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Research Article

Plant growth promotion and bacterial canker control of *Lycopersicon esculentum* L. cv. Campbell 33 by biocontrol agents

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Abstract: Endophytic and epiphytic bacteria have been reported as agents of bio-control of diseases and plant growth promotors. Here, *in vitro* and greenhouse experiments were conducted to evaluate the action of two microbial strains; *Aureobasidium pullulans* and *Pantoea agglomerans*, on biocontrol of bacterial canker and growth promotion of tomato (*Lycopersicon esculentum* cv. Campbell 33). Two frequencies of treatment were used to assess their potential effect (15 and 30 days between two subsequent treatments). The two strains were able to inhibit, *in vitro*, the growth of *Clavibacter michiganensis* subsp. *michiganensis* the causative agent of tomato canker. Also, their antagonistic effects were confirmed in greenhouse conditions. Indeed, bacterial canker incidence in tomato plants treated with *A. pullulans* and *P. agglomerans* separately or in mixture was significantly less severe (16%) compared to the positive control (83%). The treatment frequency (intervals of 15 or 30 days) and the choice of strains to inoculate (separated or combined strains) appear to be essential for obtaining significant results. Consequently, both *A. pullulans* and *P. agglomerans* strains highly reduced incidence of bacterial canker particularly when tomato plants were treated at a frequency of fifteen days.

Keywords: Tomato, Plant growth promotion, Bacterial canker, Bio-control agents

Introduction

Bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* is one of the major and serious diseases of tomato (Davis *et al.*, 1984; Yogev *et al.*, 2009). It is a highly contagious and destructive disease both in greenhouses and in the field (Utkhede and Koch, 2004). All areas of Moroccan tomato production are infested with the pathogen whose severity varies by regions (Yogev *et al.*, 2009). Infected seeds and transplants are the main source of primary inoculums of *C. michiganensis* subsp. *michiganensis* (Fatmi *et al.*,1991).

Recommended chemical treatments to fight against this disease only reduce the population of the pathogen on the surface of plants or infested seeds (Hausbeck *et al.*, 2000). Considering the less efficiency of chemical treatments and their impact on health and environment, research and development of alternative control methods are recommended.

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Biological control is very promising because many studies conducted all over the world have led to encouraging results. In particular, microbial strains (bacteria, fungi and yeasts) have been known for their positive effects and are beneficial in the fight against certain pathogens that inhibit plant growth (Achbani *et al.,* 2005; Bencheqroun *et al.,* 2006; Faheem *et al.,* 2015; Faquihi *et al.,* 2015; Sadik *et al.,* 2015). In this work, our aims were i) assessment of two micro-organisms, yeast and bacterium, *A. pullulans* Ach2-1 and *P. agglomerans* 2066-7, for their effect on promoting tomato growth in a greenhouse experiments and ii) their activity against bacterial canker; iii) and also the effect of treatment frequency on plant growth and disease incidence in greenhouse.

Materials and Methods

Biological materials and growth conditions

Seedlings of tomato *L. esculentum* were obtained from seeds of a susceptible cultivar ‘‘Campbell 33’’ cultivated in plastic containers filled with a mixture of sterile soil from garden of Regional Center of Agriculture Research and peat (1:1 v/v) in a greenhouse under a 16 h light regime and 60–70% relative humidity at 25 to 32 °C and without supplementary fertilizers such as described previously (Amkraz*et al.,* 2013). After 2 months of growth, seedlings were divided into two groups. The first was used to evaluate the effectiveness of the selected antagonists in reducing the canker infection in greenhouse and the second group of plants was used to vegetative growth promotion by these strains.

The pathogen *C. michiganensis* subsp. *michiganensis* strain (*Cmm* 1616-3) used in this study was provided by Regional Center of Research and Agriculture of Meknes, Morocco (RCRA). It was activated by three subcultures on the Yeast Peptone Glucose Agar (YPGA) medium (Yeast extract, 5g; Peptone, 5g; Glucose, 10g; Agar, 18g; and distilled water, 1 l) and its pathogenicity was confirmed by the tobacco (*Nicotiana glutinosa)* hypersensitivity test following a slightly modified method described previously (Atkinson *et al.,* 1985). Briefly, the suspension of bacterial culture was prepared in sterile distilled water and its concentration was adjusted (10^8 CFU/ml) by spectrophotometer (UV-mini 1240, Shimadzu) at 600 nm. An aliquot was then injected into tobacco leaves using a syringe without needle (the test was confirmed for three replicates). The reaction was assessed as necrotic area around inoculums that is characterized in 24 hours at room temperature (22 to 28 °C). An aliquot of sterile distilled water was injected as negative control.

Two antagonists were used in this study: a yeast *A. pullulans* (Ach 2-1) isolated from the surface of Golden Delicious healthy apples (Achbani *et al.,* 2005)and endophytic bacterium *P. agglomerans* (2066-7) isolated from olive plant (Bouaichi *et al.,* 2015). These strains were selected for their ability to promote growth and to control soft rot disease in potato caused by *Pectobacterium carotovorum* as previously provided (Faquihi *et al.,* 2015) and 2066-7 for their biocontrol effect against bacterial diseases of onion (Sadik *et al.,* 2015).

In vitro antagonism test

In vitro test for the ability of the antagonistic strains to inhibit growth of *Cmm*1616-3 strain was performed according to the spot method (Bouddyach *et al.,* 2001). Briefly, the bacterial suspension of *Cmm* 1616-3 strain (10^8 CFU/ml) was spread out on YPGA medium and excess of suspension was eliminated and inoculated plates were dried for 15 min under a laminar flow hood. Once dry, an aliquot (10 µl) of each strains suspension (10^8 and 10^7 CFU/ml, respectively for the 2066-7 and Ach2-1) was spotted in the same Petri dishes (an aliquot of sterile distilled water was used as a control). The measure of the inhibition zones was carried after incubation at 26 °C for 72h and the test was carried out in three replicates for each bio-agent strain.

Assessment of vegetative growth in greenhouses

Experiments were conducted in a greenhouse (T° = 26–32 °C, RH = 60-70%) to evaluate the effect of the tested strains on plant growth. The garden soil was subjected to two cycles of autoclaving, separated by 24 hours, at 120 °C for 20 min and was then potted (27 cm x 35 cm).

Treatment of two-month-old seedlings, previously prepared, was carried out by spraying a 50 ml of the microbial suspension (10^8 and 10^7 CFU/ml respectively for 2066-7 and Ach 2-1 strains) at the rhizosphere according to method developed by Boudyach *et al.*, (2004). Three treatments (five replicates for each) have been realized in a randomized block design: T1 (with *Aureobasidium pullulans* Ach2-1), T2 (with *Pantoea agglomerans* 2066-7) and T3 (with their combination) and a control test (T0) consisted of untreated plants. The first microbial inoculation with Ach 2-1 and 2066-7 strains was done 15 days after transplanting of seedlings. Furthermore, to assess the impact of treatment frequency, this experiment was repeated twice with 15 or 30 days between the two subsequent inoculations. The experiment was continued for three months from the first inoculation with microbial strains and all plants were irrigated to field capacity without any fertilizers.

The growth was followed by evaluation of agronomic parameters for all plants and treatments, including plant height, diameter of their main stems measured by slide calipers (Fisher Scientific HARDENED) and leaf areas measured by plan-meter (Bioscientific ADC LTD) and chlorophyll "a" and "b" content determined as described below. The first measurements were made 20 days after the first inoculation, the second after 40 days to calculate the growth rate of agronomic parameters the formula below was used (Schultz, 2001):

$$T = \frac{\Delta L}{\Delta T} \times \frac{1}{L_0}$$

ΔL: Difference between the first and the final measurements.

ΔT: Interval of time between subsequent measurements.

L_0: Value of the first measurement.

Chlorophyll "a" and "b" contents were determined at the end of experiments for the two frequencies of treatment using the method of extraction by dimethyl sulfoxide (DMSO) solvent as previously described by (Ramsay, 1974). Ten gramme of leaf tissue (Me) were placed in a test tube containing 7 ml of DMSO, and then incubated for 45 minutes at 65 °C until the disappearance of all green color. The extracted volume is recuperated in a new tube (Ve) and complemented with DMSO (until 10 ml). For analyses of chlorophyll a and b content, the optical density was determined at two wavelengths using a spectrophotometer UV-mini 1240, Shimadzu (the length of spectroscopic tubes is a = 1cm). The Chl "a" and Chl "b" amount was calculated according to the formula (UNESCO, 1966).

$$Chlorophyll\ a = 12.7(OD663) - 2.69(OD645)\frac{Ve}{1000 \times Me \times \alpha}$$

$$Chlorophyll\ b = 22.9(OD645) - 4.68(OD663)\frac{Ve}{1000 \times Me \times \alpha}$$

Where;

OD: optical density at certain wave length (645 or 663 nm).

Ve: final volume of extract; Me: weight of sample; α: length of the light path (1 cm).

Biological control of bacterial canker in greenhouse

Experiments were carried out in greenhouse under the similar conditions of plant growth experiments previously described. The aim was to assess the suppressive effect of these antagonistic strains against bacterial canker. Five treatments were performed with six replicates in a randomized block design; T0 (control, without inoculums), T'0 (infected with pathogen strains *Clavibacter michiganensis* sub. sp *michiganensis* (*Cmm*) 1616-3 (10^8 CFU/ml)), T'1 (treated with Ach 2-1 strain and infected by pathogen *Cmm*

1616-3), T'2 (treated with 2066-7 strain and infected by *Cmm* 1616-3), T'3 (treated with the combination of Ach2-1 and 2066-7 strains, and infected by pathogen *Cmm* 1616-3). This experiment was made twice for assessment of the impact of inoculation frequency (15 or 30 days) on the suppressive effect of antagonistic strains. The infection with pathogen *Cmm* 1616-3 (10^8 CFU/ml) was carried out by rhizosphere infestation (50 ml of microbial suspension) after wounding the roots of host plant (Boudyach *et al.*, 2004) on the seventh day from the initial treatment by antagonistic strains. Disease incidences (%) were calculated using the formula (Amkraz*et al.*, 2013): infected.

$$DI\,(\%) = \frac{Number\ of\ \ symptomatic\ \ plants}{Number\ of\ \ infected\ \ plants\ within\ treatments} \times 100$$

where DI is Disease incidences (%).

Statistical analysis

The data were subjected to one-way ANOVA using the SPSS 21.0 software program. Means and standard errors were calculated for five replicates and they were compared by the Duncan's multiple range test and statistical significance was determined at 5% level.

Results

In this study, two microbial strains isolated and selected at the Regional Center Research and Agriculture of Meknes (CRRA), Morocco, were evaluated for their contribution to plant growth promotion of tomato and their antagonistic effect against *Cmm*, causative agent of bacterial canker in this plant (*Lycopersicon esculentum* cv. Campbell 33). This phytopathogenic bacterium was confirmed for ability to produce hypersensitive reaction on the tobacco leaves (Fig. 1).

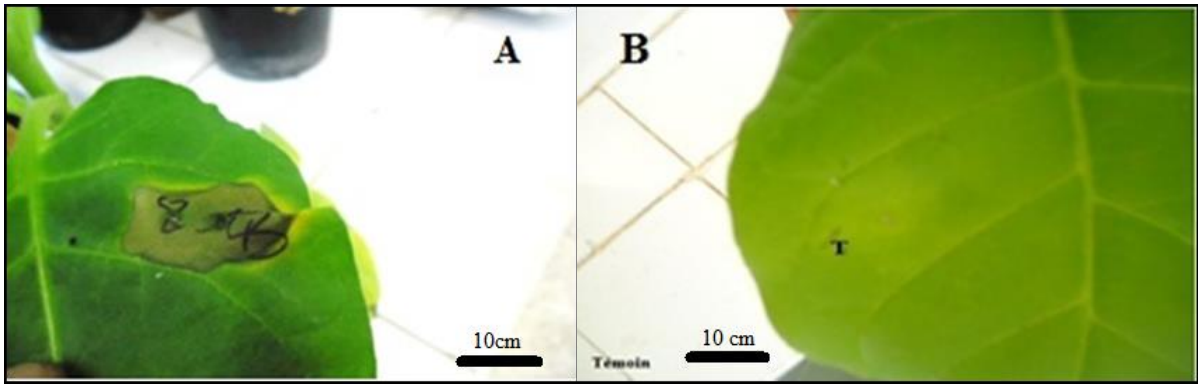

Figure 1 Hypersensitive reaction around the *Clavibacter michiganensis* subsp. *michiganensis* inoculation site (A) and negative control without necrosis (B).

***In vitro* antagonism**

The potential antagonistic strains previously isolated and confirmed as positive for hypersensitivity test (Fig. 1) were assayed against the selected phytopathogenic bacterium *Cmm* 1616-3, using *in vitro* confrontations described previously. The two antagonistic strains have shown promising results for their effect against the pathogen. Indeed, the inhibition diameters were of 21 mm and 16mm, for *A. pullulans* Ach2-1 and *P. agglomerans* 2066-7, respectively. Hence these results are promoting for using in the greenhouse experiments.

Vegetative growth in greenhouse

Growth parameters

The influence of *A. pullulans* Ach2-1 and *P. agglomerans* 2066-7 strains on plant growth rate of tomato was evaluated by measuring various parameters. Comparison between the averages of results obtained for each measurement and for the different parameters studied is presented in Figs. 2 and 3.

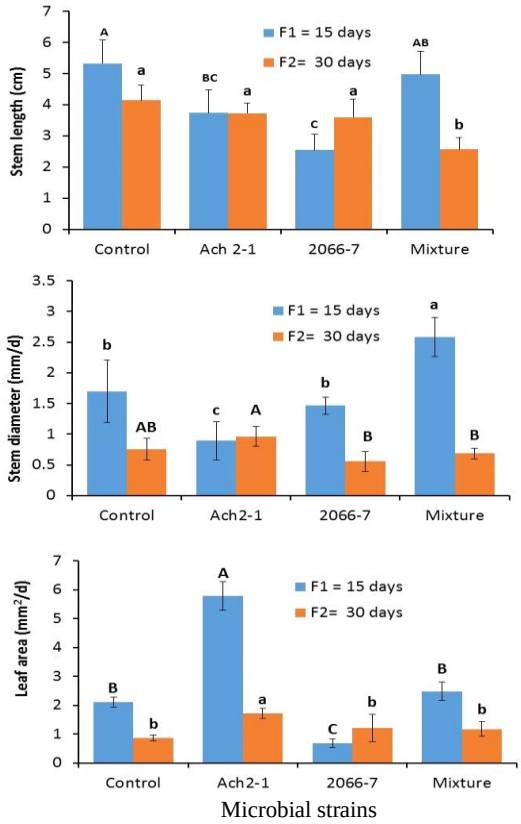

Figure 2 Growth rates of stem length, diameter and leaf area of tomato plants after treatment at intervals of 15 days or 30 days with strains of *Aureobasidium pullulans* (Ach2-1) and *Pantoea agglomerans* (2066-7) (data are averages (± standard deviation) of five replicates). (Sticks topped with the same letters do not differ significantly at 0.05%).

Both strains tested separately did not show a significant contribution to improving the growth of the plant length in either of the treatment frequencies 15 or 30 days (Fig. 2). However, in the case of the mixture of the two strains, plant diameter was increased in comparison with the control (2.59 $mm.d^{-1}$versus 1.74 $mm.d^{-1}$) while this improvement was not observed in the case of plants treated every 30 days.

Regardless of the period of time between the treatments, Ach 2-1 strain strictly contributed to the growth of leaf area, particularly when the treatment interval was short (15 days). Indeed, the growth rate of leaf area was of 5.98 $mm^2.d^{-1}$ and of 2.1 $mm^2.d^{-1}$ for treated plants and control, respectively. But, the combination of the two strains did not increase leaf area.

Chlorophyll « a » and « b » content

Influence of microbial inoculation with Ach2-1 and 2066-7 strains on the production of chlorophyll "a" and "b" was also investigated (Fig. 3).The best results were obtained with Ach 2-1 strain which increased significantly the amounts of both chlorophyll "a" and "b" when the interval between two subsequent treatments was 15 days. However, the 2066-7 strain and the combination of the two strains were unable to improve the content of chlorophylls a and b, regardless of the treatment frequency.

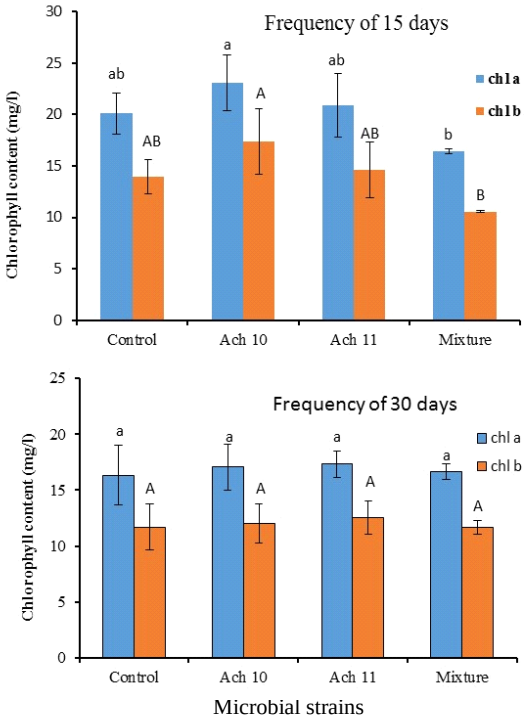

Figure 3 Influence of microbial inoculation with strains of *Aureobasidium pullulans* **(**Ach2-1**)** and *Pantoea agglomerans* **(**2066-7**)** on the production of chlorophyll a and b when the interval between two subsequent treatments is 15 days and 30 days; data are averages (± SD of five replicates). (Sticks topped with the same letters do not differ significantly at $\alpha = 0.05$).

Biological control of bacterial canker

Characteristic symptoms of bacterial canker appeared after 30 days on infected control

plants. The leaves turned yellow gradually, and then began to wilt unilaterally and bilaterally. In fruit, canker was manifested by the development of yellow spots.

Bacterial canker incidence in plants treated with Ach 2-1 or 2066-7 strains separately or with their mixture was less severe compared to the positive control, regardless the treatment frequency (table 1). However, the protective effect of two strains, used separately, was identical when the frequency of treatment was 15 days. When this frequency was of 30 days, the protective effect of the strain 2066-7 became more important while that of the strain Ach 2-1 decreased. Also it is noted that the protective effect of the mixture was higher when the frequency of treatment was 30 days.

Table 1 Bacterial canker *Clavibacter michiganensis* subsp. *michiganensis* incidence in plants treated every 15 days or 30 days by bacterial strains.

Treatments	Disease incidence (%)	
	15 days[1]	30 days[1]
Ach 2-1	49.00 ± 3.5b	66.54 ± 5.8c
2066-7	50.00 ± 5.75b	33.00 ± 2.7b
Mixture	33.32 ± 2.7a	16.67 ± 4.2a
Control	83.33 ± 5.34c	

[1] Means followed by the same letters are not significantly different (Duncan's multiple range test, $P < 0.05$).
Abbreviations: *Aureobasidium pullulans* (Ach2-1) and *Pantoea agglomerans* (2066-7).

Discussion

The first part of greenhouse experiments evaluated the contribution of strains Ach 2-1 and 2066-7 to the vegetative growth promotion of tomato plants by measurement of all growth parameters and chlorophyll "a" and "b". These results corroborated other research previously mentioned; (Satrani *et al.*, 2009) showed that the active stimulation of length of *Cedrus atlantica* rod by two *Pseudomonas fluorescens* A6RI and TGI252, was directly related to the secretion of phytohormones (i.e. the gibberellins) synthesized by these rhizobacteria. These phytohormones would act by strengthening or accelerating development of both the root system and the aerial part of plants. Our results confirmed that the response of microbial strains-treated plants depends on the specific interactions of host plant- microorganism. Zare *et al.*, (2011) and Amkraz *et al.*, 2013 reported that the stimulation of the growth of the tomato cv. Campbell 33 inoculated with *Pseudomonas* isolates was due to the fact that rhizobacteria increase the root area and hence the rate of exchange by the root system. Therefore, this allows the exploration of large soil volume and enhances water availability and nutrients to plants, especially iron, leading to a better development of the aerial and root part of the plants. For stem length no improvement was significantly mentioned. These results contrast sharply with those found by Kumar (2007) who found that the strain of *Pseudomonas* B-25 significantly improved the growth of tomato height by 49.66% compared to control plants (Kumar, 2007).

Finally, the growth parameters evaluated in our study were not affected similarly by Ach 2-1 and 2066-7 strains inoculation as so well as previously reported; such a parameter is associated with such a microbial strain or such combination, hence there is a necessity to check the compatibility between the microbial strains used in combinations (Jäderlund *et al.*, 2008).

In the second part of the study, the capacity of *A. pullulans* Ach2-1 and *Pantoae agglomerans* 2066-7 strains to protect tomatoes against canker pathogen and to improve plant growth was investigated. The two strains showed their ability to stop the growth of the *Cmm* 1616-3 pathogen. In 2006, by performing the same technique, researchers found that among 70 tested isolates, 34 showed growth inhibition more than or equal to 6 mm (Amkraz *et al.*, 2010). According to researches, the threshold for selecting antagonists was more than or equal 13 mm (Amkraz *et al.*, 2010; Xu and Gross, 1986). Thus, the two strains used in this study, would be good candidates for biological control of *Cmm* 1616-3 in greenhouse. Although, *in vitro* results do not necessarily indicate that the strains will be efficient to reduce or eradicate tomato canker in greenhouse (Amkraz *et al.*, 2010 and

Bencheqroun *et al.,* 2006). For this reason, we tested the ability of these strains to protect tomato plants against canker caused by the pathogen *Cmm* 1616-3in greenhouse.

In plants treated every 15 days by Ach 2-1 and/or 2066-7 strains, the reduction of bacterial canker (R%) was similar (39.99%). This proportion was 60% when a mixture of both strains was used; hence it seems that the interaction of the combination of strains assisted in biocontrol of bacterial canker (Amkraz *et al.,* 2010). However, in plants where the treatment frequency was higher (30 days), the Ach 2-1 strain contributed to the reduction in the incidence of canker by only 20%, while the 2066-7 strain led to a reduction of 60%. This could be due to the ability of 2066-7 strain to colonize the roots and compete against other microorganisms through antibiosis. These results were similar to those found by other researchers using bacteria isolated from tomato root or tomato rhizosphere (Amkraz *et al.,* 2010). Indeed, the incidence of bacterial canker on *L. esculentum* cv. Campbell 33 was 11.5 % in the case of RN 39and RN 69 isolates when used in combination, whereas it was 21.5% or 23.5% when the two strains were used separately.

Otherwise, promising results were obtained when using Ach 2-1 and 2066-7 strain for biological control of *P. carotovorum* pv. *carotovorum,* causative agent of soft rot in potato (Faquihi *et al.,* 2015). In addition, another strain of *A. pullulans* (Ach1-1) was involved in biological control of *Penicillium expansum*, causative agent of blue mold, postharvest disease of apples and it showed impressive growth inhibition of this phytopathogen (Bencheqroun *et al.,* 2006).

Recently, Sadik *et al.,* (2015), showed that the *Pantoae agglomerans* 2066-7 strain has an important effect against *Pseudomonas marginalis, Pseudomonas viridiflava, Pantoea ananatis* and *Xanthomonas retroflexus*, postharvest-pathogens of onion bulbs. Indeed, the inhibition percent against these pathogens on culture medium were of 24.78%, 26.66%, 25.5% and 14.44%, respectively. These results were confirmed on wounded bulbs of onion. So, the 2066-7 strain at 10^7 CFU/ml was able to reduce the diameter of the lesions; the percentages reduction were 100% and 62% against *P. viridiflava* and *X. retroflexus*, respectively (Sadik *et al.,* 2015).

These inhibitory effects could be due to antimicrobial substances such as: hydrogen cyanide (HCN), phenazines, pyrrolnitrin, 2,4-diacetylphloroglucinol, pyoluteorin, viscosinamide and tensin that are produced by antagonistic strains (Bhattacharyya and Jha, 2012).

Nevertheless, compatibility between microbial strains is a key factor that must be considered for controlling phytopathogenic bacteria or fungi. For example, a study of the interactions between bacterial strains applied for the biological control of wilt caused by *Fusarium oxysporum* fsp. *radicis* was conducted and showed that the best reduction of radish wilt was obtained when using the *Pseudomonas fluorescens* RS 111 and *P. putida* RE8 strains separately, while suppression of the disease was not as well in the case of their combination. Furthermore, application of a mutant strain of *P. fluorescens RS 111-a* in combination with *P. putida RE8* reduced more effectively tomato wilt (Boer *et al.,* 1998). Hence, when searching for antagonistic or plant growth-promoting microorganisms, it is important to test for the most suitable combination of plant, bacteria and fungi in order to achieve satisfactory plant growth benefits (Jäderlund *et al.*, 2008).

Conclusions and perspective

Improving tomato growth (*L. esculentum* cv. Campbell 33) was studied through involvement *in vitro* and *in vivo* of two microbial strains Ach 2-1 and 2066-7 previously described. Growth improvement was related to the microbial strains, growth parameter, and to the frequency of treatment. In the perspective of biological control of bacterial canker and growth enhancement of tomato, the strain 2066-7 was well adapted to the rhizosphere and root exudates of tomato and significantly decreased

the disease incidence at treatment frequency of 30 days, while the Ach 2-1 strain was effective for growth promotion of tomato, particularly for chlorophyll production, stem diameter and leaf area, hence their importance in field conditions application to study and research their behaviors in rhizosphere containing other microorganism which can be synergistic competitive or antagonistic. Also their dynamics in the rhizosphere are recommended to understand their adaptation in different soil structures. However studies concerning their antibiotic resistance and field applications as effective biocontrol strategies are suggested. Furthermore application of Ach2-1 strains in field condition was carried out and showed its potential in growth and yield increase of date palm (data not shown).

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تحریک رشد گیاه و کنترل شانکر باکتریایی گیاه *Lycopersicon esculentum* L., cv. Campbell 33 به‌کمک عوامل کنترل زیستی

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چکیده: باکتری‌های اندوفیت و اپی‌فیت به‌عنوان عوامل کنترل زیستی بیماری‌ها و افزایش دهنده‌های رشد گیاه شناخته شده‌اند. در این پژوهش، آزمایش‌های درون شیشه‌ای و گلخانه‌ای به‌منظور ارزیابی اثر دو استرین میکروبی *Aureobasidium pullulans* و *Pantoae agglomerans* بر کنترل زیستی شانکر باکتریایی و افزایش رشد گوجه‌فرنگی (*Lycopersicon esculentum* cv. Campbell 33) انجام شد. دو تیمار با فاصله ۱۵ و ۳۰ روز به‌منظور سنجش تأثیر بالقوه آنها به‌کارگرفته شد. دو استرین موجب بازداری از رشد باکتری *Clavibacter michiganensis* subsp. *michiganensis* عامل شانکر گوجه‌فرنگی در شرایط درون شیشه‌ای شدند. هم‌چنین اثرات آنتاگونیستی آن‌ها در شرایط گلخانه تأیید شد. نتایج نشان داد، وقوع شانکر باکتریایی در گیاه گوجه‌فرنگی تیمار شده با *A. pullulans* و *P. agglomerans* (هر یک به‌تنهایی یا مخلوط دو استرین) به‌طور قابل‌توجهی (۱۶٪) در مقایسه با شاهد (۸۳٪) کم‌تر بود. هم‌چنین، تکرار تیمار (با فاصله ۱۵ یا ۳۰ روز) و انتخاب نوع استرین‌ها برای آلودگی (به‌طور مجزا یا مخلوط استرین‌ها) برای به‌دست آوردن نتایج قابل توجه ضروری می‌باشند. به‌طور کلی، هر دو استرین *A. pullulans* و *P. agglomerans* وقوع شانکر باکتریایی را به‌شدت کاهش دادند، به‌خصوص زمانی‌که گیاهان گوجه‌فرنگی با فاصله ۱۵ روز تیمار شده بودند.

واژگان کلیدی: گوجه‌فرنگی، تحریک رشد گیاه، شانکر باکتریایی، عوامل کنترل زیستی