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# Selection and efficacy biocontrol agents in vitro against fire blight (Erwinia amylovora) of the rosacea

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**Abstract -** *Fire blight caused by Erwinia amylovora is a bacterial disease which affects many plant species, mainly belonging to the family Rosaceous. The following work aims to study some biological controls through the use of antagonistic bacteria and yeast, isolated from different biotopes against this bacterial disease isolated from different biotopes. The in vitro study of the effect of antagonists against E. amylovora (CFBP1430) was used 114 isolates in antibacterial activity against the pathogen in vitro. Nine bacterial strains (2328B-5, 2328B-3, 2025-1, Ach1-1, 2074-1, 2321-5, Ach2-1, 2066-7 and 2025-11) gave their satisfactory inhibition area (greater than or equal to 20mm) and the best percentage of inhibition of the growth of E. amylovora in vitro 31 to 41%, It should also be noted that these strains are generally bacteriolytic strains.*

***Key Words: Erwinia amylovora, rosacea, biocontrol in vitro, antibios, antagonists***

## 1. INTRODUCTION

Fire blight disease caused by *Erwinia amylovora* bacteria (Burill, Winslow et *al.*1920) is one of the most important diseases of pome rosaceae and ornamental maloidea grown either in the field or in nurseries [1]- [2]. In Morocco, this disease made its first appearance in 2006 in the region of Meknes. Since then, it has continued to spread in new localities. It is manifested by the loss of branches and affects the structure of the tree. In severe cases, when the bacterium progresses in the trunk or rootstock, the tree dies. The severity of the disease depends on the susceptibility of the cultivar and rootstock, the overall health of the tree, cultural practices and environmental conditions. The economic losses due to fire blight result from a decrease in the area of fruit and tree mortality [2]- [3]- [4]- [5].

Indeed, at present, there is no direct control method that can be put into practice to eliminate this pathogen. Prophylactic measures used by farmers remain preventive and non-curative alternatives; the chemical fight as for it, remains expensive, ineffective and acts in a harmful way on the ecology and consequently on the natural balances. Thus, the development of resistant cultivars takes a lot of time and remains difficult to develop, which leads us to biological control which is the most promising technique with the lowest impact on the environment because currently, the notion of the development Sustainable agriculture requires the agricultural sector to seriously address the ecological problem.

It is in order to contribute to the fire blight control strategy, a series of antagonist's selection from 114 strains tested against the bacterium *E. amylovora*.

### 1.1 Pathogens

Two pathogenic strains adopted in this work which are part of the collection of the laboratory of phytobacteriology and biological control of INRA of Meknes- Morocco, it is the strain CFBP1430 of *E. amylovora*, isolated in 1972 from Hawthorn in northern France used as reference strain. a Moroccan strain of *E. amylovora* 1416-1 (271), used as national reference, was isolated in 2008, from the region of Meknes (Agouray) Morocco from canker on pear stalk, variety Coscia. This strain is characterized and identified by biochemical and molecular methods [6].

Strains were stored at -80 ° C in a sealed tube containing 30% glycerol. After thawing of the latter, a volume of 0.1mL of this suspension is taken and spread on a Petri dish containing LPGA and Levane medium. Pure colonies were subcultured at 26 ± 1 ° C for 24 hours due to three successive subcultures at a 24-hour interval to activate. Strains were stored in LPGA petri dishes at 4 ° C for short-term use.

### 1.2 Antagonists

For the purpose of creating a collection of antagonists against the bacterium responsible for fire blight, during the research, isolation and chemical and molecular

identification tests of *E. amylovora* strain for the study of [6], all strains that identified non-*E. amylovora* were recovered for testing as an antagonist against this pathogenic bacterium. Also, some strains of the collection of the laboratory of biological control and bacteriology (INRA, Meknes- Morocco) were added. We recovered 114 strains of which 85 strains showed an antagonistic effect. Their isolation was made from infected plants: apple, pear, quince [6], olive tree [7] and compost [8].

### 1.3 Preparation of strains

The medium used as nutrient carrier for bacteria is the LPGA medium (Yeast Peptone Glucose Agar) and for yeast is PDA (Potato Dextrose Agar). Before each experiment, the strains were subcultured and incubated at 26 ± 1 ° C, the bacteria for a period of 24 hours and the yeasts for a period of 48 hours. From the strains in the exponential phase, a suspension equivalent to $10^8$ CFU / ml for the antagonists and $10^7$ CFU / ml for the pathogen is prepared. The concentration was adjusted using an Optic Density OD 600nm reader after the preparation of the Mac Farland scale suspension.

The yeast concentration is adjusted by a spectrophotometer at an optical density (OD 595 nm) of between 0.6 and 1 at a wavelength of 595 nm. Once the value of the optical density is determined, the concentration of the suspension is determined based on the following equation [9]:

$$[Ci] = 10^7 \times (OD - 0.0886) / 3$$

[Ci]: Initial concentration = number of cells per mL OD: Optical Density

The concentration of the bacteria is calculated by determining the optical density at OD 600nm, the concentration of the suspension is determined based on the following equation:

$$[Ci] = 10^8 x (OD \times 1.2)$$

[Ci]: Initial concentration = number of cells per ml OD: Optical Density

### 1.4 Tobacco Test

Also called tobacco hypersensitivity test; it generally reveals the pathogenicity or not of a bacterial strain. It is based on the defence of tobacco against phytopathogenic microorganisms. For this test, a suspension of previously prepared strains is injected with sterile syringes into a wound on the underside of a tobacco leaf. The tobacco plant is put under the favourable conditions of development for 24 hours to record the results. If there is a browning then death of the cells of the inoculated part, the strain is called tobacco-positive and therefore phytopathogenic. In the opposite case it is called tobacco-negative and can therefore have an antagonistic interest. For each strain the test was repeated twice.

### 1.5. In vitro confrontation test (Antibiosis)

For in vitro confrontation tests, the pathogen is first seeded by flooding. After drying the medium for 15min at the laminar flow we deposited the discs already submerged in the suspensions of the antagonists. The Petri dishes are incubated in the oven for 24 hours at 26 ± 1 ° C. To better monitor the effect of the antagonists, two controls are made. The first says negative with a disk on which are poured 2µl of SDW Sterile Distilled Water. The second said positive with a disk on which are poured 2µl of the antibiotic and which in our case is streptomycin (0.5g / 9ml of SDW) used for comparison. After incubation at 26 ± 1 ° C for 48 hours, the inhibition diameters around each of the disks (inhibition zone diameter and disk diameter) were measured in centimeters. The percentage inhibition is calculated according to the following formula:

$$\% \text{ Inhibition} = (D1 / D2) \times 100$$

Where D1 = Diameter of the inhibition zone

D2 = 90mm = Diameter of the Petri dish

Strains with promising results according to a zone of inhibition greater than 20mm were retested in antibiosis with three repetitions. To confirm the results, we performed a repetition of three times.

### 1.6. Bacteriolytic and bacteriostatic test

It is preferably recalled that a bacteriolytic bacterium is a bacterium that induces the lysis of other bacteria. In other words when it comes in contact with other bacteria it makes them unsustainable. On the other hand, a bacteriostatic bacterium simply inhibits the growth of other bacteria. In his presence, therefore, the latter remain in a latent state.

The mode of action test makes it possible to highlight the type of the antagonists studied. For this fact, new tests of in vitro confrontation are carried out. Then a part of the zone of inhibition (agar of the ZI) is taken by a handle then put in a tube containing liquid medium. After incubation for 24 hours with stirring (150 rpm); if the medium becomes cloudy there is bacterial growth and therefore the antagonist has a bacteriostatic effect. However, if the medium remains clear there is no bacterial growth and the antagonist is then called bacteriolytic. And to confirm this result, 100 µl of each sample are spread on the medium (presence of bacterial growth or not).

## 2. Results and discussion

### 2. 1 Experiment results

In order to focus our efforts on promising strains, a tobacco hypersensitivity test was performed. This is a test to be done in biological control to determine the possible presence of a detrimental effect on crops (Figure 1). This test shows that out of 85 selected antagonist strains, 114 strains (97.4%) were negative tobacco and 3 strains (2.6%) were tobacco positive. The tobacco positive's strains are the strains 2066-7 and 2074-1TC 2025-11.


**Chart- 1:** Positive Tobacco Symptoms (left) and Negative Tobacco (right)

In Table 1, a classification of the 114 strains tested in vitro against the Moroccan strain 1416-4 and the French strain CFBP1430 of *E. amylovora*. The result of the test revealed that 67 strains (58%) having shown an antagonistic effect against the bacterium *E. amylovora* of which 42 strains (38%) have an inhibition zone of less than 20 mm. Although 25 strains (22%) with an inhibition zone greater than 20 mm were selected for a second selection. Thus, the confirmation antibiosis test was carried out for the 25 strains with three repetitions against the Moroccan strain 1416-4.

This test revealed that the 25 strains have an antagonistic effect against the pathogenic bacterium with a zone and a percentage inhibition in a range of respectively 20 to 36.8 mm and 21 to 41% (Table 2). In parallel, the mechanism of action analysis of the antagonists allowed us to know how the antagonists used inhibit the pathogen.

At the end of the comparison between mechanisms of action and the zone and the percentage of inhibition, it follows from the absence of correlation between the inhibition of the strains and their modes of action in the test of confrontation in vitro (Table 2). However, nine strains (2328B-5, 2328B-3, 2025-1, Ach1-1, 2074-1, 2321-5, Ach2-1, 2066-7 and 2025-11) have a significant antagonistic effect in terms of Zone and percent inhibition differ in a range, respectively from 27 to 36.8 mm 31 to 41% compared to 16 strains. The variance analysis results confirm the absence of a significant effect of antagonist modes of action on the zone and the percentage inhibition

**Table- 1:** Ranking of the antagonist strains by zone of inhibition (mm)

| Zone of inhibition | | | |
|---|---|---|---|
| <10 | 10-15mm | 15-20mm | ≥ 20mm |
| 2230-8 | 1113-5 | 2066-8 | 1113-9 |
| 2236-2 | 2077-5 | 2074-4 | 2025-1 |
| 2315-2a | 2216-2 | 2083-2 | 2025-11 |
| 2315-2c | 2315-2b | 2217-3 | 2026-2 |
| 2320-1 | 2321-12 | 2234-1 | 2066-7 |
| 2320-4 | 2330-4 | 2321-11 | 2074-1 |
| 2320-6 | 2330-4 | 2321-8 | 2077-7 |
| 2324-5 | 2330-6 | 2321-8 | 2077-7 |
| 2324-6 | 2331-4 | 2321-9 | 2216-11 |
| 2330-8 | 2331-4 | 2328-6 | 2217-8 |
| 2331-1 | 2332-3 | 2330-1 | 2321-1 |
| 2331-1 | 2332A-5 | 2330-5 | 2321-2 |
| 2331-2 | | 2330-7 | 2321-5 |
| 2331-2 | | 2331-3 | 2321-6 |
| 2331-5 | | 2331-3 | 2322-3 |
| 2331-5 | | 2333-5 | 2324-3 |
| 2331-7 | | | 2328B-3 |
| 2333-1 | | | 2328B-5 |
| 2333-2 | | | 2330-2 |
| 2333-3 | | | 2330-3 |
| 2333-4 | | | 2332A-2 |
| | | | 2332A-4 |
| | | | 2332B-1 |
| | | | Ach1-1 |
| | | | Ach2-1 |
| | | | *Bacillus substilis* |

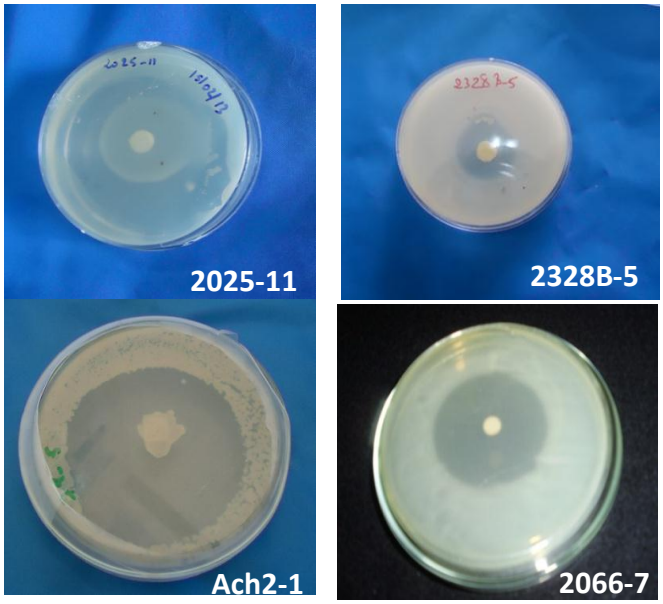


**Chart- 2:** Overview of the different categories of Inhibition zone *in vitro* confrontation test

**Table- 2:** Selected strains after *in vitro* confrontation test

| Code of strain | Date of collection | Origin | Sampling location | Specie | ZI | % | Mode of action |
|---|---|---|---|---|---|---|---|
| 1113-9 | 15/02/04 | Apple Fruit | INRA- Meknes | *Aureobasidium pullulans* | 23,4 | 26% | Bacteriostatic |
| 2025-1 | 02/02/12 | Compost | INRA- Meknes | - | 28,2 | 31% | Bacteriolytic |
| 2025-11 | 02/02/12 | Compost | INRA- Meknes | *Paenibacillus brasiliensis* | 36,8 | 41% | Bacteriolytic |
| 2026-2 | 3/02/12 | Compost | INRA- Meknes | *Bacillus* sp. | 21,0 | 23% | Bacteriolytic |
| 2066-7 | 08/03/12 | Olive tree | Meknes | *Pantoae agglomerans* | 32,7 | 36% | Bacteriolytic |
| 2074-1 | 03/04/12 | Olive tree | My Driss Zarhoun | *P.agglomerans* | 28,8 | 32% | Bacteriostatic |
| 2077-7 | 03/04/12 | Olive tree | My Driss Zarhoun | Acinetobacter | 24,8 | 28% | Bacteriostatic |
| 2216-11 | 15/05/12 | Cognassier | Fez | - | 23,7 | 26% | Bacteriostatic |
| 2217-8 | 15/05/12 | Apple tree | Meknes | - | 20,0 | 22% | Bacteriostatic |
| 2321-1 | 20/12/12 | Olive tree | Hoceima | - | 24,8 | 28% | Bacteriostatic |
| 2321-2 | 20/12/12 | Olive tree | Hoceima | - | 25,4 | 28% | |
| 2321-5 | 20/12/12 | Olive tree | Hoceima | - | 30,2 | 34% | Bacteriostatic |
| 2321-6 | 20/12/12 | Olive tree | Hoceima | - | 23,4 | 26% | Bacteriostatic |
| 2322-3 | 20/12/12 | Olive tree | Hoceima | - | 23,4 | 26% | Bacteriostatic |
| 2324-3 | 20/12/12 | Olive tree | Ouazzane | - | 24,5 | 27% | Bacteriostatic |
| 2328B-3 | 31/12/12 | Apple tree | Fez | - | 27,9 | 31% | Bacteriostatic |
| 2328B-5 | 31/12/12 | Apple tree | Fez | - | 27,9 | 31% | Bacteriolytic |
| 2330-2 | 12/02/13 | Apple tree | Meknes | - | 20,0 | 22% | Bacteriolytic |
| 2330-3 | 12/02/13 | Apple tree | Meknes | - | 22,5 | 25% | Bacteriostatic |
| 2332A-2 | 18/02/13 | Apple tree | Fez | - | 21,0 | 23% | Bacteriolytic |
| 2332A-4 | 18/02/13 | Apple tree | Fez | - | 25,2 | 28% | Bacteriolytic |
| 2332B-1 | 18/02/13 | Apple tree | Fez | - | 24,2 | 27% | Bacteriolytic |
| Ach1-1 | 15/02/04 | Apple Fruit | INRA- Meknes | *Aureobasidium pullulans* | 28,6 | 32% | Bacteriostatic |
| Ach2-1 | 15/02/04 | Apple Fruit | INRA- Meknes | *Aureobasidium pullulans* | 30,5 | 34% | Bacteriostatic |
| *B substilis* | | | INRA- Meknes | *Bacillus substilis* | 23,9 | 27% | Bacteriolytic |

ZI: Zone of inhibition; %: Percentage of inhibition

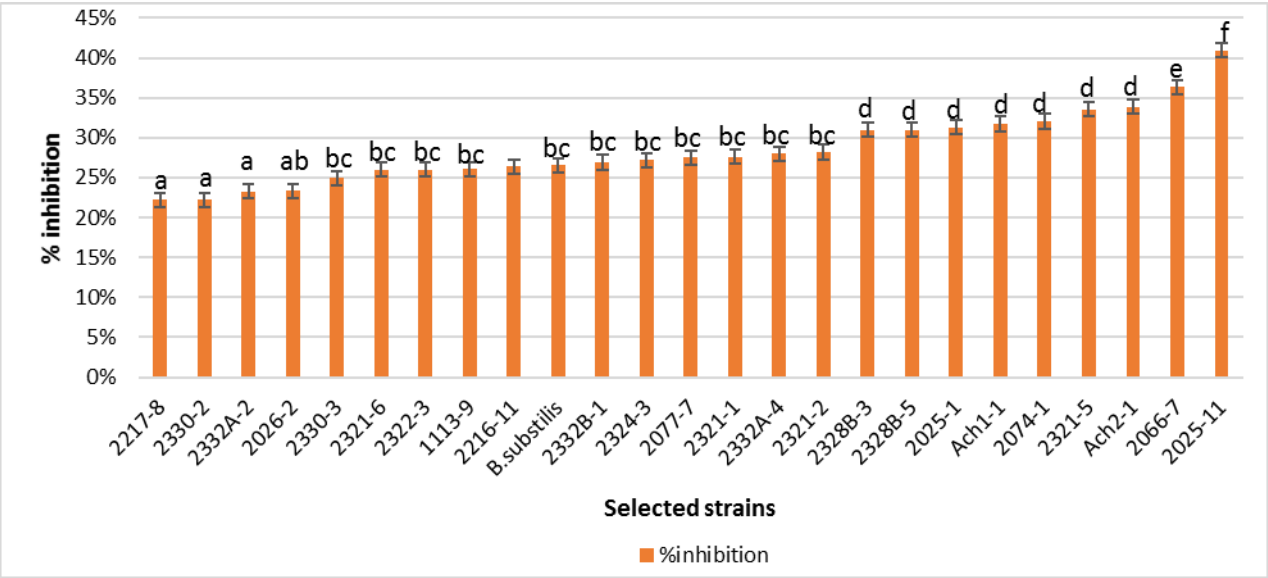


**Chart- 3:** Percentage inhibition of selected strains by in vitro test

in vitro and also the choice of nine strains by the duncan test.. This statistical test grouped the percent inhibition of strains into six groups whose group d, e and f with the results of promising strains as antagonists can limit the development of the pathogen and participate in the control strategy of fire blight (Figure 3).

## 2.2 Discussion

In Morocco, *E. amylovora* is a pathogen that is well known in orchards where it remains a quarantine pest and has recently been detected in many other Mediterranean countries [2] - [6]. According to the study of its biology, its epidemiology in Morocco, the fight against this bacterium is essential to limit its spread. In the search for control methods, a series of antibiosis tests with 114 antagonists (bacteria and yeasts) was carried out against the pathogenic bacterium *E. amylovora.* The results indicated that 67 of the antagonists studied significantly inhibit the growth of the pathogenic bacterium with distinct zones of inhibition. However, nine antagonistic strains were selected from 67 strains with a significant effect reaching 41% inhibition. The method of selection based on the in vitro inhibition zone is selected according to the previous experiences of the use of antagonists against *E. amylovora* [9]-[10] *Xanthomonas campestris, Pectobacterium carotovorum,* and *Pseudomonas syringae* [11] - [12].

Among the selected antagonists, strains 2066-7 and 2074-1 (identified *P. agglomerans*) and strain 2025-11 (identified *Paenibacillus brasiliensis*) are known by their pathogenicity which was confirmed with the tobacco test performed. However, these strains used showed a significant effect against the pathogenic bacterium arriving at 41% inhibition. In other studies, with the same strains testes as a biopesticide against other pathogens namely: Tuberculosis of the olive tree (*Pseudomonas savastanoi*)[7], onion bacterial diseases of the *Pseudomonas marginalis*, *Pseudomonas viridiflava, Xanthomonas retroflexus* and *Pantoea ananatis* [13] and also as plant growth promoting rhizobacteria (Plant Growth Promoting Rhizobacteria) [8].

*P. agglomerans* bacteria have been shown to be the most effective antagonists in all domains in vitro against *E. amylovora* compared to other antagonists [10]. The activity of this bacterium was comparable to that previously reported [14]-[15]-[16]. Similarly, *P. agglomerans* had significant potential as biological control agents against fire blight through a mechanism based on antibiotic production [10]. However, some of these latter species are ice nucleation [17]-[18] an undesirable trait that, fortunately, none of our strains possess.

Strains 2328B-5, 2328B-3 (isolated from apple tree), 2025-1 (isolated from compost), 2321-5 (isolated from the olive tree) showed antagonistic potency against *E. amylovora* are not yet identified and have no pathogenicity based on the tobacco test performed. This pathogenicity and antagonism remains to be evaluated in other tests on the other parts of plants (flowers, leaves and fruit).

Ach1-1, Ach 2-1 strains were isolated from the surface of apples "var. Golden Delicieus" [19] They showed high potency against the two main postharvest parasites (*Penicillium expansum* and *Botrytis cinerea*) on apple [19]-[20]. Other strains of *A. Pullulans* reported in other studies also exhibited yeast antagonistic activities against several pathogens, such as *Botrytis cinerea*, *Colletotrichum acutatum, Penicillium expansum, P. digitatum, P. italicum, Pecbacterium carotovorum* and *Phytophthora infestans* [21]- [22]- [23] -[24]- [25]- [26].

In addition, it is interesting to note that the Ach 1-1 and Ach 2-1 and 1113-9 strains belonging to the same yeast were isolated from the same fruit and did not demonstrate the same inhibitory capacity to inhibit in vitro *E. amylovora* bacteria. But Ach 1-1 and Ach 2-1 have shown a high degree of efficacy compared to other suspected antagonistic organisms.

In addition, antibiosis is frequently used to select potential antagonists [26] - [27] that can be used for biocontrol trials in the greenhouse and in the field. This work is a first step in the selection of control methods to inhibit this pathogen. Future work should focus on the use of its antagonists on flowers, fruit leaves, in situ to study their efficacy and behavior in the environment. In such a way that the improvement of its use as a biopesticide is extended to the protection of fire blight disease and enhances the inhibition of the development of *E. amylovora* bacteria.

## 3. Conclusion

All of the antagonists tested showed promising results in the biological control of *E. amylovora* bacterium responsible for bacterial infection. Future work should focus on the use of its antagonists on flowers, fruit leaves, in situ to study their efficacy and behavior in the environment. In such a way that the improvement of its use as a biopesticide is extended to the protection of fire blight disease and enhances the inhibition of the development of *E. amylovora* bacteria. This work is a first step in the selection of control methods to inhibit this pathogen.

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