

Potential Biocontrol Effect of the Phylloplane Bacterium *Bacillus mojavensis* A-BC-7 on the Olive Knot Disease

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Abstract

Olive knot disease, caused by *Pseudomonas savastanoi* pv. *savastanoi*, is one of the most important biotic stress for olive cultivation in most olive cultivation areas, particularly in Mediterranean countries. It conducts to intense damage in olive groves, causing heavy production losses.

Bacillus mojavensis A-BC-7, a natural colonizer of symptomless olive phylloplane, was determined as potential biocontrol agent against olive knot disease strain ITM317- Rif, a mutant strain resistant to Rifampicin (100 ppm). Bioassays on one-year-olive plants were carried out to survey the antagonism of A-BC-7 controlling knot development and pathogen populations when co-inoculated with the pathogen on stems with different ratios.

Results showed that A-BC-7 was able to decrease knot weights and pathogen population size, producing less necrotic tumors. In particular, when we applied the mixture suspensions of ITM317-Rif+A-BC-7 with the ratio 1:9 that resulted of 43.11% of overgrowth inhibition at 30 days after inoculation, which increased to reach about 59% at 60 days after inoculation and then 75% at 90 days after inoculation. That may pose epidemiological consequences.

Keywords: Biological control; Interaction; A-Bc-7; Olive knot disease; Pathogen populations

Introduction

Olive knot disease, caused by the Gram negative bacterium *P. savastanoi* pv. *savastanoi* (Psv), is proved as one of the most serious diseases attacking olive trees (*Olea europaea* L.) in most olive cultivation regions and especially in Mediterranean countries, conducting to intense damage in olive groves, causing heavy production losses [1], especially when the weather conditions (temperature and relative humidity) favor the survival of epiphytic populations of the pathogen and their entry into the bark. The disease is characterized by overgrowths formation on the stems, branches and twigs of olive plants and occasionally on the leaves and fruits [1-5]. Its symptoms persist and recur for many years in olive trees, which lead to consider it as a chronic disease [6]. Psv is characterized by its proficiency to survive as an epiphyte on olive phyllosphere [7,8], where the population size is obviously fluctuated depending on seasons: more important in spring and fall than in winter and summer [7,9-12]. It reaches about 104 bacteria/cm² (on twigs or leaves) in spring and fall, when wet weather conditions occur [7,10]. Tissues can be infected through leaf scars, wounds, and fissures on stems and twigs, caused by meteorological factors and insect, as well as by harvest and pruning practices. It was reported [13,14] that both olive quantity and quality can be reduced as a consequence of bacterial infections of the plant by the pathogen.

To study the formation and differentiation of tumors, a fundamental process is played by some bacterial virulence factors, essentially indol-3-acetic acid (IAA) and cytokinins, which conduct to an expansion in plant cell size (hypertrophy) going with an unusual cell proliferation (hyperplasia) [1,15]. In addition to the phytohormones, which play a leading role, other virulence factors are associate in disease severity which are expressed through the production of Type III secretion system (T3SS) involved in Psv virulence [16-18].

Psv is currently included in the list of transmissible agents of olive diseases, and its absence in propagating material is essential for the certification of olive mother plants.

Although the survey of olive knot disease is very complicated, the use of copper compounds may be one of the conventional practices to reduce symptoms. However, the resistance of Psv pathovars to copper bactericides [19] has required the necessity to make serious call for new control methods, such as the use of biological products to reduce also the toxic pesticide residues on fruits and to avoid environmental accumulation of chemicals and the consequent development of pathogen resistance.

As an alternative, biological control of plant pathogens using antagonistic fungal and bacterial strains is playing an important role into the management of plant diseases. *Bacillus* and *Pseudomonas* are considered as interesting classes of biocontrol bacteria that are used frequently against various plant diseases [20]. Previously, Zadeh et al. [21] and Krid et al. [22,23] reported that several fluorescent *Pseudomonas* and *B. subtilis* have high antibacterial activity *in vitro*

against Psv. After that, Kacem et al. [24,25] found that several species of *Rhizobium* displayed a potential antibacterial activity against Psv by producing bacteriocins.

Other researches demonstrated that *B. subtilis* and *B. mojavensis* have a strong antifungal activity especially against *C. albicans* [26] and *F. moniliforme* [19]. This growth inhibition is probably due to the production of cell-wall degrading enzymes and different families of lipopeptides including iturins, fengycins and surfactins according to these researches.

For over ten years, there has been a considerable interest in using *Bacillus* strains producing lipopeptide antibiotics like iturin A and surfactin as biocontrol agents due to their antagonistic and repressive activities against pathogens [27]. These amphiphilic cyclic biosurfactants have many advantages: low toxicity, high biodegradability and environment respectful characteristics [28]. The list of novel microorganisms, especially *Bacillus*, and products found in microbiologically unexplored ecosystems around the world suggest that a careful exploration of other habitats such as arid regions might continue to be useful as a source of novel microorganisms producing new compounds. *Bacillus* strains have been investigated previously for agricultural applications and have proven to be an efficient biocontrol agent against serious phyto-pathogens [28].

In the present work, we evaluated the biocontrol potentiality of a *Bacillus mojavensis* strain A-BC-7 isolated from symptomless olive phylloplane against the olive knot disease pathogen Psv strain ITM317 after creating a mutant strain resistant to the rifampicin (100 ppm) antibiotic. We examined the potential of *Bacillus mojavensis* A-BC-7 strain by regular survey of symptom expression of olive knot disease and the pathogen population into olive knots.

Material and Methods

Bacterial strains, growth conditions and inocula preparations

ITM317 rifampicin resistant (ITM317-Rif): *P. savastanoi* pv. *savastanoi* ITM317 strain was provided by the laboratory of Bacteriology (UNIBAS, Potenza, Italy) from the bacterial collection. It was isolated from active knots of naturally infected olive plants (Matera, Italy). To prepare mutant strain resistant to rifampicin (ITM317-Rif), ITM317 was subcultured on King B Agar (KBA) medium [29] supplemented with 100 ppm rifampicin and incubated for 24 h at 26°C. Resulting colonies were scraped off with sterile rod to be evaluated and comparing to the wild ITM317 for IAA production by the Salkowski test [30].

***Bacillus mojavensis* A-BC-7:** *B. mojavensis* A-BC-7 strain was provided by the laboratory of Bacteriology (UNIBAS, Potenza, Italy) from the bacterial collection. It was isolated from the phylloplane of healthy olive plants cv. Maiatica (Italy).

Growth conditions and inocula preparations: Along the experiment, both of them were isolated and subcultured on KBA + Rifampicine 100 ppm + Cycloheximide 50 ppm and KBA + Cycloheximide 50 ppm, respectively (Table 1).

Before starting this assay, ITM317-RIF colonies producing high level of IAA were selected, subcultured and prepared for bacterial inoculates. Along the experiment, ITM317-RIF and A-BC-7 were grown respectively on KBA + 100 ppm rifampicin plates and KBA at 26-28°C for 24 h. Bacterial suspensions were prepared in 10 mM

MgSO₄·7H₂O by scraping bacterial lawns off with a sterile rod, Bacterial cell densities were adjusted spectrophotometrically (A590 nm) to a concentration of 108 CFU/ml and then were diluted to 106 CFU/ml before inoculations.

Strain	Host	Origin
<i>Pseudomonas savastanoi</i> pv. <i>savastanoi</i> ITM317	<i>Olea europaea</i>	Matera (Italy)
<i>Bacillus mojavensis</i> A-BC-7	<i>Olea europaea</i>	Italy

Table 1: Hosts and origins of bacterial strains used in this study.

In planta experiments

One-year-old olive plants (*Olea europaea* L. cv. *Frantoio*) were used. Plants were incubated in a growth chamber (70% relative humidity, 16 h of light, 20,000 lux, 24-26°C; 9 h of dark). Inoculation method was performed as following. Bacterial suspension, prepared in 10 mM MgSO₄·7H₂O, containing 106 CFU/ml of strain ITM317-Rif or A-BC-7 or a mixture of both (equal volumes) was spotted on six V-shaped slits (about 1 mm deep by 3 mm wide) made in the stem in the basis and the upper part. The slits were then covered with Parafilm. Negative controls were inoculated with 10 mM MgSO₄·7H₂O solution. The experiment was carried out in duplicate on two different plants and was performed twice.

The plants were observed for symptom development for up to 10 days.

In order to quantify the reduction of symptom expression, overgrowths were excised from stems at 10, 20, 30, 60 and 90 days after inoculation (DAI) and their weights and pathogen populations size were compared.

Bacterial Isolation from the recovered knots

The points of inoculation and/or knots taken at different times were weighed and homogenized in sterile mortars in known volumes of PMS buffer. The obtained bacterial suspensions and decimal dilutions were inoculated onto plates of KB + 100 ppm of Rifampicin + 50 ppm of cycloheximide (for the isolation and enumeration of *P. savastanoi* ITM317-Rif) and KB + 50 ppm of cycloheximide (for the isolation and enumeration of *Bacillus mojavensis* A-BC-7).

The plates were incubated at 26 °C for 5 days and then the bacterial counting was carried out using the stereo microscope.

The use of *P. savastanoi* strain resistant to rifampicin is motivated by the fact that in previous assays, the test was made with the wild strain and it was impossible to survey, in particular, the population of *P. savastanoi* on KB plates because its cells were inhibited by the antagonistic strain. The use also of the two substrates allowed to separately isolate the bacterial cells of the two bacteria.

Statistics

Data were analyzed by General Linear Model Univariate Analysis of variance using duncan's multiple-comparison test. A p-value<0.05 was accepted as indicating statistical significance.

Results

Bacillus mojavensis A-BC-7 inhibits overgrowths caused by *Pseudomonas savastanoi* pv. *savastanoi* ITM317 rifampicin resistant

A-BC-7 strain, inoculated with the pathogen, affected symptom expression in olive stems. From 10 days after the inoculation of strains ITM317-Rif and A-BC-7, overgrowths were evaluated for their weights and for the pathogen populations size. Typical knots appeared clearly on the surfaces of inoculated sites at about 30 DAI with average weights of 114.80 mg \pm 0.0107 (ITM317-Rif alone), 93.20 mg \pm 0.0166 (ITM317 + A-BC-7 (5:5; v:v)) and 66.00 mg \pm 0.0028 (ITM317 + A-BC-7 (1:9; v:v)) (Figure 1).

As reported, when the A-BC-7 suspensions were added to the pathogen suspension before inoculation, formation of smaller knots was observed (Figure 1). In particular, the application of the mixture suspensions of ITM317-Rif + A-BC-7 (1:9) that resulted of 43.11% of overgrowth inhibition caused by the pathogen, which increased to reach about 59% at 60 DAI and then 75% at 90 DAI.

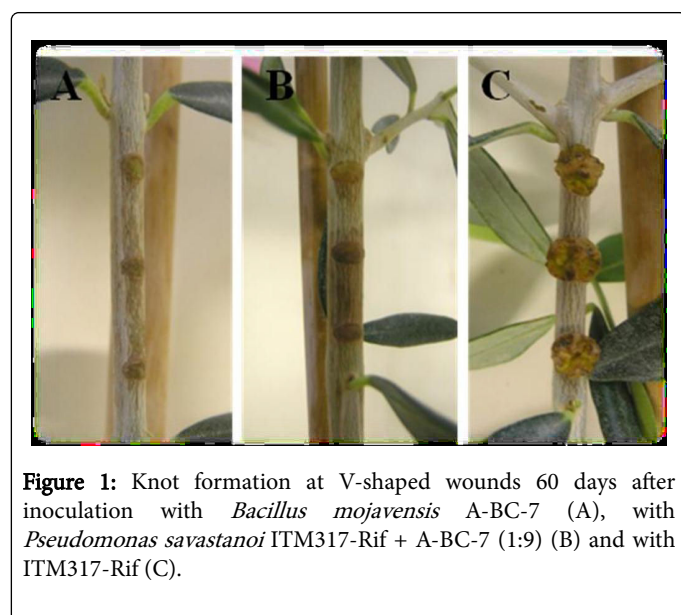


Figure 1: Knot formation at V-shaped wounds 60 days after inoculation with *Bacillus mojavensis* A-BC-7 (A), with *Pseudomonas savastanoi* ITM317-Rif + A-BC-7 (1:9) (B) and with ITM317-Rif (C).

In Figure 2, the effect of the A-BC-7 suspension with the both ratios is expressed by comparing knot weight obtained from inoculated sites.

Bacillus mojavensis A-BC-7 decreases *Pseudomonas savastanoi* pv. *savastanoi* ITM317-Rif (rifampicin resistant) populations size

Co inoculation of A-BC-7 with ITM317-Rif caused a reduction in bacterial multiplication at both ratios. In particular, ITM317-Rif/A-BC-7 (1:9). Population sizes (mean log₁₀ (cfu.g⁻¹ fresh stem tissues \pm SD) around the inoculation spots of ITM317-Rif were a quite different at the first dozen of the experiment, either in the presence with both ratios or absence of the A-BC-7. We got 9.11 \pm 0.120, 9.44 \pm 0.109 and 9.97 \pm 0.191 respectively for ITM317-Rif/A-BC-7 (1:9), ITM317-Rif/A-BC-7 (5:5) and ITM317-Rif. These differences became more significant ($p > 0.05$) in the developed knots at 30 DAI (9.61 \pm 0.247, 10.11 \pm 0.132 and 10.38 \pm 0.137) for ITM317-Rif/A-BC-7 (1:9), ITM317-Rif/A-BC-7 (5:5) and ITM317-Rif, respectively) (Figure 3).

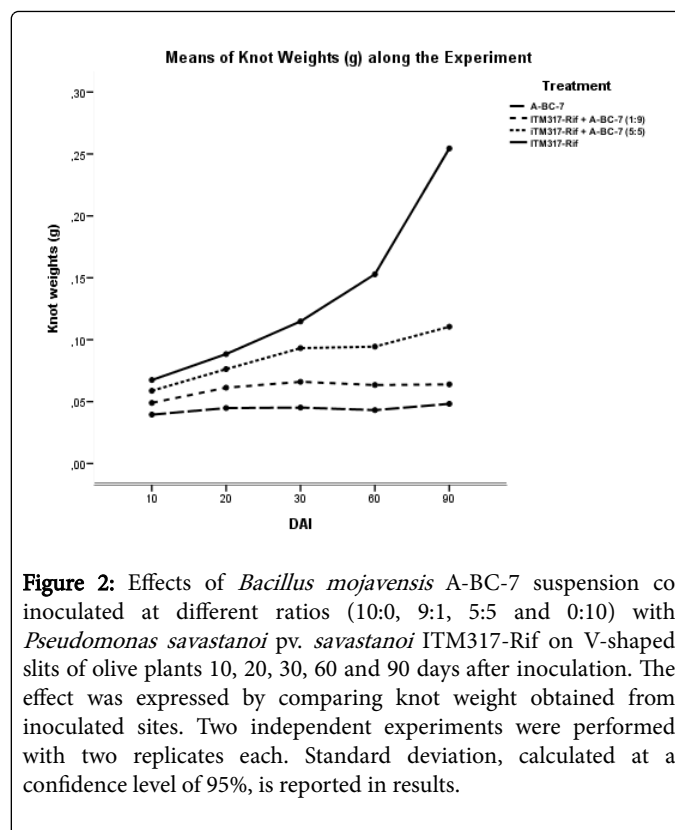


Figure 2: Effects of *Bacillus mojavensis* A-BC-7 suspension co inoculated at different ratios (10:0, 9:1, 5:5 and 0:10) with *Pseudomonas savastanoi* pv. *savastanoi* ITM317-Rif on V-shaped slits of olive plants 10, 20, 30, 60 and 90 days after inoculation. The effect was expressed by comparing knot weight obtained from inoculated sites. Two independent experiments were performed with two replicates each. Standard deviation, calculated at a confidence level of 95%, is reported in results.

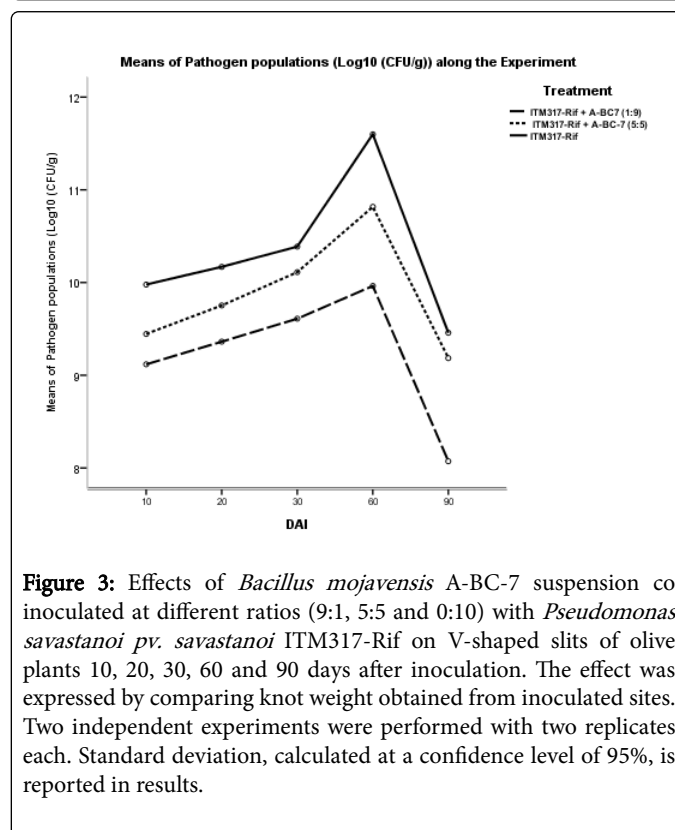


Figure 3: Effects of *Bacillus mojavensis* A-BC-7 suspension co inoculated at different ratios (9:1, 5:5 and 0:10) with *Pseudomonas savastanoi* pv. *savastanoi* ITM317-Rif on V-shaped slits of olive plants 10, 20, 30, 60 and 90 days after inoculation. The effect was expressed by comparing knot weight obtained from inoculated sites. Two independent experiments were performed with two replicates each. Standard deviation, calculated at a confidence level of 95%, is reported in results.

We noted also the positive correlation among population sizes for the different tests along the experiment. At 60 DAI, they still decreased

to rich to the size top : 9.96 ± 0.114 ; 10.81 ± 0.087 and 11.59 ± 0.067 respectively for ITM317-Rif/A-BC-7 (1:9), ITM317-Rif/A-BC-7 (5:5) and ITM317-Rif. And then, at 90 DAI, we reported an increase of population sizes. Thus, we noted 8.072 ± 0.257 ; 9.18 ± 0.390 and 9.45 ± 0.030 respectively for ITM317-Rif/A-BC-7 (1:9), ITM317-Rif/A-BC-7 (5:5) and ITM317-Rif.

The populations recovered after 60 days were 6 (5:5) and 42.54 (1:9) times lower than the control populations.

Analysis of variance (followed by Duncan's test) comparing results for control test showed significant differences ($p < 0.01$) between the different treatments and at the different times of monitoring.

Discussion and Conclusion

The swellings or overgrowths in inoculation sites were clearly observed between eight and ten days after inoculation of ITM317-RIF on olive plants. Coinoculation of *Bacillus mojavensis* A-BC-7 with the pathogen inhibited the formation of overgrowths caused by the pathogen. This effect reflects the inhibition of bacterial multiplication of the pathogen observed *in vitro* experiments (results are not shown here). The inoculation method (via V-shaped slits) was chosen to mimic damage caused by weather injuries or agronomic practices, since wounds allow epiphytic populations to invade and colonize host tissues [31]. The data obtained from experiments on olive plants lead us to conclude that *Bacillus mojavensis* A-BC-7 can be used as a biocontrol agent to survey *P. savastanoi* pv. *savastanoi*. The reduction of populations size of *P. savastanoi* pv. *savastanoi* that was obtained by combining ITM317 and A-BC-7 suspensions particularly with the ratio 1:9 is of great significance with respect to its effectiveness as a natural biological control agent on symptomless phylloplane olive plants. Data, expressed as means standard errors (calculated at a confidence level of 95%), are from two independent experiments with three replicates each (n 6).

The overall results reported in this study make the conclusion that the phylloplane bacterium *B. mojavensis* strain A-BC-7, isolated from symptomless olive plants, has the potential to control the survival of the causal agent of olive knot disease and to prevent its multiplication at inoculation sites particularly at higher ratio. Further studies on the application of this bacterium to young nursery-grown olive plants are required to evaluate the optimal ratio needed to get effective disease control.

Finally, these findings push new interest in the biological control agents that should be more investigated as an alternative measure to reduce the risk associated with the use of synthetic pesticides.

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