB) media<sup>[5]</sup> were used for fungal growth. Czapek's minimal medium<sup>[9]</sup> was used for fungal and bacterial growth.

**Antagonism Test:** The antagonistic effects of *Enterobacter cloacae* and *P. aeruginosa* were tested against both of *Fusarium oxysporum f. sp. vicia* and *Fusarium oxysporum f. sp. sesame* using different methods:

**Bacterial lysate antagonistic effect:** The capacity of *Pseudomonas aeruginosa*, and *Enterobacter cloacae* to inhibit the growth of *F. oxy. f. sp. sesame* was tested using an overlay technique<sup>[12]</sup>.

**Cultural filtrate method:** *E. cloacae* cultural filtrate was tested for antifungal activity<sup>[17]</sup> against *F. oxy. f. sp. sesame* and *F. oxy. f. sp. vicia*.

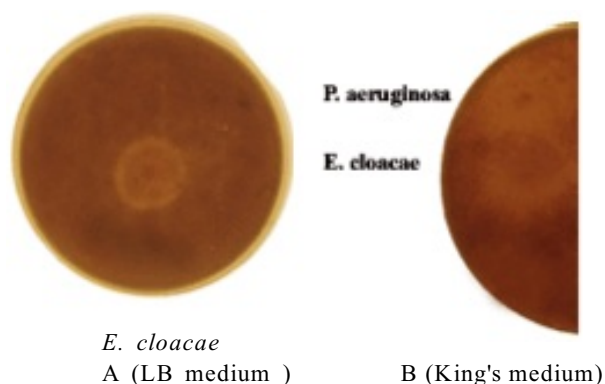
**Growth inhibition in liquid medium:** Three-ml sterilized filtrate of 5 days culture of *E. cloacae* on Czapek's medium supplemented with 1 % chitin, were mixed with PDB (1:4 ratio). As a control, 3ml filtrate of Czapek's medium was used. Each treatment was inoculated with a plug of *F. oxy. f. sp. sesame* mycelium and incubated at 28°C for 3 days. Mycelia were collected at the end of incubation periods by filtration, dried at 105°C for 24 hr and weighted.

**Antagonistic Gene(s) Expression Studies:** Antagonistic genes expression studies were carried out with *E. cloacae*. *E. cloacae* inoculated in Czapek's medium supplemented with 10% glucose and incubated at 30°C for 2 days. The cells were then collected, washed, transferred to Czapek's medium containing 1% chitin or 10% glucose and incubated at 30°C for 5 days. Also, *E. cloacae* was grown in Czapek's medium supplemented with 1 % chitin as a sole carbon source for 7 days. Culture filtrates of *E. cloacae* were obtained, filter sterilized and used for antagonism test against *F. oxysporum* on PDA plates. Radial growth was recorded. Results were expressed as percent inhibition of radial growth compared with the control plates.

**Protoplast Fusion:** The protoplast fusion technique<sup>[23]</sup> was performed between *P. aeruginosa* and *E. cloacae*. The *P. aeruginosa* and *E. cloacae* hybrids were selected by using *Pseudomonas* medium supplemented with L-arabinose, to eliminate *P. aeruginosa* and incubated at 41°C, to eliminate *E. cloacae*.

## RESULTS AND DISCUSSIONS

Biological control of soil-borne plant pathogens by antagonistic microorganisms is a potential non-chemical means of plant disease control. *P. aeruginosa* suppress disease by phenazine derivatives antibiotic produced on roots grown in soil<sup>[19]</sup>. *E. cloacae* is known to suppress different plant pathogens, e.g., *Pythium ultimum*<sup>[14]</sup>. The data related to suppression of *Fusarium* wilts is limited<sup>[24]</sup>. The present investigation deals with the biocontrol of the soilborne fungal pathogen *F. oxysporum*, which causes root wilt disease resulted in great crop losses.



**Fig 1:** Growth inhibition of *F. oxysporum f. sp. sesame* by *P. aeruginosa* and *E. cloacae* cell lysate. A: Inhibition test in LB medium. B: Inhibition test in King's medium.

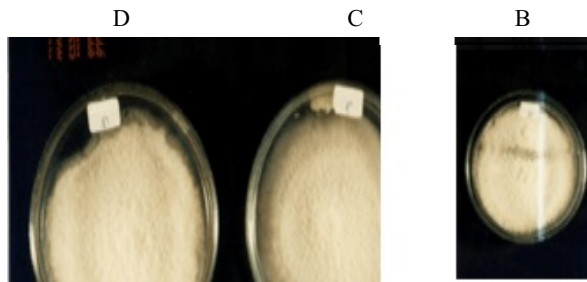
**Antagonism:** *E. cloacae* and *P. aeruginosa* colonies grown in King's or LB media were lysed using chloroform<sup>[12]</sup>. *F. oxy. f. sp. sesame* cultured in PDA medium was overlaid and incubated at 28° C for 3 days. Bacterial colonies grown in King's medium were able to inhibit *F. oxy. f. sp. sesame* growth with the same efficiency where both strains produced inhibition zone diameter of 3.1 cm (Fig. 1-B). When LB medium was used, only *E. cloacae* was able to inhibit *F. oxy. f. sp. sesame* growth (Fig 1-A). These results are in agreement with those reported by Nielson *et al.*<sup>[20]</sup> who demonstrated that both medium-independent and medium-dependent types of antagonism against two plant pathogen fungi were found among *P. fluorescens*.

In another attempt, growth of *F. oxys. f. sp. sesame* was measured after three days of incubation in PDB medium supplemented with 20% of *E. cloacae* Czapek's-chitin culture filtrate. Mycelia of *F. oxy. f. sp. sesame* were collected by filtration, dried at 105°C for 24 hr and weighted. Results showed 9 % decrease of *F. oxy. f. sp. sesame* growth, i.e. its

mycelia weighted 78 mg compared with 85 mg in the control medium, i.e. (PDB supplemented with Czapek's medium filtrate 4: 1 ratio).

**Antagonistic Gene (S) Expression of *E. Cloacae*:** Chitinase enzyme is known to degrade chitin, which is an inherent part of many plant deleterious fungi and insects. Different types of chitinase are existing in the bacterium *E. cloacae*<sup>[17,18]</sup>. To enhance the antagonistic effect of *E. cloacae*, chitinase –expression studies were carried out.

*E. cloacae* was inoculated continuously in glucose - Czapek's medium or chitin- Czapek's medium and incubated at 30°C for 7 days. The two culture filtrates were added to PDA medium at a concentration of 20% and inoculated with *F. oxy. f. sp. sesame*, the growth of both cultures was detected. Results are present in Figure 2 and Table 1. Figure 2 showed that *E. cloacae* culture filtrate grown continuously in chitin-Czapek's had effective antagonistic for *F. oxy. f. sp. sesame* while the glucose- Czapek's culture filtrate had no detectable effect.



**Fig 2:** Inhibition of *F. oxysporum f. sp. sesame* by chitin- culture filtrate of *E. cloacae*. Plate D: PDA medium supplemented with 20 % chitin- Czapek's medium filtrate (control). Plate C: PDA medium supplemented with 20% chitin- cultural filtrate of *E. cloacae*. Plate B: PDA medium supplemented with 20% glucose-cultural filtrate of *E. cloacae*.

**Table 1:** Growth inhibition of two *F. oxysporum* strains by different *E. cloacae* cultural filtrates \*.

Pathogens	Pathogen growth inhibition %		
	Filtrate A	Filtrate B	Filtrate C
<i>F. oxysporum f. sp. sesame</i>	(55%)	(0 %)	(58 %)
<i>F. oxysporum f. sp. vicia</i>	(28.5%)	(0 %)	(50 %)

\*PDA medium supplemented with 20 % culture filtrate of *E. cloacae* grown on: Continuous chitin- Czapek's medium for (A), Continuous glucose - Czapek's medium (B), Glucose -Czapek's medium for 2 days then on chitin - Czapek's medium for 5 days (C).

**Table 2:** *P. aeruginosa* and *E. cloacae* protoplast fusion.

Incubation period	No. of fusants*in 100 µl
10 min.	94.6
20 min.	76.6
30 min.	38.8

\* Fusants were selected on *Pseudomonas* medium supplemented with L- arabinose as the carbon source and incubation at 41°C for 4 days.

The inhibition of colony extension of *F. oxy. f. sp. sesame* and *F. oxy. f. sp. vicia* by *E. cloacae* culture filtrates was studied and the obtained results are present in Table 1. Table 1 showed the different effects of *E. cloacae* filtrates in inhibiting growth of *F. oxy. f. sp. sesame* and *F. oxy. f. sp. vicia*. The former pathogen was more sensitive to *E. cloacae* filtrate than the latter one. The most inhibition effect was found when *E. cloacae* was grown for two days on glucose then five days on chitin - medium, where it could inhibit 58 % of the colony extension of *F. oxy. f. sp. sesame* and 50 % of colony extension of *F. oxy. f. sp. vicia*. These finding is in agreement with those found by Blaseau *et al.*<sup>[4,16,7,10]</sup>.

Results presented in Table (1) and Fig. (2) show that no antagonistic effect was detected to any of the two strains when glucose was used as the source of carbon. This may reflects glucose repression to antagonistic gene(s) expression. Repression by glucose suggests that catabolite repression or the so-called glucose effect may be involved in the regulation of chitinase synthesis<sup>[26]</sup>.

***P. aeruginosa* and *E. Cloacae* Protoplast Fusion:** In this work, in order to combine different mechanisms for fungal biocontrol, the protoplast fusion technique was performed between *P. aeruginosa* and *E. cloacae*. Protoplasts were induced from *P. aeruginosa* or *E. cloacae* cultures<sup>[23]</sup>. Results showed that 24 % of *P. aeruginosa* cells were converted to protoplasts comparing with 42 % of *E. cloacae* cells. The difference in protoplast induction efficiencies of both strains may reflect differences in their cell walls structure as mentioned by Hopwood,<sup>[13]</sup>.

A mixture of 85 x 10<sup>3</sup> *P. aeruginosa* protoplasts and 19 x 10<sup>3</sup> of *E. cloacae* protoplasts was mixed with 25% PEG 6000. One hundred microliters of protoplast suspension were tested for hybrid fusants at 10 min interval. Fusants selection was done by inoculation on *Pseudomonas* medium supplemented with L-arabinose, and incubation at 41°C for four days. Table 2 presented the best protoplast fusion efficiency which was in the first 10 min of the treatment, where about 94 fusants were found per sample tested. In 20 min incubation, the protoplast

fusion efficiency reduced about 20 % where about 76 fusants were found per sample. Increasing the incubation to 30 min gave the least fusants efficiency where only about 39 fusants were obtained in 100µl sample, i.e. less than 60 % of the 10 min protoplast efficiency. These results may be due to that increasing incubation time increases the probability of more than two cells to fuse. This situation resulted in less viability and more instability of the fusants produced

In four days several hybrid colonies were grown. They have been subcultured at the same incubation conditions for more than a week. No fusant was able to re-grow on the selective condition. These results could be an indication for unstable fusants. This could be the result of the huge genetic difference between the two parental genera, i.e., *Pseudomonas* and *Enterobacter*. These results are in agreement with what Dale<sup>[6]</sup> has found, that viable progeny will not be obtained unless the two parents are from closely related species and disagree with what Abdel-Salam *et al.*<sup>[1]</sup> had found where they could obtain stable intergeneric fusants between *Bradyrhizobium japonicum* and *Rhizobium leguminosarum*.

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