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# Evaluation of Phytopathogenic Effect of *Pectobacterium carotovorum* subsp. *carotovorum* isolated from Symptomless Potato Tuber and Soil

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author BA performed experimental work, the statistical analysis and wrote the first draft of the manuscript. Author MT revised, completed the manuscript, author SA revised the final manuscript and author MME designed, supervised and managed the work. All authors read and approved the final manuscript.*

***Research Article***

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## ABSTRACT

**Aims:** The comparison of the four combination method to detect and isolate the *Pectobacterium* spp by using or not the step enrichment and evaluation the effect of the bacterial concentration and assessment date to symptoms expression on the infected plant potatoes.
**Place and Duration of Study:** Laboratory Virology Hygiene and Microbiology, department Biology, Faculty of Sciences and Techniques, University Hassan-II Mohammedia Casablanca.
**Methodology:** One hundred fifty samples were collected from symptomless tubers and twenty eight soil samples from the target field. Four combination procedures were performed with or without step enrichement by using DPEM and DIECA to detect and isolate *Pectobacterium*. The strains were isolated in the CVP (Cristal Violet Pectate) medium after enrichement step with DPEM. The strains S603TM3, S603TM5,P603A1,

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P303AH2, P303T1, P303K2, P303K4, P303MN2, P603GH2 were identified by biochemical and pathogenecity tests, the confirmation species was performed by PCR using primers Y1 and Y2. During this study, one strain was choice to evaluate the capacity of the *Pectobacterium* to develop symptoms non–emergence (NE) symptomless plant (SP), chlorosis (Chl); blackleg (Blg); desiccation (Dst).under the following conditions: the combination between the assement date (40 and 70 days) and bacterial concentration ( $10^4$ and $10^8$ CFU/ml).

**Results:** The isolates were performed on medium Crystal Violet Pectate. Their biochemical tests allowed us to conclude that all the isolates belonged to the *Pectobacterium.* The strains were able to develop the soft rot in the slices of potato, confirmed by PCR yielded an amplified fragment of the size (434 bp). The results of the combination test to detect the *Pectobacterium* revealed that the enrichment step was.the most efficient technique for *Pectobacterium* sp isolation comparing to direct plating on CVP medium, 23% of strains were isolated by enriched medium DPEM as compared without enrichment step 13%, detection of *Pectobacterium* sp was improved by adding Sodium Diethyldithiocarbamate (DIECA). The results of the combination conditions assessment date and bacterial concentration show no effect between the concentration $10^4$ and $10^8$ CFU/ml to develop the symptoms, in the otherwise, chlorosis and blackleg symptoms attenuated and increased after 70 days, the desiccation symptom was also significantly affected by an assessment date.

**Conclusion:** The detection and isolation of *Pectobacterium* from asymptomatic potato tubers were improve by enrichement step. Some of these strains were belong to *Pectobacterium carotovorum* subsp. *carotovorum*. The development and expression symptom (no emergence, chlorosis, blackleg and dessication) of *Pectobacterium* in the potato plant is not correlated with assessment date and inoculums level. Otherwise, some physiological and environemental conditions can affect their development.

*Keywords: Pectobacterium spp; enrichement step; development of symptoms; potato plant.*

## 1. INTRODUCTION

In many production areas, microbial contamination by the plant pathogen belonging to the *Pectobacterium* species may be serious constraint to potato production and commercialization. These phytopathogens can cause tuber soft rot and can also result in the occurrence of various field symptoms, including reduced emergence, chlorosis, wilting, stem rot, blackleg, desiccation and plant death [1]. Tuber rotting can also develop both after harvest and from storage because *Pectobacterium* is often carried as latent infection in tubers [1,2]. This can cause further spread of infection and can lead to reduced yields and increased production cost. These microorganisms can cause many different symptoms within a host, as described above [2,3,4]. Under wet conditions, they move out of the xylem and macerate parenchymatous tissues resulting in blackleg symptom. In contrast, wilting chlorosis and desiccation of plant happen predominantly under dry conditions [5,6,7]. Epidemiological studies have shown that potato seed tubers are the major source of inoculum and contamination [8,9]. The objectives of this paper were thus (i) to isolate and identified the causal agent of bacterial soft rot disease on potato and combination were performed with DPEM and DICEA to increase detection the *Pectobacterium* in the symptompless potato tubers (ii) to coordinate the effect of inoculum level of *Pectobacterium carotovorum* subsp. *carotovorum* and assessment date with the symptoms in the potato plant cultivar (cv Desiree), to this and two bacterial concentration ($10^4$ and $10^8$CFU/ml) and two date assessement (40 and 70 days) were used.

## 2. MATERIALS AND METHODS

### 2.1 Plant Material

One hundred fifty symptomless tubers samples cultivar Désirée (cv Désirée) and twenty eight soil samples from the potato's field culture were collected. Tubers were washed in tap water, dried at room temperature for 1h and stored at 4ºC.

### 2.2 Bacterial Strains and Conditions Culture

Tubers were cut with a sterile knife and grinded by mixer. four combinations A, B,C, D were performed under the following condition: combination A (20 g of mixture + 10 ml of Phosphate Buffer Saline (PBS) (Sigma-Aldritch), combination B (20 g of mixture + 10 ml of PBS + 0,2% Sodium Diethyldithiocarbamate (DIECA) (Sigma-Aldritch), combination C (20 g of mixture + 10 ml of Phosphate Buffer Saline (PBS) + 10 ml of DPEM) and combination D (20 g of this mixture + 10 ml of Phosphate Buffer Saline (PBS) + 0,2% Sodium Diethyldithiocarbamate (DIECA) + 10 ml of Double Pectate enrichment medium DPEM $MgSO_4.7H_2O$ 0.32 g/l, $(NH_4)_2$ $SO_4$ 1.08 g/l, $K_2HPO_4$ 1.08 g/l, Sodium polypectate 1.62g/l. The combinations A and B were agitated for 1 h at room temperature. The combinations C and D were incubated under anaerobiosis conditions for 48 h at 27°C. After incubation, the suspension was diluted in PBS medium and 0,1ml from diluted suspensions ($1/10^4$ and $1/10^5$) were placed on CVP medium. After the enrichment step, performed by using Double Pectate Enrichment Medium (DPEM), the strains of *Pectobacterium* were placed on Crystal Violet Pectate (CVP) medium [5], additionated with 0.1 % tryptophane. The characteristics isolates were transferred on King B medium or LPGA Agar medium for growth and purification. The strains were stored in Brain Heart Infusion broth (Biokar diagnostics) supplemented with 20% glycerol at -80ºC (Biokar Diagnostics).

### 2.3 Biochemical and Physiological Tests

Pectate lyase tests were performed in M63 glycerol minimal medium supplemented with 15% glycerol and 2% Polygalacturonic Acid (Serva Heidelberg, FRG), 0.003 % tryptophane and 0.0002% thiamine, [10,11]. Indeed, the non fluorescent colonies on King B medium (Biokar diagnostics) were transferred on M63 plates. In each plate was added acetate solution 8% after 48h at 27ºC. Pectolytic colonies were surrounded by clear haloes (Fig. 1). These pectolytic isolates were subjected to biochemical tests to identify the *Pectobacterium* species (Experiments were done according to the methods described in Laboratory Guide for Identification of Plant Pathogenic Bacteria [12,13].

### 2.4 PCR Experiments

DNA extraction was carried out according to the protocol of Li and Boer 1995. Two specific primers Y1 and Y2 (5'TTA CCG GAC GCC GAG CTG TGG CGT3' and 5'CAG GAA GAT GTC GTT ATC GCG AGT3'). PCR products were subjected to electrophoresis in 1.5% agarose minigels. A standard 1 Kb DNA ladder (Promega) was run on each gel as well as positive (reference strain of *Pectobactrerium carotovorum* 132C) and negative controls.

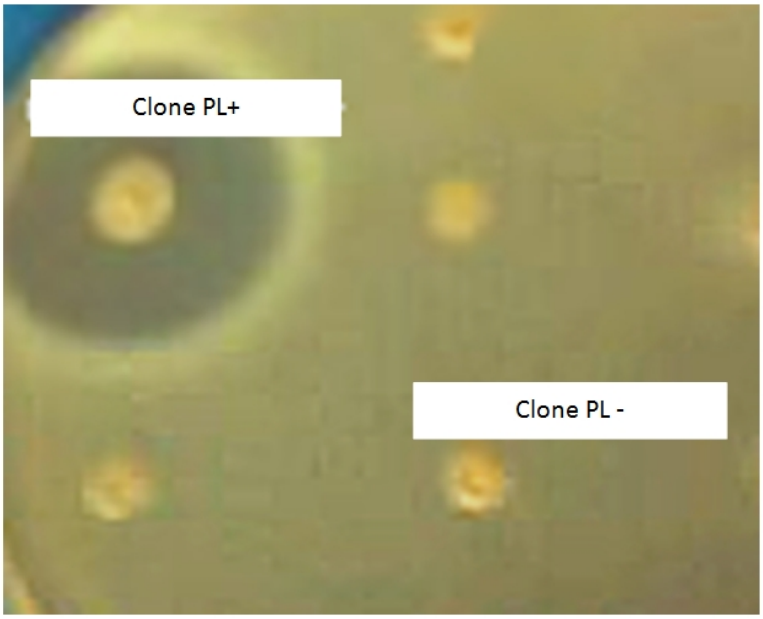


**Fig. 1. Screening test using M63 minimal medium to evaluat the pectate lyase activity (PL) of *Pectobacterium* sp (PL). The PL+ clones (positive pectates lyase activity) were surrounded by a clear haloes**

## 2.5 Analysis of Symptoms Diversity on Potato Tubers

### 2.5.1 Design of the experiment

This experimental design was a 2x2 factorial arranged as a randomized complete block design. The effect of inoculums level of P303AH2 ($10^4$ and $10^8$ CFU/ml) and assessment date (after 40 and 70 days), on disease incidence and development caused by *Pectobacterium carotovorum* were studied. Each treatment consisted of 15 replicates and each experiment was repeated three times. The sand was neutralized in 8N HCl for 1 h and rinsed with sterilized distilled water three times. It was dried and autoclaved at 121°C for 20 min. Approximately 2,5 kg of sand was used in each pot.

### 2.5.2 Inoculation of tubers

Certificated potato seeds tubers cultivar Désirée were vacuum infiltrated with bacterial suspension ($10^4$ or $10^8$ CFU/ml) of the strain P303AH2 tubers were inoculated as described previously by [14]. Five liters were needed to totally cover seed tubers in the bucket [7]. Each seed tuber was covered with a thin layer (3 cm) of sand and pots are placed in plastic bag. The controls seeds were inoculated with PBS buffer solution. All pots were placed in a greenhouse and were irrigated with Hoagland's solution [15].

### 2.5.3 Symptom assessments

The symptoms were recorded as follows: Non–Emergence (NE) Symptomless Plant (SP), Chlorosis (Chl); Blackleg (Blg); Desiccation (Dst).

## 3. RESULTS

### 3.1 Detection of *Pectobacterium* sp

The Results showed that the enrichment step was the most efficient technique for *Pectobacterium* sp detection comparing to direct plating on CVP medium (Table 1). More positive organisms (23%) were obtained by enriched medium DPEM compared without enrichment step (13 %). It should be noted that the addition of an antioxidant, Sodium Diethyldithiocarbamate (DIECA), to tuber mixture improved the detection of *Pectobacterium* sp. Indeed, incubation in PBS buffer containing DIECA followed by an enrichment method was more sensitive for *Pectobacterium* detection: *Pectobacterium* sp isolates averaged 30% of the cultivable organisms compared to 23 % obtained after incubation with PBS only.

**Table 1. Rate of detection the *Pectobacterium* sp isolated from rotted potato tuber cultivar Désirée**

| | **Enrichment step in DPEM** | | **Step without enrichment (Direct plating)** | |
|---|---|---|---|---|
| | **plating after incubation in PBS + DIECA** | **Plating after incubation in PBS** | **after incubation in PBS + DIECA** | **after incubation in PBS** |
| Rate of detection | 46/150 *(30 %)* | 35/150 (*23 %)* | 25/150 *(~17 %)* | 19/150 *(~13 %)* |

*DIECA: Diethylcarbamic Acid, DPEM: Double Pectate Enrichment Medium, PBS: Phosphate Buffer Solution*

### 3.2 Isolation and Biochemical Characterization

The strains (S603TM3, S603TM5, P603A1, P603AH2, P303T1, P303K2, P303K3, P303K4 P303HN2, P603GH2) were isolated from soil and potatoes tubers. To compare their bacteriological test, they were Gram-negative, anaerobic facultative, catalase positive and oxidase negative, able to grow on M63 medium. Of the all these strains isolated *belong to Pectobacterium* and majority was identified as *P.carotovorum* subsp. *carotovorum*. The strains designated S603TM3 and P603GH2 were tested and identified as *Pectobacterium chrysanthemi (Pch)*. The isolate P303MN2 was identified as *P. carotovurum* subsp. *odorifera (Pco*) (Table 2)

### 3.3 PCR Confirmation

All *Pectobacterium* isolates were confirmed by PCR The result showed that Y1 and Y2 are able to amplify a fragment of 434 pbcorresponding to *pel* gene from the genomic DNA of all isolates (Fig. 3). Therefore all of the studied strains were identified as *P. carotovorum*.

**Table 2. Biochemical and physiological characteristics of pectinolytic *Pectobacterium* strains**

| | The isolates from potato tubers and soil | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **S603TM3** | **S603TM5** | **P603A1** | **P603AH2** | **P303T1** | **P303K2** | **P303K3** | **P303K4** | **P303HN2** | **P603GH2** |
| **Utilisation of:** | | | | | | | | | | |
| Lactose | + | + | + | + | + | + | + | + | + | + |
| Malonate | - | - | - | - | - | - | - | - | - | - |
| Tréhalose | + | + | + | + | + | + | + | + | - | + |
| α-méthyl-d-glucoside | - | - | + | - | - | + | + | + | + | nd |
| Melibiose | + | - | + | + | + | + | + | + | + | nd |
| D-arabitol | - | - | - | - | - | - | - | - | + | nd |
| Inuline | + | - | - | + | - | + | + | + | + | nd |
| Citrate | + | - | + | - | + | - | + | - | + | nd |
| Maltose | + | + | + | - | + | + | + | + | + | nd |
| **Production of :** | | | | | | | | | | |
| Indole from tryptophan | + | - | - | - | - | - | - | - | - | + |
| **Lecithinase** | - | - | - | - | - | - | - | - | - | - |
| **Reducing substances from sucrose** | - | - | - | - | + | - | - | - | + | nd |
| **Growth on 5 % NaCl** | + | - | + | + | - | + | + | + | + | + |
| **Growth at 37 °C** | + | + | + | + | + | + | + | + | + | nd |

## 3.4 Symptoms Development

Each infected plant and controls were scored for four symptoms: non-emergence, chlorosis, blackleg and desiccation. The effect of inoculum concentration ($10^4$ and $10^8$ CFU/ml) on expression and development the different symptoms was studied (Fig. 2). For control plants treated with PBS (84 % of symptomless plants were obtained), no differences were observed between healthy plant after 40 or 70 days. The difference of symptomless plant was observed between the concentration $10^4$ and $10^8$ CFU/ml after 40 days (F=25,00; P=0,0075) and after 70 days . Results revealed significant effects of inoculum concentrations on symptomless plant after 40 and 70 days. The development symptoms were described as follow: no emergence symptom has not been affected by assessment date (18% and 24% of the plants without ermergence symptom were observed at $10^4$and $10^8$ CFU/ml respectively) after 40 days and 70 days. Data in the Table 3 indicated that there is no effect of inoculum concentration on frequencies of the chlorosis and blackleg symptoms effect. However, for the assessment date, chlorosis symptoms attenuated after 70 days as compared with 40 days. The frequency of plants with blackleg symptom increased at the final assessment (70 days). Analysis of Anova indicated that desiccation was also significantly affected by an assessment date (F=16,00; P=0,0161).

**Table 3. Effect of *Pectobacterium carotovorum* subsp. *carotovorum* to develop the symptoms in the plant potato in plot**

| Plant statue | | | | | | |
|---|---|---|---|---|---|---|
| **After 40 days** | | | | | | |
| **Inoculation** | Parameter | SP | NE | Chl | Blg | Dst |
| **PBS** | Plant frequency (%) | 84 | 2 | 13 | 0 | 0 |
| | Mean | 12,66 | 0,33 | 2 | 0 | 0 |
| | Standard deviation | 0,57 | 0,57 | 1 | 0 | 0 |
| **$10^4$ CFU/ml** | Plant frequency (%) | 11 | 18 | 67 | 4 | 0 |
| | Mean | 1,66 | 2,66 | 10 | 0,66 | 0 |
| | Standard deviation | 0,57 | 0,57 | 0 | 0,57 | 0 |
| **$10^8$ CFU/ ml** | Plant frequency (%) | 0 | 24 | 71 | 4 | 0 |
| | Mean | 0 | 3,66 | 10,66 | 0,66 | 0 |
| | Standard deviation | 0 | 1,52 | 1,52 | 0,57 | 0 |
| **After 70 days** | | | | | | |
| **Inoculation** | **Parameter** | **SP** | **NE** | **Chl** | **Blg** | **Dst** |
| **PBS** | Plant frequency (%) | 84 | 2 | 7 | 0 | 7 |
| | Mean | 12,66 | 0,33 | 1 | 0 | 1 |
| | Standard deviation | 0,57 | 0,57 | 1 | 0 | 0 |
| **$10^4$ CFU/ml** | Plant frequency (%) | 7 | 18 | 51 | 16 | 9 |
| | Mean | 1 | 2,66 | 7,66 | 2,33 | 1,33 |
| | Standard deviation | 0 | 0,57 | 0,57 | 0,57 | 0,57 |
| **$10^8$ CFU/ml** | Plant frequency (%) | 0 | 24 | 51 | 20 | 4 |
| | Mean | 0 | 3,66 | 7,66 | 3 | 0,66 |
| | Standard deviation | 0 | 1,52 | 0,57 | 1 | 0,57 |

*In each treatment 15 potato tubers were inoculated with PBS (Phopsphate Buffer Saline), $10^4$ CFU/mlor $10^8$ CFU/mlof bacterium and the experiment was repeated 3 fold. SP: symptomless plant; NE: non-emergence; Chl: chlorosis; Blg: blackleg; Dst: desiccation*

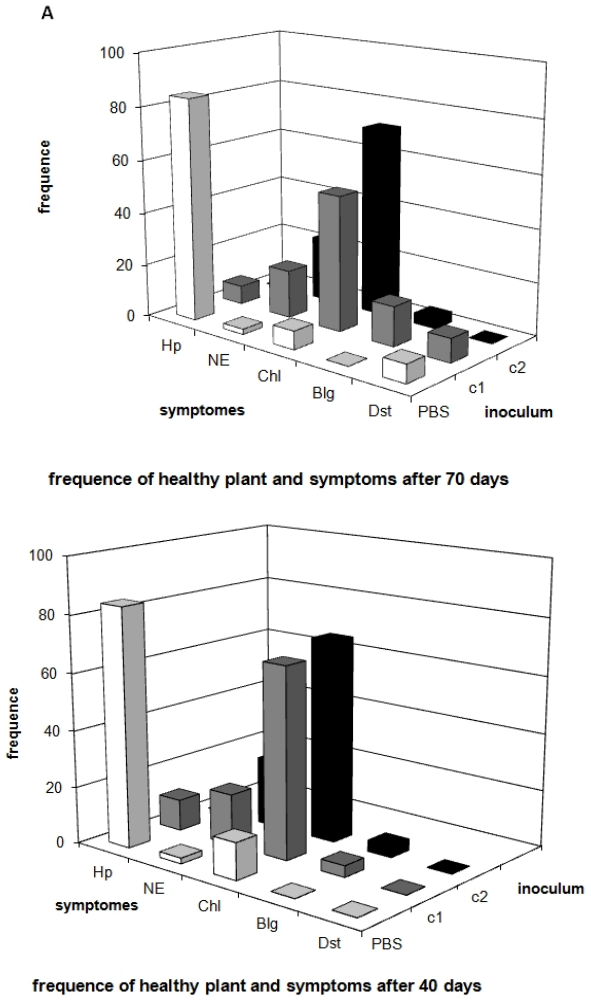


**Fig. 2. Percentage of healthy (symptomless) (Hp) and diseased plants: NE (non emergence);Chl (chlorosis); Blg (blackleg); Dst (Desiccation) . Development of symptoms was recorded after 40 days (A) and after 70 days (B)**

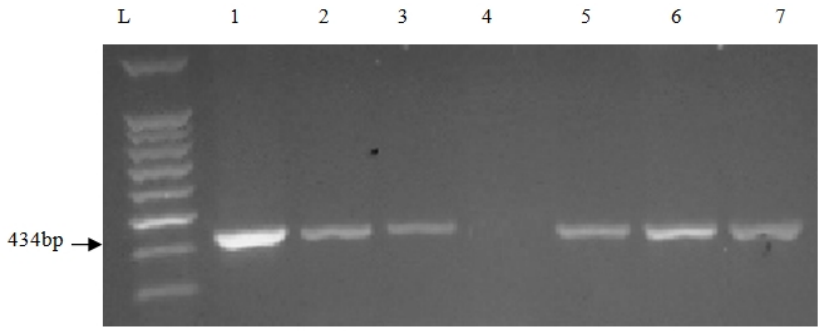


**Fig. 3. Gel electrophoresis in 1.5% agarose of PCR product from genomic DNA with primers Y1/Y2. Lane L, DNA ladder 100bp; lane 4, negative control; lane 5, positive control and lanes 1(S603TM3), 2(S603TM5), 3(P603A1), 6 (P603AH2) and 7(P303T1) isolates of the collection. The arrowhead indicates the 434 bp amplified fragment**

## 4. DISCUSSION

One of the aims of this study is to determine whether Polypectate Enrichment Medium was able to improve *Pectobacterium* detection. Four combinations were tested using DPEM and DIECA. . The highest recovery rates of *Pectobacterium* was obtained by step enrichment with DPEM as shown in Table 1, indicating that the enrichment step was the most efficient technique to isolate *Pectobacterium* comparing to direct plating on the CVP medium. In addition, results revealed that the detection of these target bacteria in naturally infected potato samples was more sensitive when DIECA was added to mixtures; similar observation has been reported by previous studies [16,17]. The enrichment step is a sensitive, easy to perform and cost effective method for the detection of *Pa* and *Pch* in potato plant. In the soil and the irrigation water samples, detection of *Pectobacterium* sp especially *Pa* was frequently improved by an enrichment step [18,19,20,3], because *Pa* will be inhibited by saprophytic bacteria in the same sample. On the basis of biochemical profiles obtained, isolates were identified as *Pectobacterium* sp. biochemical and physiological analyses revealed a considerable variation among *Pectobacterium* isolates. This diversity has been previously reported [21,22,23,24,25,26]. Although there were some atypical characteristics among the strains, they could not be classified as any of the proposed subspecies. One isolate of *P. carotovorum* subsp. *odoriferum* (P603GH2) was isolated from a potato tuber. it is difficult to define its role [27]. These results were consistent with others [12,24],phenotypic diversity of *Pcc* strains will be result from their large host range, their presence in the soil and on the surfaces of plants differently than the strains of *Pca* [2,10,26]. The geographical distribution of *Pca* was systematic influenced by environmental conditions especially the temperature. They are more sensitive to the high temperatures than *Pcc* and *Pch* [19,27,28]. Development of soft rot symptoms was also observed on inoculated potato slices by identified strains. On the basis the pathogenicity tests, these strains revealed that they were pectinolytic, and showed differences in aggressivity against potato tuber. The strains collected from soil were less aggressive than strains isolated from potato tuber. Similar results were reported by other studies [29,9]. In the present work, we followed the development of symptoms in potato plants grown in a greenhouse from seed tubers artificially inoculated with *Pectobacterium carotovorum* subsp. *carotovorum* strain (P303AH2). The pathogenicity biotest provide that symptom intensity did not differ significantly between the two bacterial concentration used. These results differ from those obtained by Helias et al. [1] and Yahiaoui-Zaidi et al. [26]. The authors reported that the inoculm concentration and the date assesement were affected the aggressiveness and the disease symptoms expression in the potato plants. The aggressiveness of *Pectobacterium* is due to several factors, including role of cell wall degrading enzymes, motility, adhesion, lipopolyssacharide, siderophores secrection, quorum sensing, causing the soft rot potato [30,31]. *Pectobacterium* must be able to overcome several barriers present in the potato plants. Otherwise, the plant age, the factors related to cultivar, nutritional status, environmental, physiological, physical or chemical factors can be involved in increased tubers resistance. The others studies were demonstrated that size and maturity tubers affect the susceptibility of potato tubers to soft rot, the smaller potato tubers size and mature were most resistant of disease, which complicates analysis of tuber resistance to maceration. It was showed that the weight of potato tuber can affect the soft rot tubers [32,33]. It was shown that the resistance of foliar late blight of potato has been show to change as a plant age. Potato plants have a defensive arsenal to protect against pathogens infection, they produce defense proteins PR, and they attacked the agent directly or stopped their invasion**,** consequently they decreased their aggressiveness [34,35]. The plant cell wall solidification by lignification (lignin, suberin, phenolic compounds, glycoproteines (hydroxyproline)) may disrupt the growth of the pathogen and therefore the symptoms expression [34,36,37]. Otherwise, Moh et al. [38] has conducted experiments to study the effect of environmental factors influencing the growth of *Pectobacterium* spp on potato tubers.

## 4. CONCLUSIONS

Their results showed a significant effect of the two parameters *aw* (relative humidity) and temperature on the maximum specific growth (μmax) of *Pectobacterium* spp. There is not a linear relationship between the density of the inoculums, disease severity and symptoms development, the disease severity generally increases with the inoculum. The concentration $10^2$CFU/ml can trigger diseases if the conditions are optimum. However, the aggressiveness level of pathogen remains in close relationship with biotic and abiotic conditions.

## ACKNOWLEDGMENTS

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### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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