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Aureobasidium pullulans (De Bary) G. Arnaud, a Biological Control against Soft Rot Disease in Potato Caused by *Pectobacterium carotovorum*

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Abstract: ***In Morocco, Pectobacterium carotovorum is well known as causal agents of potato soft rot. Actually, there are no efficient bactericides used to protect potato against Pectobacterium spp. biological control using antagonistic could be an interesting approach to manage this disease. Thus, a collection of thirty antagonists isolated from compost, rhizospheres and endophytes of various crop plants in Morocco were evaluated for their ability to inhibit the phytopathogenic agent Pectobacterium carotovorum in vitro, in vivo and in greenhouse. The in vivo test showed that only ten antagonistic isolates have significantly inhibited the growth of P.carotovorum (P512C9), among them, eight isolates were completely efficient against the strain P512C9. In addition, seven isolates having the greatest pathogen inhibitory capabilities were subsequently tested for their ability to control soft rot potato in greenhouse. The results showed that the treatment by Ach inhibited the development of the soft rot with a reduction of an incidence of disease nearly 100%. The treatments by the others isolates 2331-5, 1113-9, 2066-7, 2339-9, 2321-9 and 2015-1 have reduced up to 70.3%, 64.5%, 87%, 85.9%, 55.6% and 89% the disease of the pathogen (P512C9). The isolate Ach which had the strongest antagonistic activity against P. carotovorum, belongs to the yeast-like species Aureobasidium pullulans (De Bary) G. Arnaud. As well, the treatment with strain Ach has proved a high action on potato plant growth compared to others treatments. In conclusion, the results showed that the black yeast A. pullulans would be an interesting microorganism to be used as a bio-control agent against soft rot disease.***

Keywords: Potato soft rot, Pectobacterium carotovorum, Aureobasidium pullulans, Bio-control, Antagonistic

1. Introduction

Soft rot, a major bacterial disease of potato, is caused by bacteria belonging to *Pectobacterium* sp. [1], which belongs to the family of *Enterobacteriaceae*. This species contains two subspecies *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc)*, *Pectobacterium atroseptium* (*Pa*) [2]-[3]. In Morocco, approximately 95% of the *P. carotovorum* isolated from potato plants with tuber soft rot are *Pcc* [4]-[5]. These bacteria are responsible for significant economic losses to potato each year, since they have the ability to infect the tuber in both the field, and after harvest [6]-[7]. Infection may occur at any stage of post-harvest handling including washing, grading and packing [8]. Effective control methods have not yet been developed. Various strategies have been developed to control plant diseases, such as chemical antibiotics and copper, which have been used for many years [9]-[10]. In addition, disease management involving cultural practices, plant activators, and plant resistance genes are extensively used to control various bacterial diseases. However, copper resistance has been reported in many bacterial pathogens, for this reason,

biological control remains the most suitable means to limit losses due to this disease. Therefore, recent studies have reflected a great interest in biological control agents in agriculture, especially by use of antagonistic microorganisms. Recently, numerous studies based on use of antagonistic agents were conducted in order to develope a biological control agent against *Pectobacterium* sp. and revealed significant results such as; *Bacillus subtilis* [11]-[12], *Pseudomonas* sp [13]-[14]-[11], *Streptomyces* sp [15], *Lactobacillus* sp [14].

The goals of this research were to (1) evaluate the bio-control potential of thirty antagonists against *P. carotovorum* in Morocco, (2) investigate the effect of these antagonists on the growth of potato plant as plant growth-promoting rhizobacteria (PGPR).

2. Materials and Methods

2.1 Pathogen Preparation

The pathogen *P. carotovorum* strain P512C9, used throughout this study had been isolated from tuber potato rotted during the 2012 season in Casablanca. The strain was identified as *Pcc* by phenotypical and biochemical tests, confirmed by PCR using the primers Y1/Y2 as *P. carotovorum* and conserved at - 80°C in glycerol 30% in the Laboratory of Virology, Microbiology and Quality / Eco-Toxicology and Biodiversity. The activation of the strain was realized on LPGA medium and was sub-cultured three times prior to use.

2.2 Antagonistic strains

The antagonists used in this study are the yeast *Aureobasidium pullulans* (Ach1-1, Ach et 1113-9) and the bacteria (2331-5, 2321-5, 2027-2, 2236-2, 2321-10, 2330-3, 2322-3, 2066-7, 2321-6, 2216-11, 2321-11, 2015-1, 2339-9, 2321-9, 2077-5, 2328B-3, 2332A-2, 2217-3b, 2074-1TC,

2330-4, 2320-4, 2026-2, 2328B-5, 2339-7, 2217-3a, 2332A-4). These antagonists belong to the collection of laboratory of Phytobacteriology and Biological Control of the National Institute of Agronomic Research (INRA) of Meknes. The strains Ach1-1, Ach and 1113-9 were isolated from the surface of Golden Delicious healthy apples [16], while other strains were isolated from compost and two cultures; olive tree and apple tree (Table 3). The strains were conserved at -80°C in glycerol 30%, the reactivation was carried out on PDA and LPGA media for yeast and bacteria respectively and were sub-cultured at 25°C, three times with a 24h interval.

2.3 Antagonistic Activity Test

2.3.1 Test for antibiosis

In vitro test for the ability of the thirty one antagonistic strains to inhibit growth of *P. carotovorum* strain (P512C9) was carried out. Briefly, the bacterial suspension of P512C9 strain with a cell density of about 10^8 CFU/ml was spread out on LPGA medium. Excess of suspension was eliminated and the agar plate was dried for 15 min. Once dry, the disks with the cream of antagonistic strains were deposited on the middle and the dishes were incubated at 27°C for 24 h – 48h. The antibacterial and antifungal activity was assayed by observing inhibitory zones in the background of tested strains after 18-24 h of incubation. Each assay was performed in triplicate. Degree of antagonism shown was determined by measuring the average diameter of clear zone of inhibition.

2.3.2 Slices of potato test

The antagonistic activity of the bacterial and fungal isolates was tested *in vivo* using the method "slices of potato" used by [17] with some modifications. In brief, potato tubers (var. Desiree) were soaked in a solution of NaClO (10%) for 10 min and rinsed with sterile distilled water. Then the tubers were aseptically cut into slices (2 cm), in each slice well of 1 cm diameter and 1 cm deep is carried. The potato slices were put in Petri dishes containing sterilized filter paper impregnated with 2 ml of sterile distilled water. Thereafter, 100 µl of antagonistic suspension (10^8 CFU/ml) was injected in the well, and the test was incubated at 27°C for 24h. Afterwhile 24 h, 100 µl of pathogen suspension (10^6 CFU/ml) was added to each treatment. Four repeats for each treatment are chosen with a positive control (P512C9) and a negative control (only sterile distilled water). All the treatments were incubated at 27°C for 24 h.

2.3.3 Pots experiments in greenhouse

To evaluate the effectiveness of the selected antagonists in reducing soft rot potato infection in greenhouse, seven isolates having the largest activity *in vitro* and *in vivo* against *P. carotovorum* (P512C9) were selected for further study. Experiments were carried out in greenhouse to evaluate the suppressive effect of tested strains and its integrated treatments on soft rot. The soil was sterilized in an autoclave at 120 ° C for 20min for two times [18] or in an incubator at 70 ° C for 3 days in order to avoid any contamination. After sterilizing, the soil is put in 26-27cm pots and they are arranged in the direction south-north for soak up the sun for photosynthesis.

The potato seeds (var. Desiree) have been disinfected before use by deceiving in a solution of NaClO (10%) for 10 min and rinsing twice with sterile distilled water. Seed tubers were dipped before planting in an antagonistic suspension (10^8 CFU/ml) for 2 hours, and then they were planted in soil well-drained to a depth of 10 cm. One day after planting, 10 ml of the suspension of pathogen (P512C9) 10^6 CFU/ml was added into each treatment. Thereafter, the second reminder of the antagonistic agent was carried one week after planting. Seven treatments were realized; Ach + Pathogen, 2339-9 + Pathogen, 2015-1 + Pathogen, 1113-9 + Pathogen, 2321-9 + Pathogen, 2331-5 + Pathogen, 2066-7 + Pathogen, positive control (P512C9) and negative control (only water treatment) with five repeats for each treatment. The temperature in greenhouse was maintained at 24°C along the experiment.

In addition, plants were watered daily before reaching the lifting. After emergence, the tubers were irrigated with 500 ml of water every 3 days (the field capacity). Also nutrients such as phosphorus, nitrogen and potassium were added to ensure adequate plants nutrition during mid-growth and tuberization. Data on soft rot incidence was recorded during the crop cycle in October 2013 - February 2014. Number of soft rot infected tubers were recorded and expressed in percentage using the following formula described by [19]:

$$Infection\% = \frac{Number\ of\ infected\ tubers}{Total\ number\ of\ tubers} X\ 100$$

Percentage of disease reduction (PDR) was calculated according to the following formula described by [20]:

$$PDR = \frac{Ack - Atr}{Ack} X\ 100$$

Where Ack and Atr represent the severity of the disease in control and treated specimens, respectively.

2.4 Evaluation of the State of Culture

Seven strains (Ach2-1, 2339-9, 2015-1, 1113-9, 2321-9, 2331-5, 2066-7) showing antagonistic effects against *P.carotovorum* were used to investigate the growth of potato plant. The development of plants was followed by evaluation of parameters of growth and development, these parameters were determined during the stages of culture; Stadium emergence, tuberization and maturation. For this reason, effect of the applied antagonists to vegetative growth was evaluated by measurement the diameter of the stem; measured by a slide caliper "Fisher Scientific HARDENED", the length of the plant with a graduated rule, Leaf area was measured on all leaves cut with almost the same length selected randomly per treatment. After cutting, leaves were placed in plastic bags and were transported immediately to the laboratory. The area of each leaf was measured using planimeter (Bioscientific ADC LTD). Chlorophyll content index was measured per 15 days, in the morning at 11 h, using a SPAD chlorophyll-meter. The leaves were selected on shoots exposed to the north and having approximately equal lengths [21]. In addition, fresh weight, root volume and length were also investigated.

Furthermore, aboveground biomass of each plant has been assessed; briefly, the plants (stems and leaves) were placed in an incubator at 80 ° C for 72 hours, and then were weighed. Caliber of the tuber was determined by a slide

caliper "Fisher Scientific HARDENED", the average weight of tubers is also evaluated.

2.5 Statistical Analysis

Data were subjected to analysis of mean comparisons performed using Wilcoxon test to compare between the treatments by antagonists and the negative control, and between the plants infected by pathogen and treated by antagonists and the plants untreated.

3. Results

In this study, antagonistic microorganisms isolated from the surfaces of fruit and vegetable as well as the compost were used. Thirty probable bio-control agents were tested for their antagonistic property against *P. carotovorum*.

3.1 *In vitro* inhibition

In the *in vitro* tests, 21 antagonists have restricted the growth of the soft rot pathogen of potato, *Pc* strain P512C9. The diameter of inhibition zones ranged from 11 to 25 mm. Maximum inhibition zones of 25 mm diameter was found with strain 1113-9 (Table 1, Figure 1).

Table 1: Inhibitory activity of antagonistic isolates *in vitro* against *P. carotovorum* (P512C9)

Inhibition zone	Antagonistic strain
6 to 10 mm	2332A-2, 2077-5, 2332A-4, 2216-11, 2321-11, 2328B-5, 2321-9.
11 to 15 mm	2328B-3, 2027-2, 2339-9, 2015-1, 2074-1TC, 2339-7, 2217-3b, 2217-3a, 2331-5, 2266-7, 2236-2, 2330-3, Ach.
16 to 25 mm	2330-4, 2321-10, 2321-5, 2322-3, 2321-6, 1113-9.

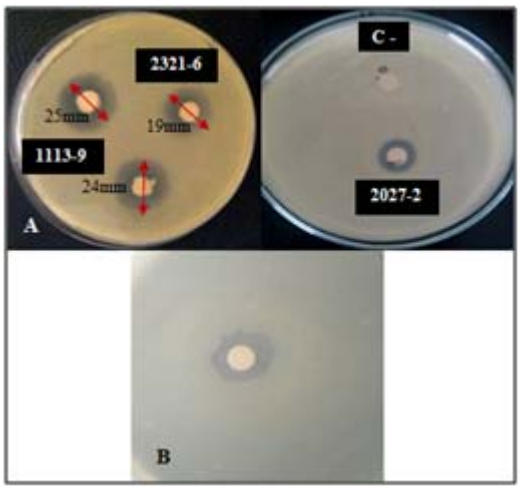


Figure 1: Antagonistic activity of isolates 1113-9 and 2321-6 showing inhibition zones against potato soft rot bacterial strain *Pc* (P512C9). (A) and (B) are representatives of positive inhibition as shown by the encircled inhibition zones, and (C-) is presenting negative inhibition as demonstrated by the no-inhibition zone.

3.2 Slices of potato test

The treatment in *vivo* with the yeast strains and bacteria antagonistic has shown a protection against the development of *P. carotovorum*. For the two yeasts (Ach and 1113-9) and the antagonistic bacteria (2339-9, 2321-9, 2077-5, 2331-5, 2321-5, 2321-6, 2066-7, 2015-1) the protection reached 100%, whereas for other strains the protection was varied between 0% and 75% compared to the negative control and those of the positive control was showed a spongy texture and necrosis at the boundaries of holes (Figure 2).

Figure 2: Confrontation test between *P. carotovorum* and the antagonistic strains on slices of potato. Line 1: positive control (P512C9), Line 2: negative control (sterile distilled water), line 3 and line 5 the antagonistic strains have effect against *Pc*, line 4: antagonist hasn't effect against strain *Pc*

3.3 Essay *in situ* of antagonistic activity against the soft Rot disease

On the basis of the results of the confrontation test on slices of potato, the isolates 1113-9, Ach, 2339-9, 2015-1, 2321-9, 2331-5 and 2066-7 were selected for the control trial in greenhouse against the soft rot bacterial strain P512C9. In our experimental conditions, the essay of biological control of *P. carotovorum* showed that the treatment by Ach inhibited the development of the soft rot and the incidence of disease was reduced by nearly 100%. The treatments inoculated by strains 2331-5,1113-9, 2066-7 ,2339-9, 2321-9 and 2015-1 have reduced up to 70.3%, 64.5%, 87%, 85.9%, 55.6% and 89% respectively compared to inoculation with pathogen strain P512C9 (Figure 3).

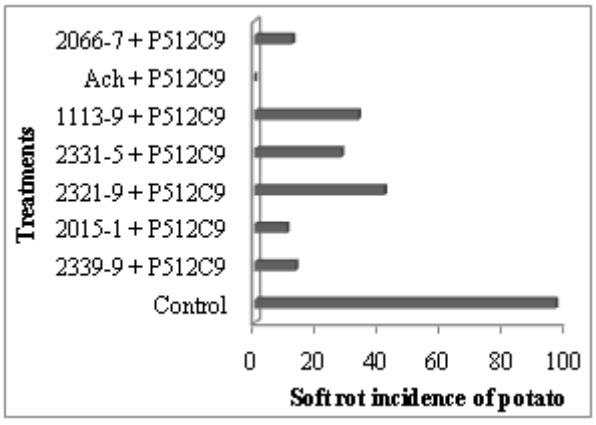
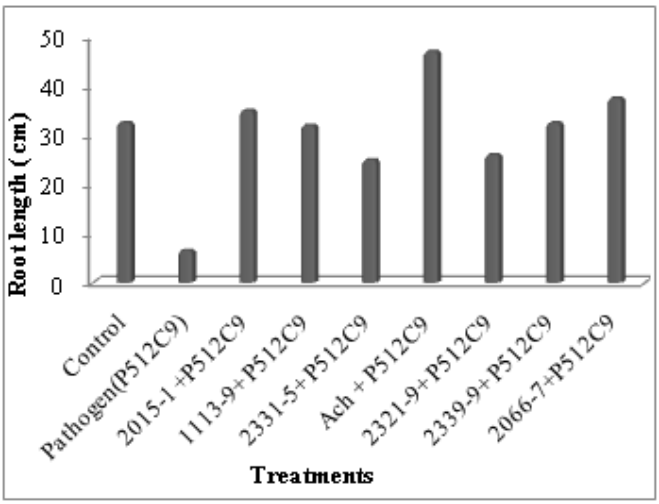
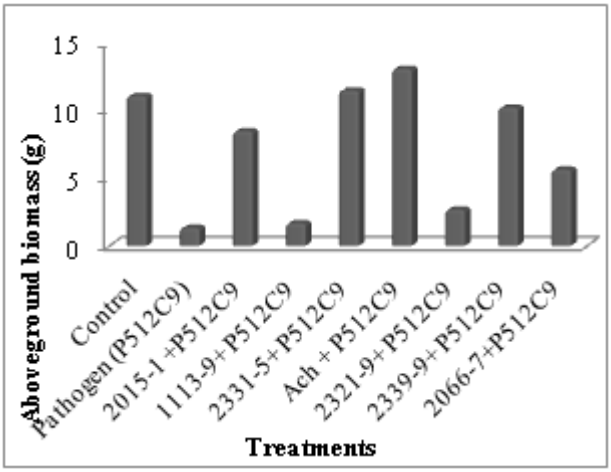
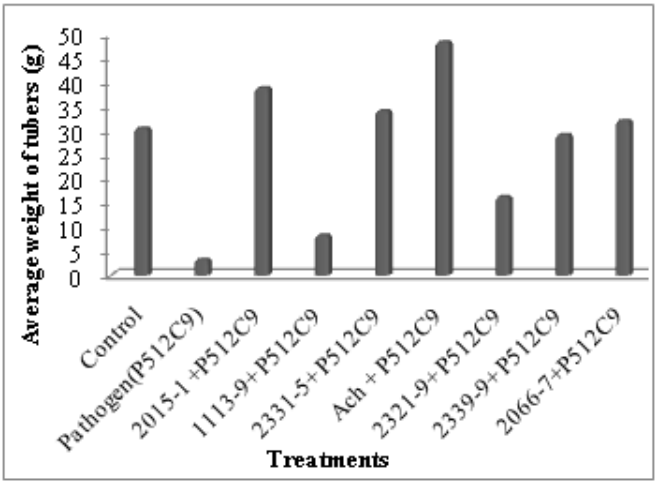


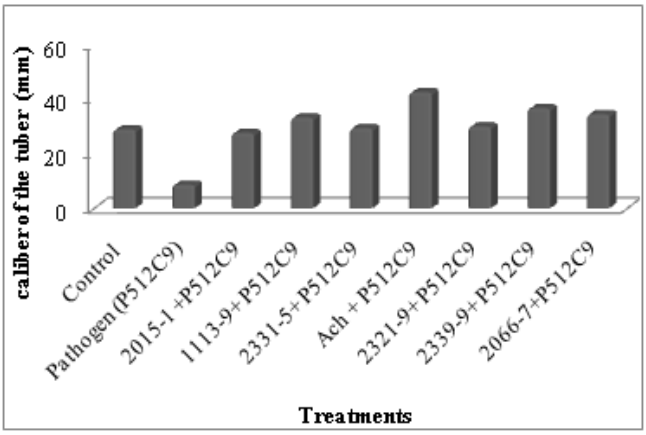
Figure 3: Incidence of soft rot disease on potato in greenhouse experience. Control: pathogen strain of *P. carotovorum* (P512C9).

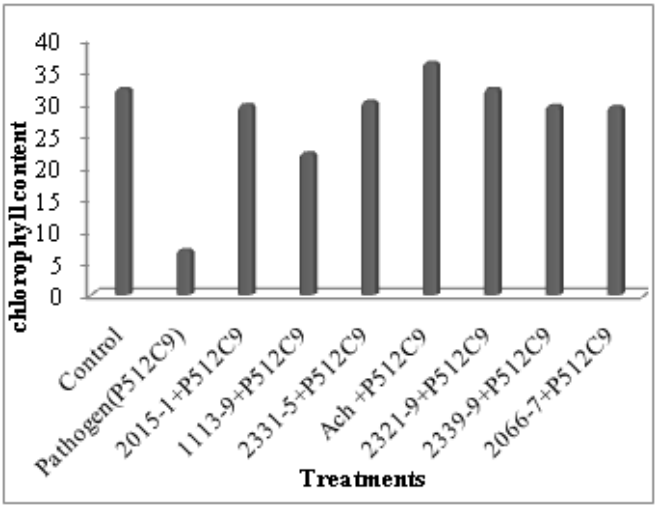
The results of the activity antibacterial and antifungal the antagonistic strains against *P. carotovorum* were showed that all physiologic parameters have a significant difference; this signification has varied from one agent to another (Figure 4). Furthermore, the analysis of mean comparisons of the evaluated factors (diameter of the stem, length of the plant, Leaf area, chlorophyll content, aboveground biomass, root volume and length, average weight of tubers and caliber of the tuber) have revealed a effect of the treatment on the biological control experiment. In particular, the treatment by the strain Ach has demonstrated that this yeast-like can significantly inhibit the growth of soft rot bacteria in situ. The pretreatment of potato tubers with antagonistic yeast successfully prevented the initial infection and reduce soft rot disease of potato and multiplication of soft rot bacteria. (Table 2).











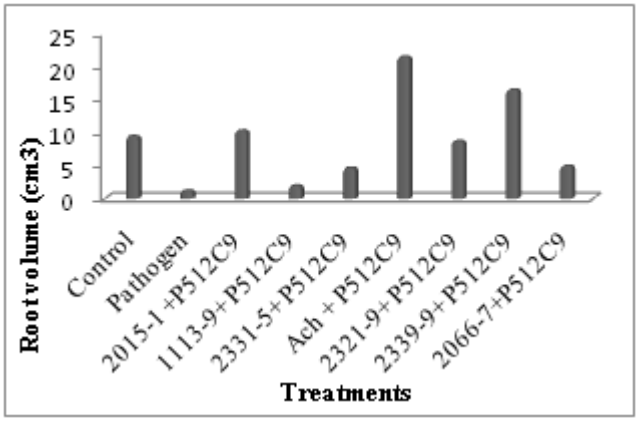


Figure 4: Physiologic parameters to evaluate the activity of the antagonistic strains against *P. carotovorum* in greenhouse.

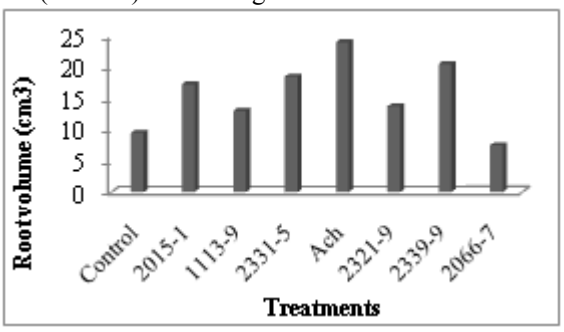
Table 2: Degree of significance of treatment compared to the control pathogen (strain P512C9)

Treatment / Parameter	2015-1 + Pathogen	1113-9 + Pathogen	2331-5 + Pathogen	Ach + Pathogen	2321-9 + Pathogen	2339-9 + Pathogen	2066-7 + Pathogen
Diameter of the stem	0.01	> 0.05	0.01	0.001	0.01	0.01	0.01
Length of the plant	0.04	0.01	0.02	0.01	0.04	0.01	> 0.05
Chlorophyll content	0.005	0.03	0.005	0.001	0.01	0.01	0.01
Leaf area	0.01	> 0.05	0.02	0.001	0.02	0.02	> 0.05
Average weight of tubers	> 0.05	> 0.05	> 0.05	0.02	> 0.05	> 0.05	> 0.05
Root volume	> 0.05	> 0.05	> 0.05	0.05	> 0.05	> 0.05	> 0.05
Root length	0.02	0.05	0.05	0.005	0.01	0.01	> 0.05
Caliber of the tuber	0.01	0.05	0.005	0.005	> 0.05	0.005	0.005
Aboveground biomass	> 0.05	> 0.05	> 0.05	0.05	> 0.05	> 0.05	> 0.05

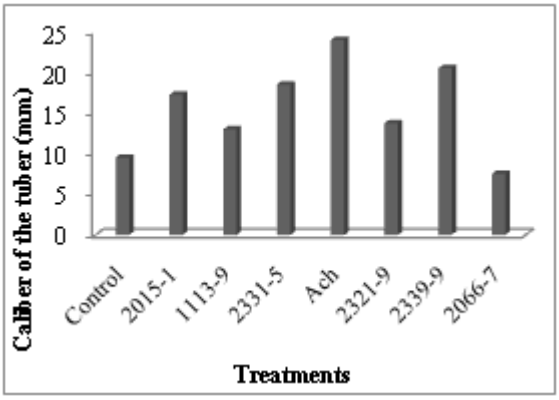
3.4 Effect of PGPR on the potato crop

In aim to evaluate the multiple activities of antagonistic agents to promote the potato plant growth, it was found necessary to control the parameters of the development and growth of the crop; thereby different parameters described previously were measured on the treatments with only the antagonistic strains during the crop cycle in October 2013 - February 2014.

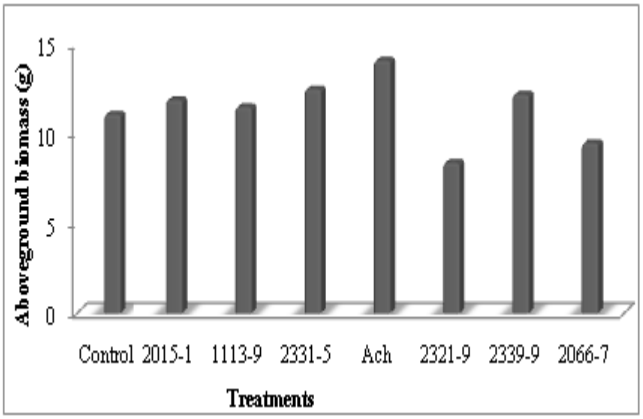
The data of the effect of PGPR strains showed slight differences with regard to the above ground biomass (Figure 5-c), the plant height, the foliar area, and the root length compared to sterile distilled water-treated controls. However, in treatment with strain Ach, the caliber of tuber (Fig 5-b), the root volume (Figure 5-a) and the chlorophyll content (Figure 5-d) weresignificantly higher than that of control. However, the statistical analysis doesn't showed significant results compared to negative control. Except, the treatment by antagonist strain Ach which showed a high degree of significance to the physiological parameters; chlorophyll content (P = 0.03), the caliber of tuber (P = 0.01) and the root volume (P= 0.05) than to negative control.



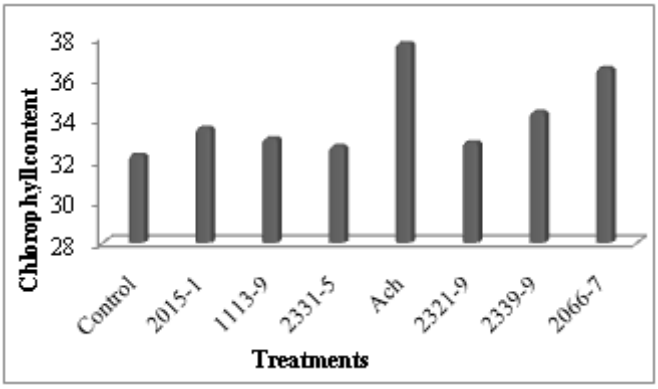
(a)



(b)



(c)



(e)

Figure 5: Effect of the agents benefic (PGPR) on chlorophyll content (d), the volume and length of the root (a-e), aboveground biomass (c) and caliber of tuber (b) evaluated during the cycle of potato crop.

4. Discussion

In Morocco, *P. carotovorum* is a well known pathogen of potato and was detected in many other Mediterranean countries [22]. To evaluate the activity of thirty antagonists (bacteria and fungus) against *P.carotovorum*, three experiments were carried out; the antibiosis test, slices of potato test and *in situ* greenhouse test. Results indicated that the investigated antagonists significantly reduced potato soft rot disease compared to untreated controls. However, among the antagonists, strain 2321-9 had a slight effect against *P. carotovorum*, the strains (2339-9, 2015-1, 2331-5, 1113-9 and 2066-7) presented an average effect. Furthermore the yeast-like Ach showed a high activity, and was the most effective and stable antagonist. This is consistent with data of a previous study in which strain Ach1-1 of same species,

Aureobasidium pullulans, among different potential antagonistic microorganisms isolated from apple surfaces, has shown a protective level of more than 90% against several pathogens, such as*; P. expansum* [16]-[23]. Other strains of *A. pullulans* reported in other works also displayed high antagonistic activities not only against *P. expansum* on apples but also against major postharvest pathogens on several important crops [24]-[25]-[26]-[27]-[28]-[29]-[30].

On the other hand, the results of the present study demonstrated that the antagonistic yeast strain Ach can completely inhibit the development of soft rot disease *in vivo* and *in situ*, with regard to other antagonists (1113-9, 2015-1, 2066-7) that have significantly inhibited the growth of soft rot bacteria *in vitro* (test antibiosis) than the strain Ach. Thus, those data are consistent with several studies reporting the lack of correlation between *in vitro* antibiosis and bio-control. For example, [31] proved that no significant correlation was observed between the *in vitro* growth of *Phytophthora cactorum* in presence of a bacterium and the protection from infection of apple seedlings by that bacterium in the green-house [31]. Additionally, *in vitro* screening for antibiosis is frequently used to select prospective antagonists [32] which may be used for bio-control essays in greenhouse as well in field.

In addition, it is interesting to note that, some strains were isolated from the same fruit and have not prove the same capacity to reduce the soft rot disease. Such as, the strains 2330-3, 2328B-3, 2217-3a, 1113-9, Ach, all were isolated from apple fruit but the yeast-like *Aureobasidium pullulans* (Ach) has demonstrated a high degree of efficacy compared to other presumed antagonistic organisms. Consequently, the present work is in agreement with other previously published research that found those no relationship between the ecological origins where the antagonist was isolated and its effectiveness against diseases. Moreover, the efficacy in the reduction of soft rot produced by *P. carotovorum in vivo* and *in situ* has varied between the strains antagonists [33]-[34]-[35].

In the current work, the effect on the stimulation of the growth of the potato plant by the PGPRs strains was also evaluated. Results showed that the strains tested didn't have significant effects, in particularly *A. pullulans* strain Ach which produce significant differences in variation of chlorophyll content, root volume and caliber of the tubers indicating that the strain Ach had an important effect on the assimilation of nutritive element by plant. In conclusion, the present work has provided strong evidence from both *in vivo* and *in situ* of the bio-control activity of *A. pullulans* strain Ach against *P.carotovorum*. The observed reduction of soft rot by *A. pullulans* is suggesting a complex mode of action still poorly understood. The ability of the yeast antagonistic to outcompete the pathogen for nutrients and space can be one of the mechanisms in the bio-control as previously suggested in other studies. Therefore, further investigation will be carried out in this direction in order to (1) find the main mechanisms involved in the bio-control activity of *A. pullulans* strain Ach against *P. carotovorum*, (2) search for the formulation of *A.pullulans* as biofungicides to control of soft rot disease caused by *P. carotovorum.*

Table 3: list of the antagonistic strains selected, examined for biological control against soft rot of potato

Code of strain	Date of collection	Origin	Sampling location	specie
2015-1	24/11/2011	Compost	Meknès	*Bacillus cereus*
1113-9	15/02/2004	apple	Market	*A. pullulans*
Ach	15/02/2004	apple	Market	*A. pullulans*
2066-7	08/03/2012	olive tree	Taounate	*P. agglomerans*
2339-9	5/03/2013	Apple tree	El Hajeb	-
2321-9	20/12/2012	Olive tree	ketama	-
2331-5	12/02/2013	Apple tree	Meknès	-
Ach1-1	15/02/2004	Apple	Market	*A. pullulans*
2321-5	20/12/2012	Olive tree	Ketama	-
2027-2	3/02/2012	Olive tree	INRA Meknes	*Bacillus cereus*
2236-2	27/06/2012	Cognassier	BeniMellal	-
2321-10	20/12/2012	Olive tree	Ketama	-
2330-3	12/02/2013	Apple tree	Meknès	-
2322-3	20/12/2012	Olive tree	Ketama	-
2321-6	20/12/2012	Olive tree	Ketama	-
2216-11	15/05/2012	Cognassier	Bouderbala	-
2321-11	20/12/2012	Olive tree	Ketama	-
2077-5	03/04/2012	Olive tree	My Driss Zarhoun	-
2328B-3	31/12/2012	Apple tree	Fès	-
2217-3b	15/05/2012	Apple tree	Tifrit-Bouderbala	-
2217-3a	15/05/2012	Apple tree	Tifrit-Bouderbala	-
2332A-2	18/02/2013	Apple tree	Fès	-
2074-1TC	03/04/2012	Olive tree	My Driss Zarhoun	*P.agglomerans*
2330-4	12/02/2013	Apple tree	Meknès	-
2339-7	5/03/2013	Apple tree	El Hajeb	-
2328B-5	31/12/2012	Apple tree	Fès	-
2026-2	3/02/2012	Compost	INRA Meknes	-
2332A-4	18/02/2013	Apple tree	Fès	-
2320-4	12/12/2012	Apple tree	El Hajib	-

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