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Evaluation of biological control agent *Pantoea agglomerans P10c* against fire blight in Morocco

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The bacterial antagonist *Pantoea agglomerans P10c* was evaluated for efficacy to control fire blight in Morocco. It was evaluated in the laboratory on detached blossoms (pre and post-inoculation of pathogen) and under field for its ability to suppress growth of *Erwinia amylovora* on blossoms of pear trees (*Pyrus communis*); and for its aptitude to establish on and to colonize the blossoms of apple tree (*Malus domestica*) under field conditions. Results revealed that the installation of *P. agglomerans* P10c strain on detached blossom before the inoculation with the *E. amylovora* (preventive treatment) allows an efficient control of the disease (96%), with an incidence that never exceed the level 2.5%. but for curative treatment the incidence increased to 40% and the efficiency was only about 32%. Under field, *P. agglomerans* P10c applied a twice (10 and 60% bloom time) at 10^8 CFU/ml reduced significantly the incidence of fire blight by 64.7 to 76.9%. The experiment of the establishment and the survivability of *P. agglomerans* P10c in the stigma showed a positive evolution which increased from 4.5×10^4 to 7.6×10^5 CFU/flower. Results from this work illustrate that this antagonistic strain is able to control efficiently fire blight in apple and pear trees under the conditions of the arias of Meknes.

Keys words: *Erwinia amylovora*, *Pantoea agglomerans* P10c, antagonist, blossom, preventive treatment, apple tree, pear tree, Meknes.

INTRODUCTION

Fire blight, caused by the bacterium *Erwinia amylovora* (Burrill, 1883), is a devastating disease of rosaceous plants (Van der Zwet, 1979). This plant pathogen has been reported from several regions of the world including North and Central America, New Zealand, United Kingdom, Europe, Middle East and Central Asia (Smits et al., 2010). The disease was introduced in Morocco in 2006, in a commercial orchard in the rural area of Ain Orma (Saoud, 2008), and it has spread rapidly throughout the most important pome fruit production regions causing significant and serious economic losses (Fatmi, 2009; Yaich et al., 2011). Fire blight can affect all

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aerial parts of the plant, but blossoms are the most important site of infection for epidemic blossom blight (Norelli et al., 2003). Therefore, the suppression of the infection during blossom stage is the pillar of the strategy to control the disease. In addition, the pathogen needs to multiply on the stigmatic surfaces before penetrating and infect the flower (Johnson and Stockwell, 1998). Indeed, if the multiplication of *E.amylovora* is inhibited, the probability of the infection of the blossom and the dispersion of the bacteria to other blossoms will be reduced. The elimination of the pathogen in this period couldn't be ensured by the chemical products currently registered in Morocco against fire blight because of their adverse phytotoxic effects on blossoms and fruits. Whereas, the use of antagonists of *E. amylovora*, such as *P. agglomerans* (Stockwell et al., 2002; Vanneste, 2011) and *Pseudomonas fluorescens* (Cabrefiga et al., 2007, 2014), has been widely demonstrated as an alternative for the control of fire blight in several countries. The objective of this study was to test the efficacy of a product based on the strain *P. agglomerans* P10c under laboratory and field conditions in the country of Morocco.

MATERIALS AND METHODS

Bacterial strains

The bacteria used in this study were the bacterium *P. agglomerans P10c*, approved in Italy under the name of PomaVita® (10^{11} CFU/g) and in New Zealand under the name of Blossom Bless®. This antagonistic bacterium is resistant to streptomycin (100 ppm) and rifampicin (20 ppm). *P. agglomerans P10c* was supplied in lyophilized form, the identity and purity of strain was verified by biochemical tests using API 20 E system after 10 serial dilution plating on LB agar of the suspension of 1 g of PomaVita in 1 ml of sterilized water. The pathogen *E. amylovora* was isolated from a commercial pear orchard (cv Cossia), located in the region of Meknes, referring to the EPPO Bulletin (2004). The identity of *E. amylovora* was checked by amplification of 1 kb fragment of the plasmid pEA29 by the PCR technique (Bereswill et al., 1992). Thereafter, the *E. amylovora* quantity was increased using the Levane culture medium at a temperature of 25°C.

Detached blossoms assay

Evaluation of the efficacy of *P. agglomerans P10c* was conducted in laboratory conditions on detached blossoms pear cv. Duke of Bordeaux. blossoms were collected at the white button stage from pear orchard (planting 2004) in the region of Meknes, putted in plastic bags on ice, and transported to the laboratory. In the laboratory, collected flowers were maintained in submersion in water contained in 2 ml tubes. Tubes containing water were supported by a polyester rack and each rack contains 20 to 24 blossoms constituting the experimental unit. The later were placed in a growth chamber (2.5 m length, 1.5 m in width and 1 m in height). The temperature, humidity and lighting were: 22°C day / 10°C night, 90 ± 10% and 17 h, respectively (Wilson et al., 1992). Treatments were assigned to 20 newly opened blossoms per treatment in a randomized complete block design with four replications. The blossoms were grouped into blocks, based on their availability in the field. The total amount of flowers was 320 (20 × 4 × 4). In order to simulate appropriate conditions to test the effectiveness of *P. agglomerans P10c*, four treatments were applied: (1) *E. amylovora* alone as a control (E0); (2) *P. agglomerans P10c* before *E. amylovora*: Pre-inoculation (PE); (3) *E. amylovora* then *P.agglomerans P10c*: Post-inoculation (EP) (4) *P. agglomerans P10c* alone (P). The antagonist was prepared by diluting 1 g of the lyophilized product in 1 L of distilled water to produce recommended doses and it was applied on blossoms using a micro-sprayer. 24 h later, the pathogen was suspended at a concentration of 5×10^8 CFU / ml and was applied to flowers using a brush. The concentration was adjusted using an optical density reader at 600 nm after preparation of the suspension across 5 Mac Ferland. After four days of pathogen inoculation, daily tracking for a period of 5 days of the number of blossoms, at each treatment, showing symptoms of fire blight was performed. To compare the effect of different treatments, the area under the curve progression disease (AUDPC) was calculated using the formula of Shaner and Finney (1977):

$$\mathbf{AUDPC} = \sum_{i=1}^{n} \left[\frac{y_i + y_{i-1}}{2}\right] \times (t_i - t_{i-1})$$

yi is the incidence of the control disease for treatment i at times t and n the number of reading dates incidence.

The effectiveness of the two treatments: *P. agglomerans P10c* then *E. amylovora* and *E. amylovora* then *P. agglomerans P10c* was calculated relative to the control at the end of the trial by the following formula:

$$E = (y_0 - y_i)/y_0$$

With *y0* the incidence of the control and *yi* the incidence of the treatment *i.*

Biological control of fire blight on pear trees

The trial was conducted in a commercial orchard of pear variety Cossia planted in 2004, it is located Toulale in the region of Meknes. The antagonist was prepared referring to the instructions contained in the data sheet of the commercial product (10 g of the lyophilized product in 10 L of water, that is, 10^8 CFU/ml). The application on the trees was carried out using a knapsack sprayer with pressure maintained until runoff. Two applications of the antagonist was performed (Stockwell et al., 2010), one at 10% bloom time and the second a week later (60% bloom time) under conditioned temperature 20°C and low wind. Treatments were assigned to a tree by treatment in a completely randomized design with five replicates. The trial was carried out under natural inoculation. For the evaluation of treatment, the disease incidence on blossoms was calculated. It was measured by calculating the number of blighted blossoms relative to the total number blossoms of observed branch. Ten branches were randomly observed for each tree, in total 50 branches considered by treatment with an average of 30 blossoms per branch. This evaluation of was carried out one week after the second application of the antagonist.

Establishment and survival of P. *agglomerans* P10c on apple flowers under field conditions

The trial was conducted over a period of two weeks of blooming

period, on apple trees (cv. Golden). To test the establishment and survival of *P. agglomerans P10c* on apple blossoms under field conditions, newly opened blossoms (less than 24 h) were selected and marked by a plastic string. The marked blossoms were randomized in two identical blocks to have 80 blossoms per block, with additional blossoms to prevent the risk of loss by wind or insects. In total, 200 blossoms were marked. Flowers of one of this blocks were sprayed with a suspension of *P. agglomerans* P10c using a micro-sprayer. The suspension was prepared by diluting 1 g of the lyophilized product in 1 L of distilled water. In parallel, the group of blossoms of the other block was sprayed with water only. These blossoms were considered as control. Weather conditions were monitored using a thermo-hygrograph in the shadow, placed 2 m from the ground. After spraying, 12 flowers per block were taken every day for 7 days. The 8^{th} and 9^{th} sampling were done at a rate of 3 days after the 7th sampling. The collected flowers were transported to the laboratory in small and sterilized bags. Analysis and determination of *P agglomerans* P10c population were done according to the following protocol (Francesco et al*.,* 2011): Under the laminar flow hood, each blossom was treated individually; the five stigmas were removed using sterile forceps and were washed in 1 ml of 10 mM MgSO4. Washing was done in an Eppendorf tube with vortexing for a few seconds. Solutions were subject to a series of dilutions of 1:10 th in 10 mM $MgSO_4$. (that is, 100 µl of lavat in 900 µl of 10 mM $MgSO_4$). 50 ml of each dilution was plated on LB agar supplemented with streptomycin (100 ppm) and rifampicin (20 ppm). For the blossoms treated with water, plating of the solutions was performed directly without dilution. Petri dishes were incubated at a temperature of 25°C and colony counting was made after 24 h; the bacterial concentration by CFU/ml was obtained by multiplying the number of CFU/Petri dish by 1000 ml/50 ml. Finally, the CFU/blossom concentration was obtained by calculating the initial concentration.

Statistical analysis

The calculated efficiencies was transformed using arcsin√. The data obtained, AUDPC and arcsin√E, were analyzed by ANOVA. The Student-Newmen-Keuls test was used to separate means for significant effect at the 0.05 probability level. All statistical analyses were performed with SPSS 17 (IBM) software.

RESULTS

Detached blossoms assay

The fire blight symptoms were observed in the control treatment. The average number of infected flowers varied during the test period. Indeed, four days after inoculation, the average incidence of the disease on the affected flowers is about 40% for the control (E0) and 20% for the treatment (EP). This incidence increased to a maximum of 60% for the control and 40% for the treatment EP. Furthermore, preventive treatment (PE) expressed an incidence that does not exceed the threshold of 2.5% during the entire test period (Figure 1). Analysis of variance of AUDPC shows a highly significant effect between treatments and the comparison of means by Student-Newmen-Keuls test ($p < 0.05$) showed the presence of three classes as indicated in Table 1.

In terms of efficiency, treating flowers with *P. agglomerans* P10c preventively significantly reduced the incidence of the disease by about 96%. This efficiency was only about 32% in the curative cases.

Biological control of fire blight on pear trees

The incidence of blossom infection in control pear trees naturally inoculated with the pathogen was high (65.7% in a trial 1 and 72% in a trial 2). The performance of P10c for disease control varied insignificantly among trials (Figure 2). In trial conducted in 2013, *P. agglomerans* P10c significantly reduced the percentage of blighted blossoms on commercial pear orchard by 64.7% compared to water-treated control. In trial conducted in 2015, this percentage of reduction increased to 76.9%.

Establishment and survival of *P. agglomerans* P10c on apple flowers under field conditions

In all samples (for 9 days), *P. agglomerans P10c* was isolated from flowers inoculated by spraying. The isolation success rate at the first sample (D0 + 1) was above 90%. This rate was generally maintained throughout the test period. The size of the populations of *P. agglomerans P10c* isolated from flowers shows a growing tendency to change over time ranging between 10^4 and 10^8 CFU/flower suggesting multiplying of the antagonistic bacteria on the flower surface (Figure 3). Indeed, the initial size of the population of *P. agglomerans P10c* was estimated between 1×10^4 and 1×10^5CFU/flower. Subsequently, the population has grown to stabilize around 5.5×10^5 CFU/ flower during the three days. Then, a slight decrease was noticed and that is mainly due to the recording night temperatures below 10°C , then the multiplication of the bacteria took over eventually reaching 7.6×10^5 CFU / flower. In terms of non-inoculated flowers (control), no colonies of *P. agglomerans P10c* was obtained on the culture media during the entire test period.

To explain the influence of climatic conditions on the biotic potential of *P. agglomerans P10c*, correlations between key climate parameters and population levels of the antagonist were established (Figure 4). A positive correlation between daily mean temperature and the average size of the established population was observed. A strong influence of temperature on the development and survival of *P. agglomerans* P10c was recorded. For cons, the minimum relative humidity was negatively correlated with the studied parameter.

DISCUSSION

To be effective, the biological control agent should be

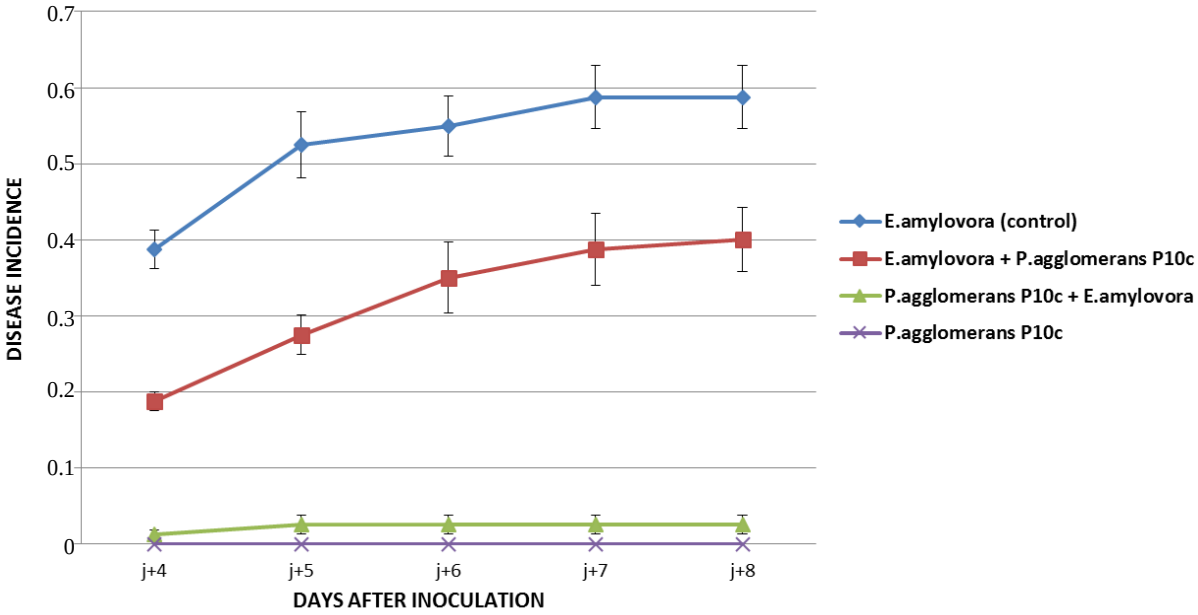

Figure 1. Evolution of the average incidence of symptoms of *E. amylovora* inoculated to pear blossoms cv. Duke of Bordeaux before or after de application of P10c at 10^8 CFU/ml under laboratory conditions (growth chamber [2.5 m length, 1.5 m in width and 1 m in height]. The temperature, humidity and lighting were 22°C day/10°C night, 90 ± 10% and 17 h, respectively).

Table 1. Area under the curve progression disease (AUDPC) of different treatments (detached blossoms assay).

Traitement	AUDPC
E. amylovora (E0)	2.25^a
E. amylovora than *P. agglomerans P10c* (EP)	1.25^b
P. agglomerans P10c than *E. amylovora* (PE)	0.12^c
P. agglomerans P10c (P)	0.00^c

Treatments followed by the same letter are not significantly different according to the least significant difference test at the 0.05 level.

able to multiply at the same ecological niche of the pathogen. This results in competition on the space and nutrients. It was determined that the competition on the sites and on the nutritional substrates limiting growth is the main mechanisms by which *P. agglomerans* suppresses the growth of *E. amylovora* on the stigma (Özaktan and Bora, 2004). This explains the result obtained when *P. agglomerans* P10c was applied to flowers before infection with the pathogen (preventive treatment). Otherwise, the result of curative treatment could be explained by mechanisms other than competition on the ecological niche. Wodzinski et al. (1994) report that *P. agglomerans* inhibits *E. amylovora* by acidification of the medium, producing bacteriostatic substances or bacteriocin herbicolin or induction of hypersensitivity reactions. Stokwell et al. (2002) show that *P. agglomerans* has a capacity to produce antibiotic contributing to the suppression of fire blight.

Under field conditions, Johnson et al. (1993) found that early establishment of populations exceeding 10^5 CFU per blossom of *P. fluorescens* Pf A-506 and *P. agglomerans* Eh C9-1 on pear blossoms suppressed establishment and growth of *E. amylovora*. The incidence of fire blight on blossoms was reduced by about 60% with two applications of bacterial antagonists in experimental plots in the Pacific Northwest (Nuclo et al., 1996) and California (Lindow et al., 1996).

The population level may be influenced by temperature and humidity, our results are agree with those obtained by Johnson et al. (2000) and Stockwell et al. (2002) for *P. agglomerans* strain Eh252 under the conditions of the State of Oregon (USA). Finally, to be effective, the antagonistic bacteria must settle the entire stigmatic surface in the orchard conditions (Johnson et al., 1993; Lindow and Suslow, 2003), and the size of the population of the antagonist on the stigma must be between 10^5 and 10^6 CFU / flower (Wilson et al., 1992; Wilson and Lindow, 1993). In this study, detectable average population size of *P. agglomerans* P10c ranged from 4.5×10^4 to 7.6×10^5 CFU / flower under real conditions during the 12 days after the date of inoculation (Figure 3). This result shows that *P. agglomerans* P10c has colonized the flowers in the real conditions and could contribute significantly to reduce the *E. amylovora* installation.

In conclusion, the installation of the strain *P.*

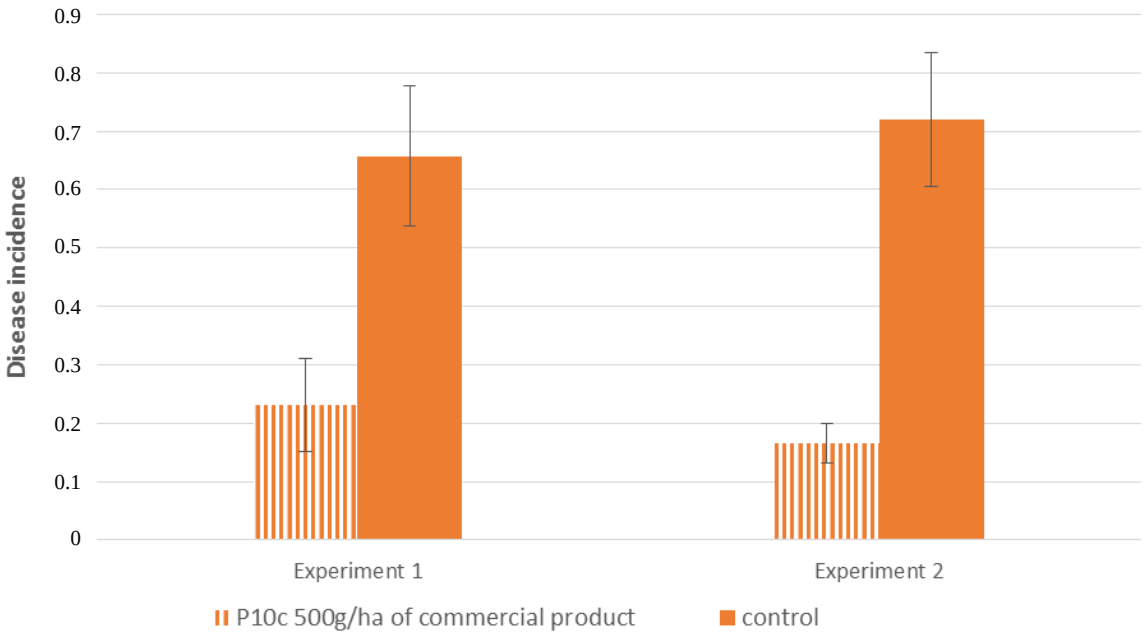

Figure 2. Effect of Antagonist strain P10c on the occurrence of fire blight on pear blossoms one week after the second application of the antagonist. Tow application of 500 g/ha of commercial product were applied at 10 and 60% bloom time (Experiment 1 in 2013, Experiment 2 in 2015)

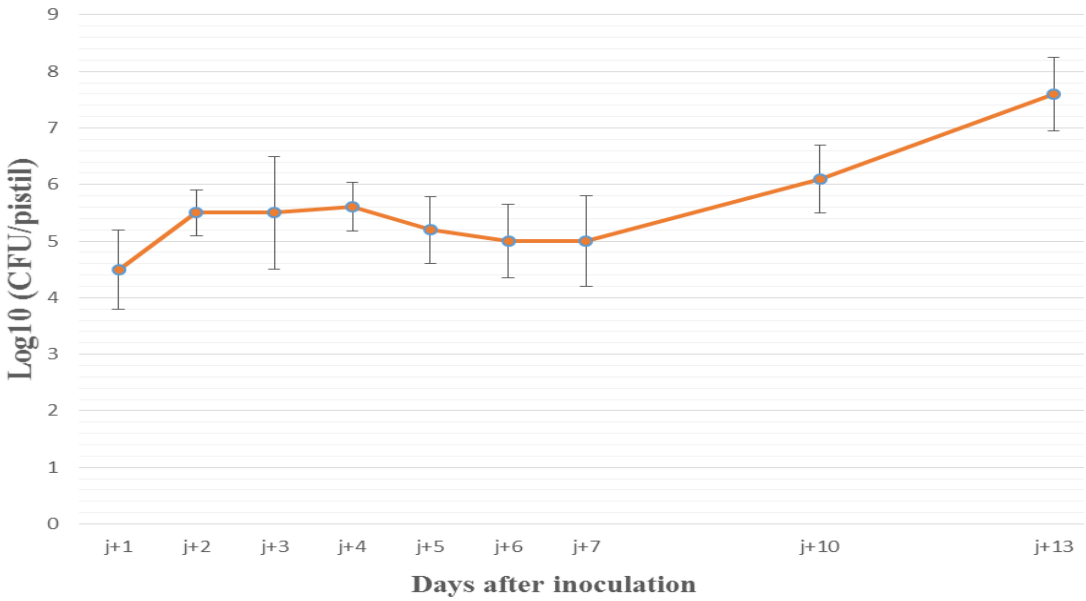

Figure 3. Mean population level of *P. agglomerans P10c* under real conditions on apple blossoms cv. Golden.

agglomerans P10c before inoculation with the pathogen has significantly slow the disease. Under field conditions, the monitoring of its dynamics shows an increasing tendency to evolution indicating the reproduction of this antagonist on the flower surface. The estimated size of the population of *P. agglomerans P10c* on the blossoms and the efficacy trials shows that this antagonistic strain would be able to control effectively fire blight in the

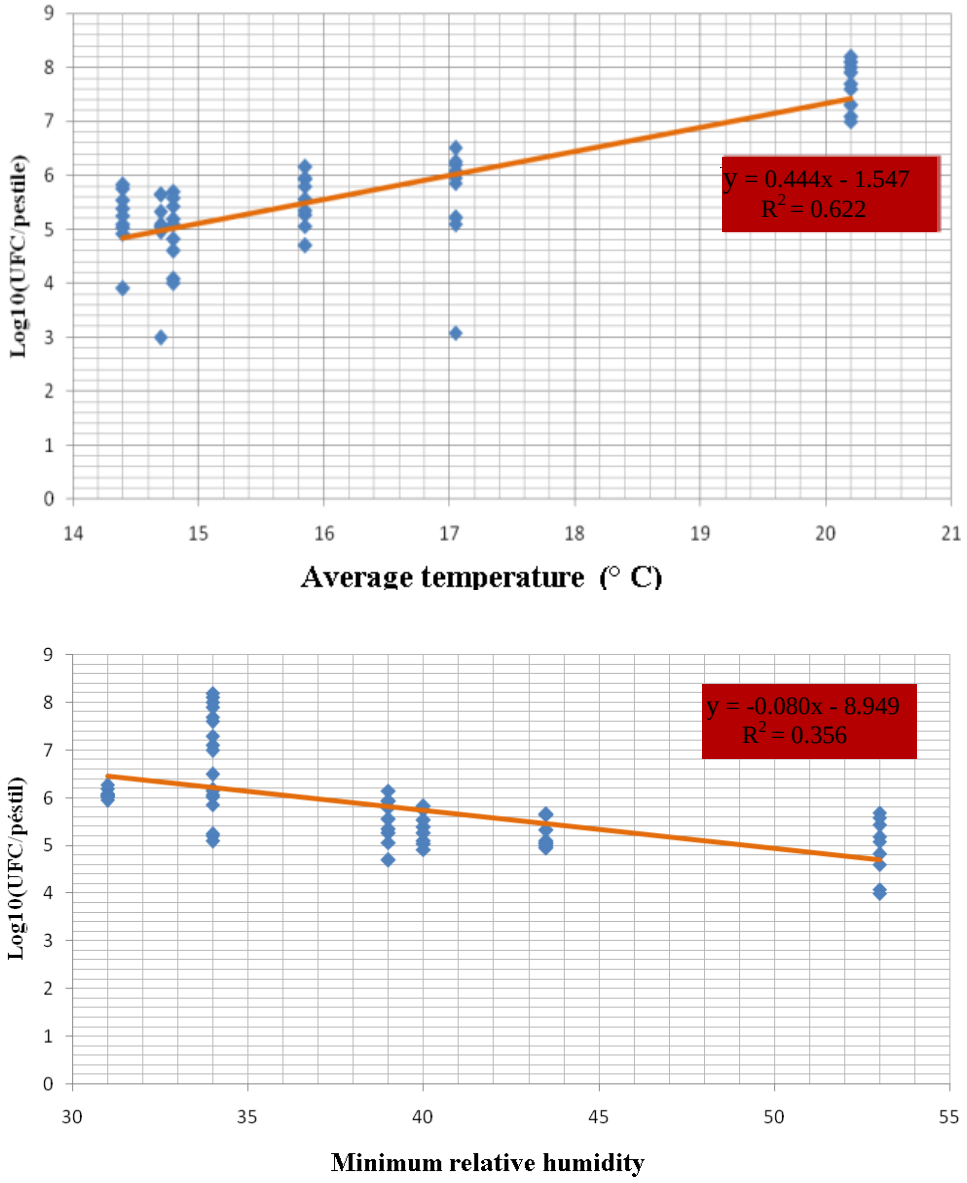

Figure 4. Correlations between population levels of *P. agglomerans* P10c and the average temperature (top), relative humidity (below).

orchard conditions in Meknes region.

Conflict of Interests

The authors have not declared any conflict of interests.

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