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Biological Control of Bacterial Onion Diseases using a Bacterium, *Pantoea agglomerans* 2066-7

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Abstract: ***Epiphytic microorganisms, isolated from the olive knots, apple fruits and trees, quince, compost and water from different areas were screened for antagonistic activity against Pseudomonas marginalis, Pseudomonas viridiflava, Xanthomonas retroflexus and Pantoea ananatis on onion bulbs. From 77 microorganisms tested for antagonistic properties against bacterial onion diseases, the strain 2066-7 of Pantoea agglomerans was selected. Complete control against Pseudomonas marginalis and Pseudomonas viridiflava at 10^7 CFU.ml^{-1} concentration of 2066-7 and an inhibition percent higher than 90% against Xanthomonas retroflexus and Pantoea ananatis were obtained on wounded onion bulbs inoculated with 10^5CFU.ml^{-1} of pathogens under cold conditions. The inhibitions percent were decreased under 25°C and 30°C.***

Keywords: Morocco, onion bacterial diseases, *Pantoea agglomerans* and biological control

1. Introduction

In Morocco, the onion crop (*Allium cepa* L.) has a high economic importance, representing 11% of nationally produced vegetable crops [1]. This culture is susceptible to a number of diseases caused by fungi and bacteria [2]. Thereby the most onion diseases begin on plants growing in the field and continue to develop on the bulbs during storage and transit, when symptoms become evident [2]. Moreover, bacterial diseases are the most important postharvest diseases. Among whish soft rot bacterial disease caused by *Pseudomonas marginalis* and *Pantoea ananatis*, onion bacterial streak and bulb rot caused by *Pseudomonas viridiflava* and *Xanthomonas* leaf blight caused by *Xanthomonas retroflexus*. These disease accounts for significant levels of postharvest losses. Notwithstanding, disease severity is influenced by climate, crop rotations, drying and storage conditions and disease control measures [3]-[4]. The effective control of postharvest diseases begins with the understanding that these diseases originate in the field. Appropriate cultural practices, including crop rotations, removal of infected onion debris and culls and proper cultivar selection are essential for controlling onion diseases. However, these strategies of control are not efficiency in all conditions. Moreover, the synthetic products has been used as an effective antibiotic; nonetheless, resistance of bacteria to these products has become very common in many production areas [5]-[6]. More importantly, this has also raised concerns about the potential impacts of agricultural use of antibiotics on human health and environment [7]-[8]-[9]. Thus, the biological control of field and storage decay using a microbial antagonist have been developed as potential alternatives to chemicals or as part of integrated crop management systems to reduce the input of pesticides and residues on postharvest fruits and crops.

In previous studies, a screening program was carried out to identify, from different Morocco areas (Tab.1), bacteria and yeasts that would be effective against onion bacterial diseases in onion bulbs. The most effective antagonist was the bacterium *Pantoea agglomerans* 2066-7.

2. Materials and Methods

2.1 Antagonist isolation

Putative antagonists were isolated from olive knots, apple fruits and trees, quince, compost and water from different areas (Tab.1). The samples were placed in sterile conditions, transported to the laboratory and processed immediately. The vegetable samples were washed under tap water, disinfected by being dipped into 2.5% sodium hypochloride for 2.5 mins, rinsed three times with Sterile Distilled Water (SDW) dried on sterile filter paper, and then cut into small pieces in few drops of SDW in a sterile petri dish. The resulted suspension was left to stand for 30 mins, and streaked with a sterile loop onto the surface of Kings B (KB) medium (protease peptone, 20 g; K2HPO4·3H2O, 2.5 g; MgSO4·7H2O, 6 g; glycerol, 15 ml; agar, 15 g; and distilled water, 1 l) [10] and YPGA medium (yeast extract, 5g; peptone, 5g; glucose, 10g; of agar, 18g; and distilled water, 1 l). The petri dishes were incubated at 26°C for 3–5 days. For compost samples, they were suspended in SDW. Single colonies were collected and checked for purity[11].

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Table 1: Microbial antagonists used for the control of soft rot disease of onion bulbs

Region	*Host Plant*	*Code*
Market	Appel	1113-5
INRA-Meknes	Compost	2015-1, 2025-1, 2025-11, 2026-2
Meknes	Olive tree	2027-2, 2066-7, 2066-8
My Drisszarhoun	Olive tree	2074-1F, 2074-1TC, 2074-1, 2077-5, 2077-7, 2077-6
Bouderbala	Olive tree	2083-2
Bouderbala	Quince	2216-11, 2216-2
Bouderbala	Apple	2217-3, 2217-8
Ifran	Quince	2221-12
Midelt	Quince	2234-1
Beni-Mellal	Quince	2236-2
El Hajjeb	Apple	2320-4, 2320-6
Ketama	Olive tree	2321-6, 2321-9, 2321-10
Taounat	Olive tree	2323B-3
Fez	Apple	2328B-7
Meknes	Apple	2330-5, 2330-6, 2330-7, 2331-1, 2331-2, 2331-5, 2331-7
Fez	Apple	2332-3, 2332A-4, 2332A-5, 2332A-1, 2332A-3, 2332B-1
El Hajjeb	Apple	2333-5
Casablanca	Water farm E_1 153955	2340-1, 2340-2, 2340-3, 2340-4, 2340-5, 2340-6
Casablanca	Water farm BA3 153961	2343-1, 2343-2, 2343-3, 2343-4
INRA-Meknes	Apple	ACH $_{1\text{-}1}$, ACH $_{2\text{-}1}$
INRA-Meknes	-	*Bacillus subtilis*
Casablanca	Water A1+2	2342-1, 2342-6, 2342-3, 2342-2, 2342-4
Casablanca	Water farm E_2	2341-2, 2341-1, 2341-3
Fez	Apple	2317-5
Meknes	Onion bulb	2278-9
Fez	Apple	2315-2a, 2315-2c
El Hajjeb	Apple	2339-2, 2339-8, 2339-9, 2339-6, 2339-5, 2339-3, 2339-7, 2339-4, 2339-1

2.2 Production of antagonist and pathogens

For screening of potential antagonists, 77 microorganisms were tested against bacterial onion diseases caused by *P. marginalis*, *P. viridiflava*, *P. ananatis* and *X. retroflexus*. Microorganisms were prepared with three loopfuls of cultures grown for 24–48 h on YPGA medium. For further *in vitro* experiments, antagonist suspensions were prepared by growing cultures in YPGA medium.

Strains of *P. marginalis* and *P. ananatis* were isolated from onion soft rot and *P. viridiflava* and *X. retroflexus* were isolated from symptomatic onion leaves. Desired concentrations were obtained by adjusting the suspension according to a standard curve with a spectrophotometer UV-mini 1240, Shimadzu by measuring the optical density at 420 nm[12].

2.3 Bulbs

Onion reed bulbs, from El Hajjeb region, Meknes, Morocco, were used immediately after harvest.

2.4 Screening potential antagonists

Bacterial confrontation was performed at petri dishes, 77 strains were used for the first screening of antagonist potential (Tab.1). The suspension of bacterial pathogenic strains were spread on YPGA medium. After drying of petri dishes, the pastilles imbibed by suspension of antagonist bacteria were placed in the middle of dishes. The dishes were incubated at 25°C. Results were read after 24h. The inhibition percent was calculated with following reaction [13]:

$$Percent\ inhibition = \frac{\text{Rate without inhibitor} - \text{Rate with inhibitor}}{\text{Rate without inhibitor}} \times 100$$

2.5 Secondary Screening

The isolate used in this study was *P. agglomerans* strain 2066-7, which provided the most effective results in the preliminary experiments.

To determine the minimum effective concentration of *P. agglomerans* against bacterial onion diseases, surface-sterilized bulbs were wounded Surface- (3X3X3 mm^3). Then, 25 µl of aqueous suspensions of strain 2066-7 (10^8, 10^7, 10^6 and 10^5 CFU ml^{-1}) were applied to each wound. After 24 h, the wounds were inoculated with 20 µl of an aqueous suspension of each pathogen (10^5 CFU ml^{-1}). Eight bulbs constituted a single replicate and each treatment was repeated twice. Lesion diameters were measured after seven days of incubation, at 7°C, 25°C and 30°C in moist chamber. The test was repeated twice.

2.6 Calculation of severity and incidence

Incidence of disease was calculated as the percentage of plants with symptoms and severity was determined according to a modified numerical scale (0-5) of [14]-[15]-[16], as follows; where 0 represents no symptoms, 1= less than 10%, 2= 11-25%, 3= 26-50%, 4= 51-75%, and 5= 76-100% of the symptoms.

2.7 Statistical analysis

The results were subjected to the Duncan's multiple range test for separation of means using the SPSS 20.

3. Results

3.1 Screening potential antagonists

From more than 150 microorganisms isolated, 77 were tested in primary in *in vitro* screening against onion bacterial diseases. More than 43%, 23%, 17% and 8% of these bacteria showed some antagonist activity against *P. marginalis*, *P. viridiflava*, *P. ananatis* and *X. retroflexus* respectively (Fig.1).

More than 85% of these bacteria showed an antagonist activity with an inhibition percent above than 10%. The

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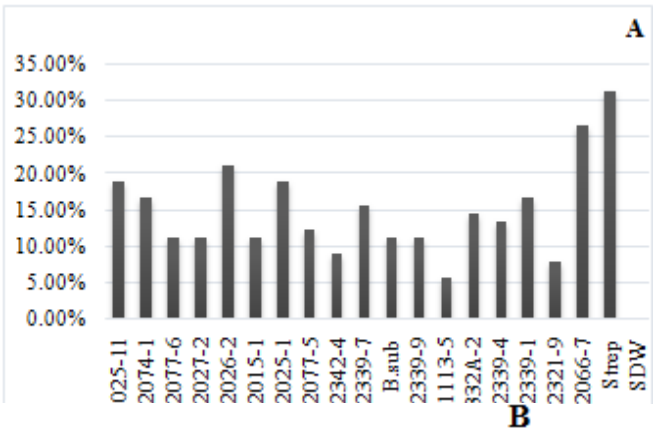
inhibition percent showed that the strain 2066-7 has the most important effect against *P. marginalis*, *P. viridiflava*, *Pantoea ananatis and X. retroflexus* with an inhibition percent of 24.78%, 26.66%, 25.5% and 14.44% respectively; this strain was identified like *Pantoea agglomerans* by the National Centre for the scientific and technical research, Rabat, Morocco.



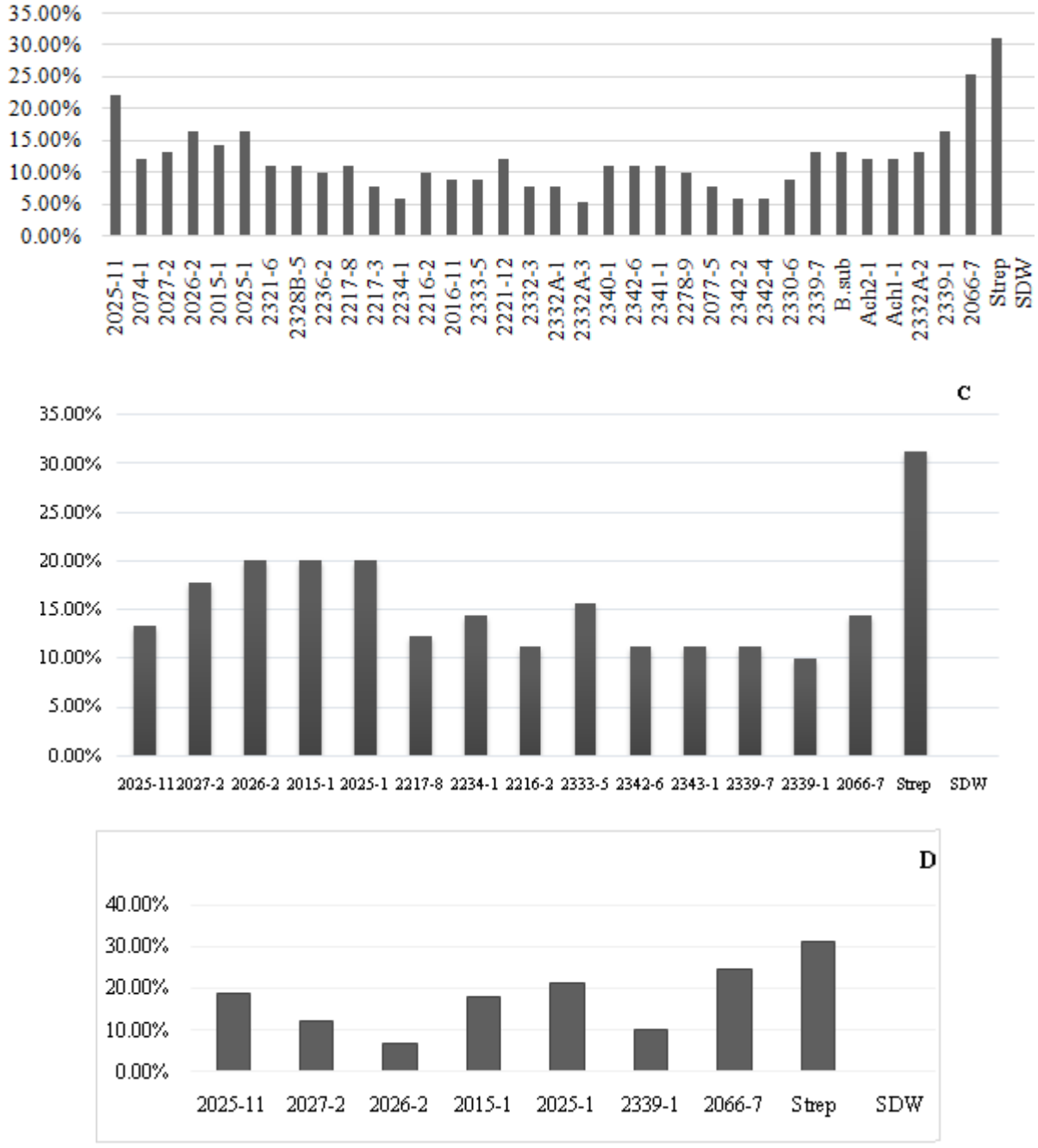


Figure 1: *In vitro* percent of inhibition caused by bacterial antagonists against: (A). *P. viridiflava*, (B). *P. ananatis*, (C). *X. retroflexus* and (D). *P. marginalis*. SDW: Sterile Distilled Water, Strep: streptomycine antibiotic, *B. sub: bacillus subtilis*.

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Secondary Screening

The *in vivo* test showed that strain 2066-7 of *P. agglomerans* degrease the lesion diameter caused by *P. marginalis*, *P. viridiflava*, *P. ananatis* and *X. retroflexus* (Tab. 2, 3, 4 and 5 and Fig. 2, 3 and 4).

Table 2: Lesion diameters (cm) on onion bulbs protected with different concentrations of *P. agglomerans* 2066-7 and wounds inoculated with suspensions of 10^5 of *P. marginalis*.

Temperature concentration of *P. agglomerans (CFU ml^{-1})*					
0	10^5	10^6	10^7	10^8	
0	0	0	2.675	3.2	7°C
1.92	12	12	12	12	25°C
1.92	12	12	12	12	30°C

Table 3: Lesion diameters (cm) on onion bulbs protected with different concentrations of *P. agglomerans* 2066-7 and wounds inoculated with suspensions of 10^5 of *P. viridiflava*.

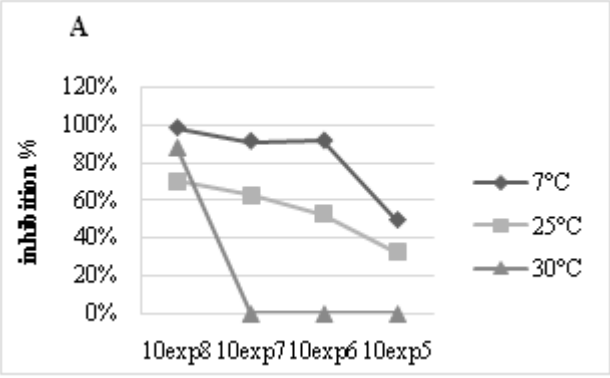
Temperature concentration of *P. agglomerans (CFU ml^{-1})*					
0	10^5	10^6	10^7	10^8	
0	0	0.6875	3.016	14	7°C
0	0	1.0525	10	10	25°C
0	0.9375	1.0625	15	15	30°C

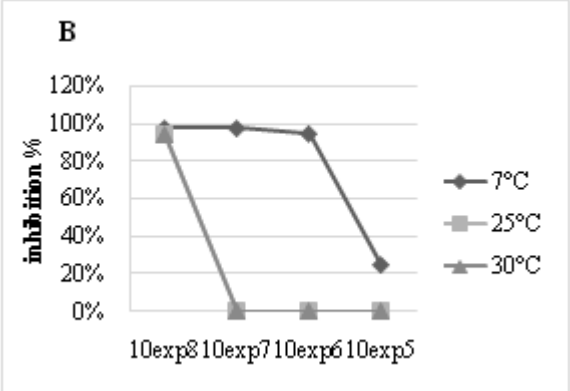
Table 4: Lesion diameters (cm) on onion bulbs protected with different concentrations of *P. agglomerans* 2066-7 and wounds inoculated with suspensions of 10^5 of *Pantoea ananatis*

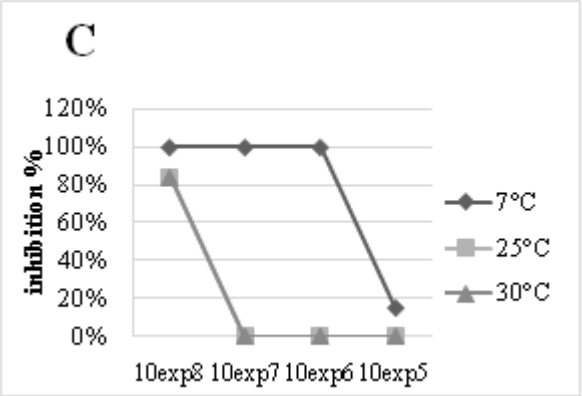
Temperature concentration of *P. agglomerans (CFU ml^{-1})*					
0	10^5	10^6	10^7	10^8	
0.5	0.5	1.0625	1.8375	19.5	7°C
1.205	15	15	15	15	25°C
1.205	15	15	15	15	30°C

Table 5: Lesion diameters (cm) on onion bulbs protected with different concentrations of *P. agglomerans* 2066-7 and wounds inoculated with suspensions of 10^5 of *X. retroflexus*

Temperature concentration of *P. agglomerans (CFU ml^{-1})*					
0	10^5	10^6	10^7	10^8	
0.2375	0.1125	0.8375	4.8625	9.6	7°C
1.125	1.43	1.78	2.55	3.787	25°C
0.3375	2.9	2.9	2.9	2.9	30°C







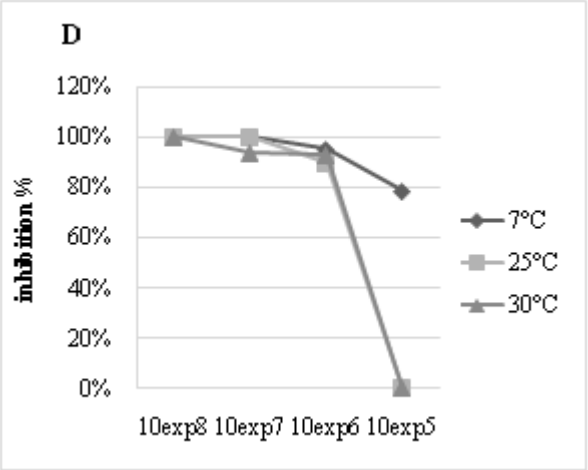


Figure 2: Suppression of lesion diameter caused by (A). *X. retroflexus*,(B). *P. ananatis*, (C). *P. marginalis* and (D).*P. viridiflava* , using *Pantoea agglomerans* (2066-7) with different concentrations. Under 7°C, under 25°C and under 30°C. Onion bulb were wounded (3 x3 x 3 mm). *Pantoea agglomerans* (2066-7) was applied to each wound with concentrations of 10^5, 10^6, 10^7 and 10^8 CFU ml^{-1}, followed by inoculation of the pathogens at 10^5 CFU ml^{-1}.

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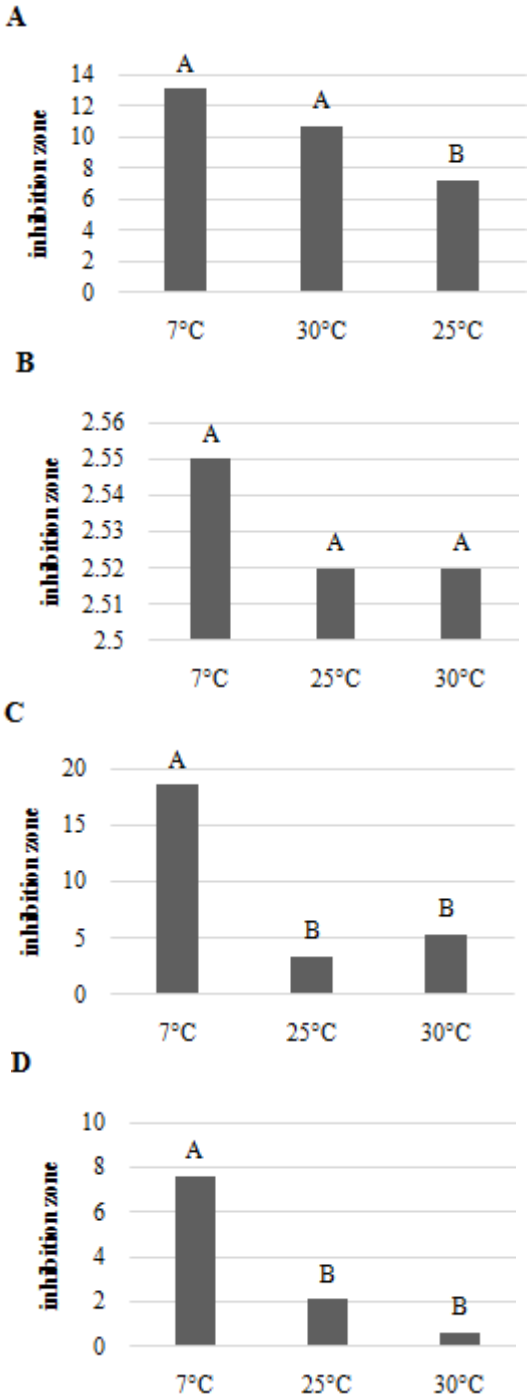

Figure 3: Influence of temperatures (7°C, 25°C and 30°C) on Zone of inhibition caused by *Pantoea agglomerans* (2066-7), with different concentrations, against; (A). *P. viridiflava*, (B). *P. marginalis*, (C). *P. ananatis* and(D). *X. rétroflexus*. Onion bulb were wounded (3 x3 x 3 mm). *Pantoea agglomerans* (2066-7) was applied to each wound with concentrations of 10^5, 10^6, 10^7 and 10^8 CFU ml^{-1}, followed by inoculation of the pathogens at 10^5 CFU ml^{-1}. The statistical analysis was performed by the Duncan's range multiple test. The treatments having same letters are not significantly different (P <0.05).

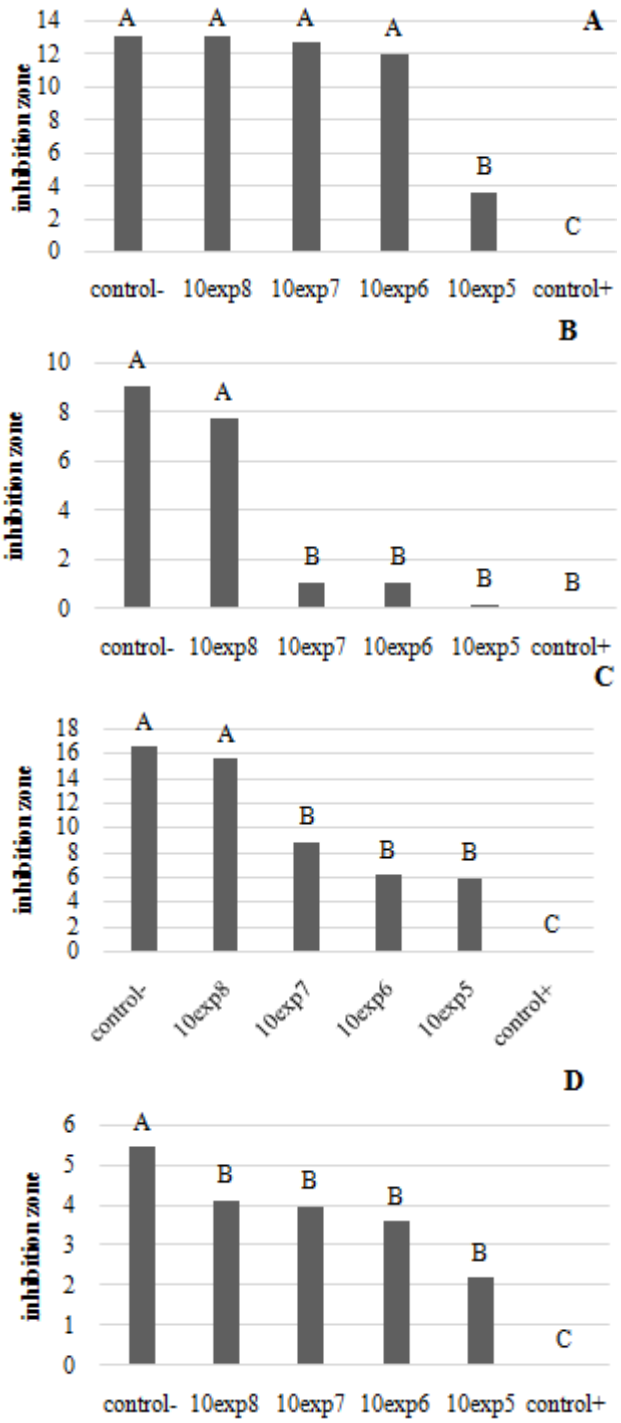

Figure 4: Influence of antagonist concentration on zone of inhibition caused by *Pantoea agglomerans* (2066-7), against; (A). *P. viridiflava*, (B). *P. marginalis*, (C). *P. ananatis* and (D). *X. retroflexus* under 7°C, 25°C and 30°C. Onion bulb were wounded (3 x3 x 3 mm). *Pantoea agglomerans* (2066-7) was applied to each wound with concentrations of 10^5, 10^6, 10^7 and 10^8 CFU ml^{-1}, followed by inoculation of the pathogens at 10^5 CFU ml^{-1}. Different letters in the same row indicate significant differences between means using Duncan's Multiple Range Test (P< 0.05).

Under cold temperature, the antagonist bacterium, *P. agglomerans* (2066-7) greatly inhibited development of *P. ananatis*, *P. viridiflava*, *X. retroflexus* and *P. marginalis* decay (Tab.2, 3, 4 and 5) at all tested concentrations. No lesions developed on onion bulbs protected with *P.*

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agglomerans at the three tested concentrations 10^8, 10^7 and 10^6 UFC.ml^{-1} when 10^5 UFC ml^{-1} of *P. marginalis* was applied (Tab.2). When *P. agglomerans* at 10^5 CFU ml^{-1}was challenged, the reduction of disease was 15.10% (Fig.2).

At 10^8 and 10^7 CFU ml^{-1} concentrations, *P. agglomerans* completely controlled rot development of *P. viridiflava* at 10^5 CFU ml^{-1} (Tab.3). At an antagonist concentration of $10^5$and 10^6CFU ml^{-1}, the inhibition percent were 78.45% and 95% respectively (Fig.2).

At all studied concentrations, *P. agglomerans* controlled rot development caused by *P. ananatis* by an inhibition percent of 97% at 10^8 and 10^7 CFU ml^{-1}, 95% at 10^6 and 25% at 10^5 CFU ml^{-1} (Fig.2).

Against *X. retroflexus* and at 10^8, 10^7 and 10^6 and 10^5 CFU ml^{-1} of *P. agglomerans* an inhibitions percent of 98, 91% and 49.34% were noted respectively (Fig.2).

Under 25 °C and at 10^8 CFU ml^{-1}, *P. agglomerans* reduced lesion diameter and infected wounds of *P. marginalis* by 84%. No lesions inhibition on onion bulbs at others concentrations.

On onion bulbs, all treatments with *P. agglomerans* significantly inhibited development of soft rot caused by *X. retroflexus* (Fig. 2). At the concentration of antagonist of 10^8, 10^7, 10^6 and 10^5 CFUml^{-1} an inhibitions of lesions diameter of 70%, 63%, 53% and 33% were noted respectively. Against *P. viridiflava*, the strain of *P. agglomerans* 2066-7 reduced completely the lesions diameter at a concentrations of 10^8 and 10^7 CFUml^{-1}, at 10^6 CFUml^{-1}the antagonist reduced the lesion diameter whit inhibitions percent of 89%. Although the suppression of lesion diameter caused by *P. ananatis*, with a percent of 94% at 10^8 CFUml^{-1}was noted, no lesion inhibition at others concentrations of *P. agglomerans* was observed.

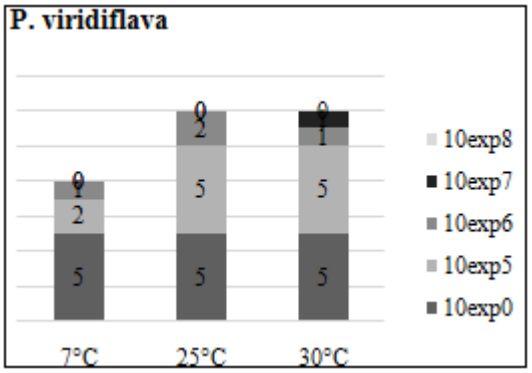
Under 30°C we obtained the same results that we found under 25°C against *P. ananatis* and *P. marginalis* at all concentrations and against *P. viridiflava* at 10^8 CFUml^{-1} of *P. agglomerans*.

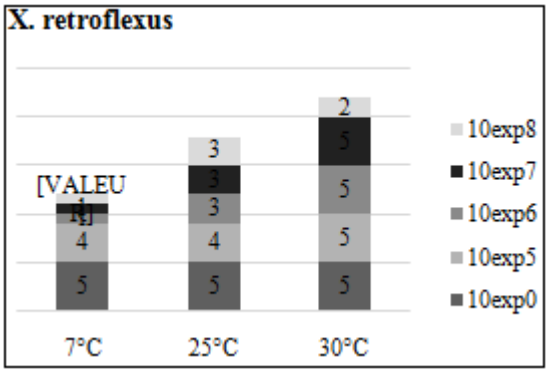
At 10^7 and 10^6 CFU.ml^{-1}, antagonist reduced the lesions diameter, caused by *P. viridiflava*, with 94% and 93% respectively, no lesion inhibition at 10^5 CFUml^{-1}. Against *X. retroflexus* an inhibition of lesion diameter with a percent of 88% was noted at 10^8 CFUml^{-1} of *P. agglomerans*. No lesion inhibition at other concentrations of antagonist was detected.

The variance analysis, using the IMB SPSS statistics 20, showed that pathogen strain and concentration were a highly significant effect on the lesion development diameter. Thus, apropos of concentrations, the difference was significant between high concentration (10^8 CFUml^{-1}) and others antagonist concentrations against *P. marginalis* and *P. ananatis*, no observed difference between the tree concentrations 10^5,10^6 and 10^7 CFUml^{-1}. Against *P. viridiflava* and *X. retroflexus*, no significant difference between the tree concentrations 10^8, 10^7 and 10^6 CFUml^{-1}(Fig. 4). Relating to the temperatures, the best result was obtained under cold conditions (Fig. 3). Thus, against *P. ananatis* and *X. retroflexus*, the analysis showed that we have a highly significant difference between low (7°C) and high (30°C) temperatures, no difference showed between 25°C and 30°C. No different showed between the 7°C and 30°C against *P. marginalis* and *P. viridiflava* (Fig.3).

4. Calculation of Severity and Incidence

The results of the present study showed that *P. agglomerans* 2066-7 strain degrease the severity and incidence of bacterial onion pathogen under all tested temperatures. Thus, the most important effect of antagonist stain was noted under cold temperature (7°C) by a severity of; 0 at 10^6 and 10^7 UFC.ml^{-1} against *P. marginalis* and *P.viridiflava* respectively and 1 at 10^6 UFC.ml^{-1} against the four bacterial pathogen. Under 25°C a severities of 0, 1, 2 and 3 were noted at 10^7 UFC.ml^{-1} against *P. viridiflava*, at 10^8 UFC.ml^{-1} against *P. ananatis*, at 10^8 UFC.ml^{-1} against *P. marginalis* and at 10^6 UFC.ml^{-1} against *X. retroflexus* respectively. A severity of 0 was noted against *P. viridiflava* at 10^8 UFC.ml^{-1}, of 1 at 10^8 UFC.ml^{-1} against *P. ananatis* and of 2 against *P. marginalis* and *X. retroflexus* at 10^8 UFC.ml^{-1} under 30°C (Fig.5). Apropos of the incidence, the variance analysis showed that it was degreased at high concentration (10^8 and 10^7 UFC.ml^{-1}) under cold temperature (7°C) against the onion bacterial pathogens (Tab.6 and Fig.6and 7).





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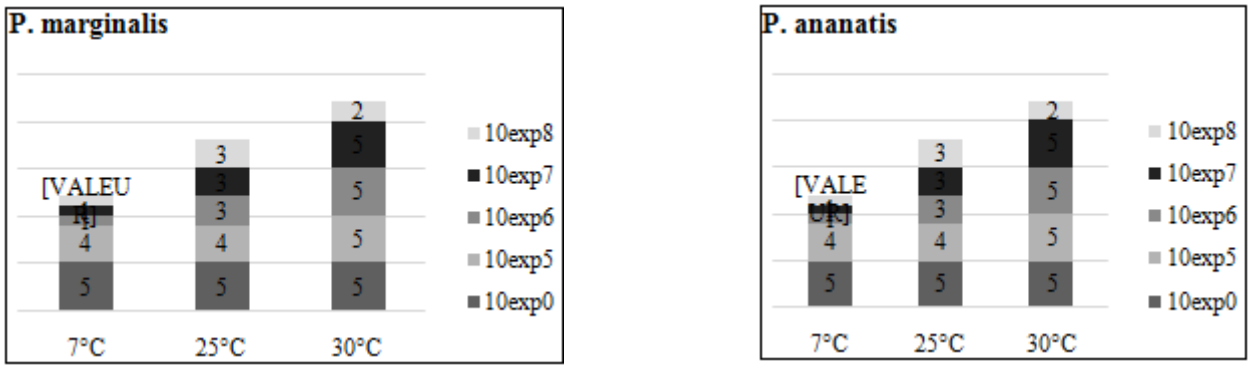

Figure 5: Severity caused by onion bacterial diseases in the presence of differences concentrations of *Pantoea agglomerans* 2066-7.

Table 6: Incidence (percentage of infected wounds) on onion bulbs protected with different concentrations of *Pantoea agglomerans* (2066-7) on the presence of 10^5 UFC $ml^{-1}$of *Pseudomonas viridiflava*, of *Pseudomonas marginalis*, *Pantoea ananatis* and of *Xanthomonas retroflexus.*

*P. agglomerans*2006-7																				
X. retroflexus					*P. ananatis*					*P. marginalis*					*P. viridiflava*					
10^8	10^7	10^6	10^5	10^0	10^8	10^7	10^6	10^5	10^0	10^8	10^7	10^6	10^5	10^0	10^8	10^7	10^6	10^5	10^0	
50	25	75	50	100	25	25	25	50	100	0	0	0	100	100	0	0	75	100	100	7°C
100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0	0	75	100	100	25°C
25	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0	75	75	100	100	30°C

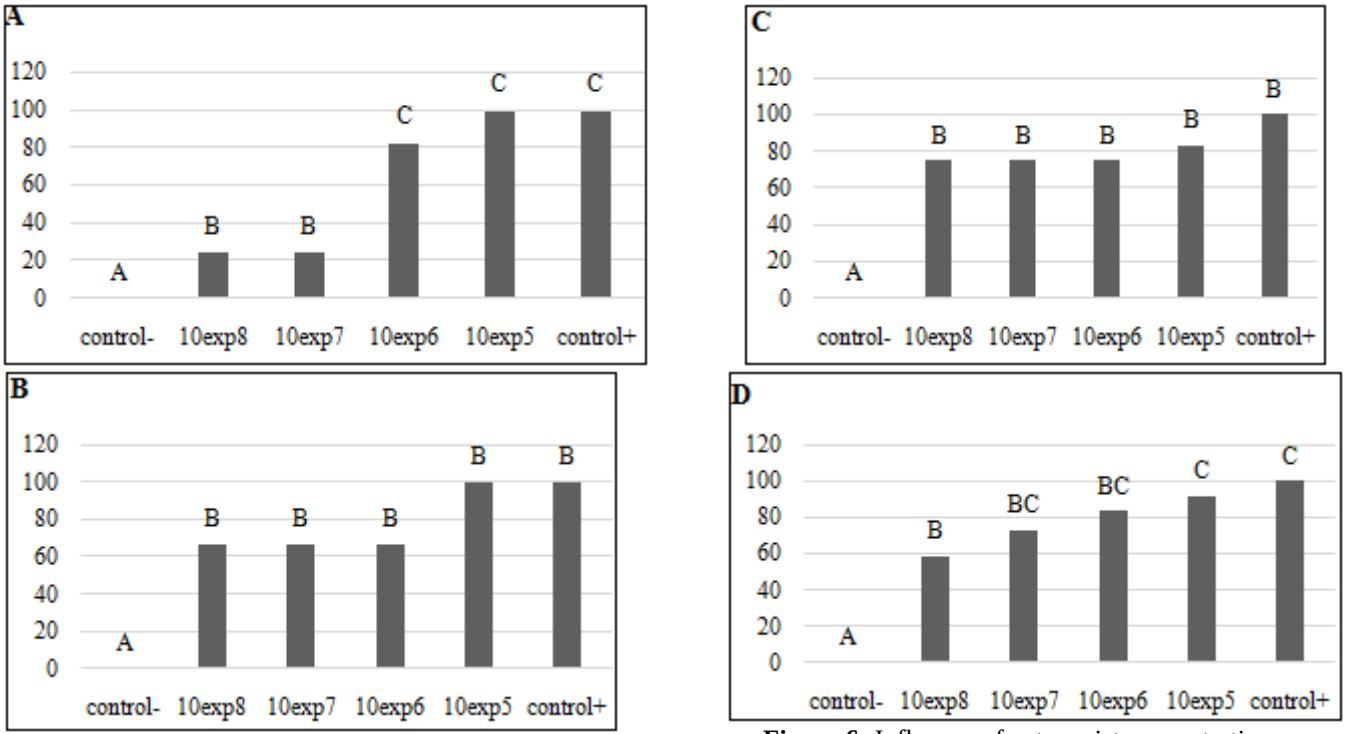

Figure 6: Influence of antagonist concentration on incidence caused by *P. viridiflava* (A), *P. marginalis*(B), *P. ananatis* (C) *and X. retroflexus*(D). The treatments having same letters are not significantly different (P <0.05).

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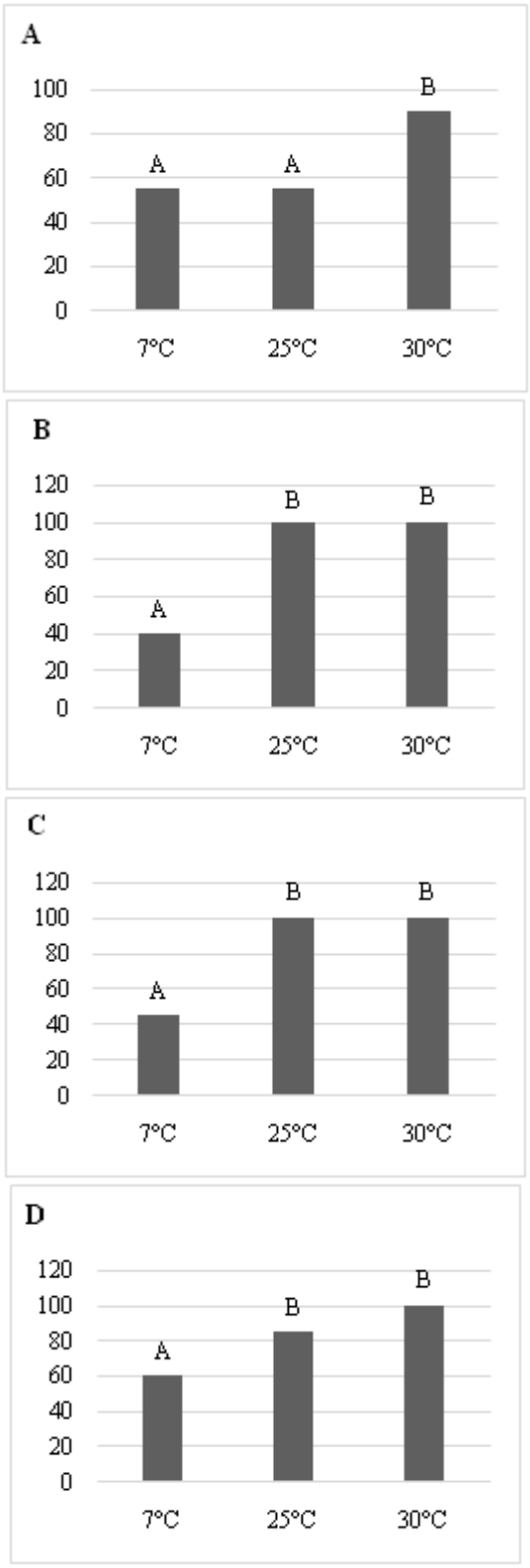

Figure 7: Influence of temperature on incidence caused by *P. viridiflava* (A), *P. marginalis*(B), *P. ananatis*(C) *and X. retroflexus* (D)in the presence of *P. agglomerans.* The tested temperatures were 7°C, 25°C and 30°C. The treatments having same letters are not significantly different (P <0.05).

5. Discussion

From 77 tested microorganisms, only 43%, 23%, 17% and 8% reduced *P. ananatis*, *P. viridiflava*, *X. retroflexus* and *P. marginalis* growth in vitro respectively. Then, numerous studies have indicated a potential for biological control of post-harvest diseases using microbial antagonists [17]-[18]-[19]-[20]-[21]. Moreover, the results of the present study demonstrate that *P. agglomerans* (2066-7) is the most effective biocontrol agent against onion bacterial diseases caused by *P. marginalis*, *P. ananatis*, *P. viridiflava* and *X. retroflexus* with a percent inhibition of 24,78% 25.55%, 26.66% and 14.44% respectively. Thus, strains of *P. agglomerans* have been previously reported as being effective in suppressing bacterial and fungal diseases [22]-[23]-[24]-[25]-[26]-[27]

An important attribute of a successful biocontrol agent is the ability to be efficient at low concentrations [7] and the effectiveness of the biocontrol agent is related to the number of viable cells [20]-[21], and to reach effective control it is sometimes necessary to use very high concentrations of the antagonist.

In vivo and under cold conditions the 2066-7 strain of *P. agglomerans* conformed to this prerequisite by being effective against onion bacterial diseases in Morocco with an inhibition percent higher than 90% at a concentration of 10^6 CFU ml^{-1}, (complete control against *P. marginalis* and *P. viridiflava* at 10^7 CFU.ml^{-1}). Sharma *et al.*, 2009 demonstrated that, for its effective control, a microbial antagonist should have the ability to grow, multiply, and suppress the pathogen at low temperature. These results indicates an excellent adaptation of 2066-7 to cold storage temperatures, a necessary feature for a postharvest biocontrol agent [7]. However, under the temperatures of 25 and 30°C, control of pathogen was reported against the four pathogens only at 10^8 CFU.ml^{-1}. These results confirmed the results obtained by McLaughlin *et al.*, 1990; El-Ghaouth *et al.*, 2004 and Nunes *et al.*, 2001 [28]-[29]-[4], who improve that in general, microbial antagonists are most effective in controlling postharvest decay on fruits and vegetables when applied at a concentration of 10^7–10^8 CFU/ml, and rarely, higher concentrations are required.

The biocontrol activity of microbial antagonists with most harvested commodities increased with the increasing concentrations of antagonists and decreasing concentrations of pathogen, this qualitative relationship, however, is highly dependent on the ability of the antagonists to multiply and grow at the wound site [30]. In this study, we found that *P. agglomerans* 2066-7 strain has an antagonist effect against onion bacterial disease; also, the most important effect of this antagonist was observed under 7°C at 10^8 CFU ml^{-1}.

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