

Variability of aggressiveness and virulence of *Erwinia carotovora* subsp. *carotovorum* causing the soft rot on potato tubers in the western of Algeria

M'hamed Benada,^{1,2}

Boualem Boumaaza,^{2,3}

Sofiane Boudalia,^{2,3} Omar Khaladi,²

Bettache Guessas¹

¹Laboratory of Applied Microbiology, Department of Biology, Faculty of Natural and Life Sciences, University Oran 1, Ahmed Ben Bella; ²Department of Ecology and Environment, University 8 May 1945, Guelma; ³Laboratory of Biology, Water and Environment, University 8 May 1945, Guelma, Algeria

Abstract

Soft rot symptoms were observed on potato plants in several potato cultivars in the western part of Algeria. A total of four strains of *Erwinia* are divided as follow: i) three strains of bacteria isolated from diseased tissues and soil, identified as *Erwinia carotovora* subsp. *carotovorum* using conventional bacteriological and biochemical methods; and ii) one strain as *Erwinia* sp, not pathogens. *In vitro* tests, on tuber slices were set up to determine slices weight lost, which allows to find differences in cultivar susceptibility and isolate aggressiveness. Among the five cultivars, Laura was the most susceptible than the others tested cultivars. Moreover, it was found that MAI isolate was the most virulent than the other bacterial isolates.

The results of this study should allow an optimization of the potato storage, after considering the susceptibility of a given cultivar to soft rot development and the aggressiveness.

Introduction

In Algeria, potato (*Solanum tuberosum* L.) is the most important crop and is becoming an increasingly important source of food. However, production has been affected by the serious damages caused by the number of fungal and bacterial diseases. *Erwinia carotovora* subsp. *carotovora* is a gram-negative bacterium that causes soft rot disease of economically important

crops, such as potatoes, tomato, broccoli and cucumbers.¹⁻⁴ This pathogen is a wide-spread and economically important disease of potato, which causes soft rot of stem and tubers before and after harvest, and greatly reduces yields.²

Nowaday *E. carotovora* is a problem in the eastern regions Algerian, due to the environmental conditions. Thus, temperature and water availability are two of the main impact on the development of this bacterial disease.⁵ The effect of these factors may result from structural and physiological changes in the plant, increased incidence and severity of diseases when T and RH were highest (20°C and 100%).⁵ The disease also has a negative impact on the storage potential of a crop, which constitutes the major source of primary inoculum in potato crops and a site where the pathogen can remain latent. The disease is related to the level of seed contamination by the bacterium under favorable conditions. Planting of seed tubers contaminated with *Erwinia* can result in reduced emergence, reduction in crop cover and rotting of tubers in the field, all of which can lead to significant yield losses.⁶ The bacteria enter plant tissue mainly in wounds. They spread via and multiply in the intercellular spaces of suberized or parenchymatous tissues. Plant cell walls are degraded and tissue is macerated by Pectolytic enzymes released from the bacteria. Macerated tissues are wet, cream to tan in color, with a soft, slightly granular consistency.

The taxonomy of soft rot bacteria of vegetables has been studied intensively in order to clarify their classification.^{7,8} Gardan *et al.*⁹ proposed that detection of pectolytic activity of *Erwinia* should be included in a separate genus, Pectobacterium. However, both classifications are still valid in the scientific literature. Three bacteria can cause soft rot and can be distinguished by temperatures; *Erwinia carotovora* subsp. *atroseptica* with optimum temperature 20°C can infect potato plants and tubers, *Erwinia carotovora* subsp. *carotovora* and *Erwinia chrysanthemi* with a high temperature of infection 20 to 35°C.¹⁰

The availability of genetic resistance to disease has permitted the production of many crops in areas. However, selection of genotypes is the only control unappropriated for many diseases, for which genetic resistance is the extreme importance in disease resistance and of disease control by means of host resistance. Over the last few years, genetic resistance of plant pathogens have been accepted as a key practice in sustainable agriculture and is one of the best methods to control bacterial pectolytic of

Correspondence: M'hamed Benada, Laboratory of Applied Microbiology (LMA), Department of Biology, Faculty of Natural and Life Sciences, University Oran 1, Ahmed Ben Bella; Department of Ecology and Environment, University 8 May 1945, Guelma, Algeria.
E-mail: mhenada@yahoo.fr

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potato tubers.¹¹ Several studies have demonstrated the ability of several genotypes of potato tubers to possess the source of resistance for all potato diseases. Resistance resources have been identified in most related wild species of potato.

This study aimed to isolate and identify the bacterium associated with recently occurred soft rot on potato in western of Algeria, for one hand to identify the sensitivity to soft rot caused by *E. carotovora* in a collection of five potato genotypes. On the other hand to assess the resistance of selected genotypes to four isolates varying in virulence, using *in vitro* test to determine slices weight lost. Four different bacteria of *Erwinia* on five varieties of potato have been studied. The bacteria have been selected because they are known of their aggressivity and strong resistance to the *in vitro* test.

Materials and Methods

Isolation of bacteria

Samples of potato plants showing symptoms of soft rot were collected in the harvest of potato heads in April and June of 2014. Isolation of the pathogen was carried out using fragments cut out with a sterile scalpel from the border area between apparently healthy tissue and diseased dark brown tissue. The samples were homoge-

nized in a few drops of sterile distilled water using pestle and mortar. After a few minutes, a loopful of resulting homogenate was streaked on nutrient agar medium and incubated for 2-3 days at 27°C.

After purification and different test of identification, the strains are stored in glycerol 30% at -80°C.¹²

Biochemical tests

The bacterial strains that were confirmed as *Erwinia carotovora* by several Biochemical tests. Five biochemical tests were carried out prior to characterization of bacteria which were oxidase, catalase, Gram staining, arginine dihydrolase and facultative anaerobic.

Gram staining

Transfer a loopful of the liquid culture to the surface of a clean glass slide. A loopful of tap water on a slide. Allow the film to air dry. Fix the dried film by passing it briefly through the Bunsen flame. Flood the slide with crystal violet solution for up to one minute. Wash off briefly with tap water (not over 5 seconds). Flood slide with Gram's Iodine solution, and allow acting (as a mordant) for about one minute. Wash off with tap water. Flood slide with 95% alcohol for 10 seconds and wash off with tap water. Flood slide with safranin solution and allow counterstain for 30 seconds. Wash off with tap water. Drain and blot dry with bibulous paper.

Oxidase test

Place a piece of filter paper in Petri dish and add 3 drops of 1% solution of tetramethyl-p-phenylene diamine dihydrochloride. Using a sterile glass rod, remove a colony of test organisms from a culture plate and smear it on the filter paper.

Catalase test

A clean microscope slide was taken. 2-3 mL of hydrogen peroxide was placed at the centre of the slide. Take a colony of test organism with sterile wooden and immerse it into hydrogen peroxide solution. Observe for generation of bubbles. This indicates oxygen production.

Arginine dihydrolase test

The medium of Arginine dihydrolase is sterilized by autoclaving at 120°C for 20 min, distributed in 2 mL in test tubes, and stored at 4°C. Both tubes were inoculated by stabbing the media one half way to the bottom. A control medium without L-arginine was also used. The medium is inoculated by stab and the surface then covered with a 5 mm of sterile liquid paraffin, tightly

capped, and incubated at 35°C for 7 days. The tubes were observed on days 1, 2, 5, and 7 for positive or negative results. A positive test was indicated by a deep pink to red color, whereas a negative test was remained unchanged or turn yellow.

Pathogenicity test

Pathogenicity of the isolated strains was tested by inoculation in tobacco plant (*Nicotiana tabacum*), to know hypersensitivity with the method of Shaad *et al.*,¹³ head tissue fragments. The inoculum was prepared from a 24 h old culture of tested strains grown on nutrient agar (NA) and suspended in sterile distilled water. Concentration was adjusted to approx. 10⁹ CFU/mL of cell density were prepared for verifying their ability to cause soft rot disease. For each isolate, 10 µL of the above-mentioned suspension was inoculated in a petiole by injection. Sterile distilled water was used as a negative control, while *P. carotovorum* subsp. *carotovorum* was used as a positive control. The inoculated plants were kept in a glasshouse (temperature range: 25-30°C, photoperiod: 12 h). Symptoms were examined daily for 3 days. A pathogenicity test on Leaf area (five to six leaves) was performed as described by Wright.¹⁴ After observation of disease symptoms, which were soft rot-like. Necrosis (the tissues dry up and take a light brown color to beige) of the infiltrated area after 24, 48 and 72 h was considered a positive reaction. A reaction positive shows the pathogenicity of the bacterium.

Selection of potato genotypes resistant to soft rot

Potato cultivars (Spunta, Kondor, Laura, Margarita and Desire are commercially variety in Algeria) were selected free of wounds and homogeneous in size. They were stored at 4°C until use. Tubers were surface disinfected by soaking in 70% ethanol, then washed with sterile distilled water. The bacteria suspension was prepared as described above.

Aggressiveness and virulence test

A total of 75 slices with about 5 mm thick were used in this study. The experimental protocol used in this work has been inspired of Haynes *et al.*^{15,16} Each slice was cut in the center of the tuber and then placed in a Petri box containing 1 mL of sterile water. A sterile filter paper of 1 cm² is placed at the wafer center. The initial weight is assessed, and then 100 µL of bacterial suspension (10⁸ UFC/mL) is deposited on filter paper. For the control group, Phosphate Buffered Saline (PBS) is applied in place of the bacterial suspension. Petri box was

wrapped with parafilm to limit gas exchange and incubated at 28°C for 48 h. For this experiment, four strains (SOL, SA, MAI, and JELLY) that exhibited various degrees of virulence were selected (Figure 1).

Statistical analysis

The results were expressed in the form of the mean±SEM (standard error mean). All results were analyzed based on a comparison of the mean and an analysis of variance (ANOVA), followed by the test of Tukey when the conditions of normality and homogeneity of the variances are observed. ANOVAs were performed by considering the effect of *strain*, *variety* as well as the interaction *strain x variety*. Significance was considered at P<0.05 using MiniTab software [Minitab, Ltd, United Kingdom (Version 16)].

Results

Morphological and biochemical characteristics

The isolated bacteria were identified as *Erwinia carotovora* sp. *carotovora* by standard bacteriological techniques; disease symptoms, pathogenicity tests, colony morphology (Figure 2) and biochemical tests. Based on our morphological analysis of the bacterial colonies, the colonies appeared to be shiny and creamy white after 48 h incubation at 27°C. According of these biochemical tests, bacteria obtained from purified colonies, were Gram-negative, oxidase negative, arginine dihydrolase negative, catalase positive, and facultative anaerobic, fermentative in Hugh Leifson and no fluoresces in UV confirmed our bacterial isolates as *Erwinia carotovora* sp. *Carotovora*.



Figure 1. Slices of potato infected with SA strain.

Pathogenicity test

In leaves inoculation test, four bacterial isolates were used. Results of the pathogenicity test showed that within three days after inoculation, yellow halo on the leaves was observed on inoculated tobacco plants (Figure 3). Control plants inoculated with water remained healthy. The strains Sol, SA and MAI are positive result (Presence of necrosis), which reflects that these strains are pathogenic, so have the capacity to cause the disease when they are contacted with host plant in favorable condition. Among these, one isolate JELLY gave a negative result; it is not pathogen strain (Table 1).

Weight loss

After 48 h and after removing the decay from the slices, a second weighing is carried out. The results showed wide variation in susceptibility of potato cultivars to the bacterial inoculation.

Five cultivars had high disease-symptom severity scores when assessed for soft rot resistance when tested with method Haynes *et al.* and Czajkowski *et al.*^{15,16}

Jelly strain is in the last with the same results as negative control, which tends to 0.

The virulence results of *Erwinia carotovora subsp. carotovora* in different varieties of potatoes, we saw there is a significant difference between strains in same varieties and between different varieties; variations among the strains in virulence and sensitivity were significant ($P < 0.05$). The strain MAI is the most virulent than other strains in Spunta and Kondor varieties. MAI strains consistently macerated potato tuber tissue more rapidly than strains of SOL and SA in Spunta and Kondor varieties for three replications of the experiment with 41.93%, 36.43% and 22.09% successively of weight loss in Kondor and 55.23%, 29.53 and 31.15% in Spunta (Figure 4). In virulence, the three strains SOL, SA and MAI induced a susceptible reaction on Desire varieties, while the strain Jelly has the same result than negative control, which tends to 0.

As results have shown, the potato Desire variety was less damaged by an artificial infection of *E. carotovora sp. carotovora* than other varieties. Therefore, this variety is more resistant to the disease. Strains SOL and SA have same degrees of virulent in Spunta and SOL was more virulent and aggressive than SA in Kondor. In Laura and Margarita varieties, the strain SOL is more aggressive than MAI, who is more aggressive than SA with 57.28% of weight loss in Laura and 32.09% in margarita, and SA is more aggressive than Jelly with $P < 0.05$. If we compare the results

between different varieties, we see that the Desire varieties is the most resistant than other varieties (Kondor, Margarita, Spunta, and Laura) with a loss that is at a maximum with the strain MAI, which is 20.21%, and the Laura varieties is the most sensitive, with maximum of loss which is with 55.23% in strain MAI.

Discussion and Conclusions

Bacterial soft rot is one of the most important factors, which cause crop losses in tubers and stem both in the field and greatly reduce yield during storage. Over the last years, these bacteria cause serious damage and are involved in soft rot diseases of several cultivars in Algeria.¹⁷ Due to the inefficiency of chemical control, development of alternative control is great importance. The resistant cultivar is one of the best methods to control bacterial pectolytic of potato tubers.¹¹ The comparison of virulence and aggressiveness of four strains *Erwinia carotovora subsp. carotovorum* was studied by using 5 genotypes of potato tubers arising the western of Algeria.

The present results suggest that four isolates were pathogenic to eggplant with different degrees of aggressive and caused varying degrees of soft rot. As observed by several authors, when a critical number of *Pectobacterium spp.* is reached at the infection site, Ecc produces an assortment of degradative enzymes including pectate lyase isozymes (Pels), polygalacturonase (Peh), cellulase (Cel), protease (Prt) and pectin lyase (Pnl).^{6,18-20} This production is controlled by a quorum-sensing process that relies upon the production of N-acylho-

Table 1. Results of pathogenicity test.

Strain	Result
MAI	+
SOL	+
SA	+
JELLY	-
Negative control	-



Figure 2. A) *Erwinia carotovora* in LPGA medium. B) *Erwinia carotovora* in King B medium in ultra violet radiation.

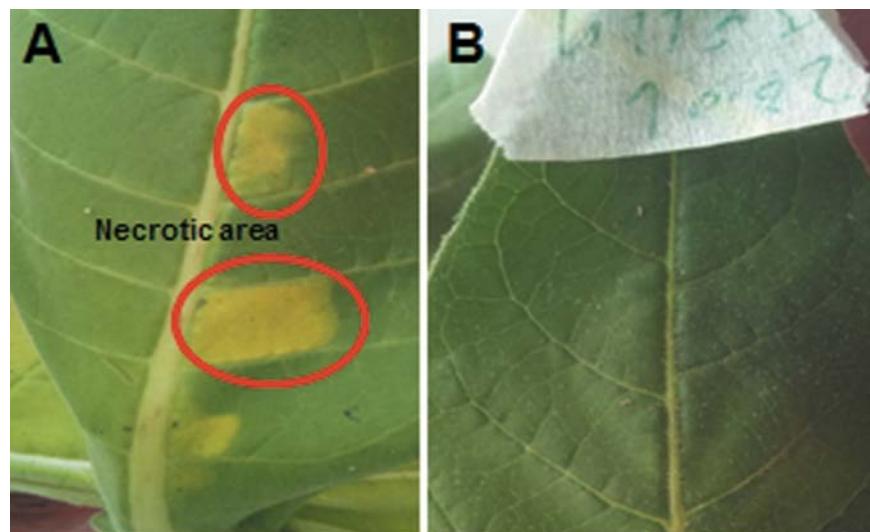


Figure 3. Yellow symptom was appeared on tobacco leaves after inoculation with Pa isolate; A) Positive result; B) Negative result.

moserine lactones (HSL).^{12,21}

In pathogenicity tests, variation in host resistance (potato) and in the ability of pathogens (*Erwinia carotovora* subsp. *carotovorum*) to infect and aggressiveness are essential underlying factors influencing soft rot disease. In previous studies, it is interesting to note that, some strains were isolated from the same tubers and have not proved the same capacity to produce the soft rot disease, such as the strains JELLY. These results were in line with those of Smith *et al.*,²² showed that significant difference ($P \leq 0.05$) were found among the strains for aggressiveness on tuber but the relative aggressiveness in one host was not always associated with aggressiveness or pathogenicity in another host.

Because of artificial inoculation, four isolates were chosen, three isolates (SOL, SA, and MAI) were highly pathogenic,

while one isolate (JELLY) were no pathogenic. However, in our tests, the pathogenicity of certain isolates differed between cultivars, and varied among isolates that were isolated at the same time. Among potatoes variety, Desire presented the lowest mean of the weight loss showing the effect of this variety of resistance to soft rot. In fact, the ability to macerate potato tuber tissues at 27°C was lower for Desire, than for other varieties. This variety is grown in most regions of western Algerian and our results have shown that this pathogen is less widespread in these areas. The resistance of tuber tissue of cultivars Desiree to soft rot caused by PCA isolates could be related to the presence of phenolics compounds Wegener and Jansen,²³ in coloured potato. Our results observed in agreement with the conclusions of several authors.²⁴⁻²⁶

In the present research, the inability of

some strains (JELLY) to induce disease can be explained by the absence of enzyme production, particularly pectate lyase (PL). Maher and Kelman²⁷ show that inability of the bacteria to degrade the lignified in tuber tissue is more related to the toxic substances, such as phenolics and phytoalexins could inhibit pectolytic enzymes of *Erwinias*.

In addition, the genotypes Kandor, Margarita, Desire, Spunta, and Laura have been determined as the most resistant to strains JELLY, which corresponds as potential sources of soft rot resistance; highlighting the need for continuing studies to identify sources of non- pathogenicity.

In conclusion, the results presented here show that Pcc strains can induce disease symptoms on potato and other vegetables. The resistant and sensibility of variety against soft rot caused by *Erwinia carotovora* subsp. *carotovora*, remains one of the means of fighting this disease, with using the resistant varieties who is the subject of our study. Among varieties used in Algeria, five of them were tested; which are Spunta, Kondor, Laura, Margarita. The Desire variety is the most resistant against soft rot disease; also, we think that we must use this variety in potato growing in Algeria.

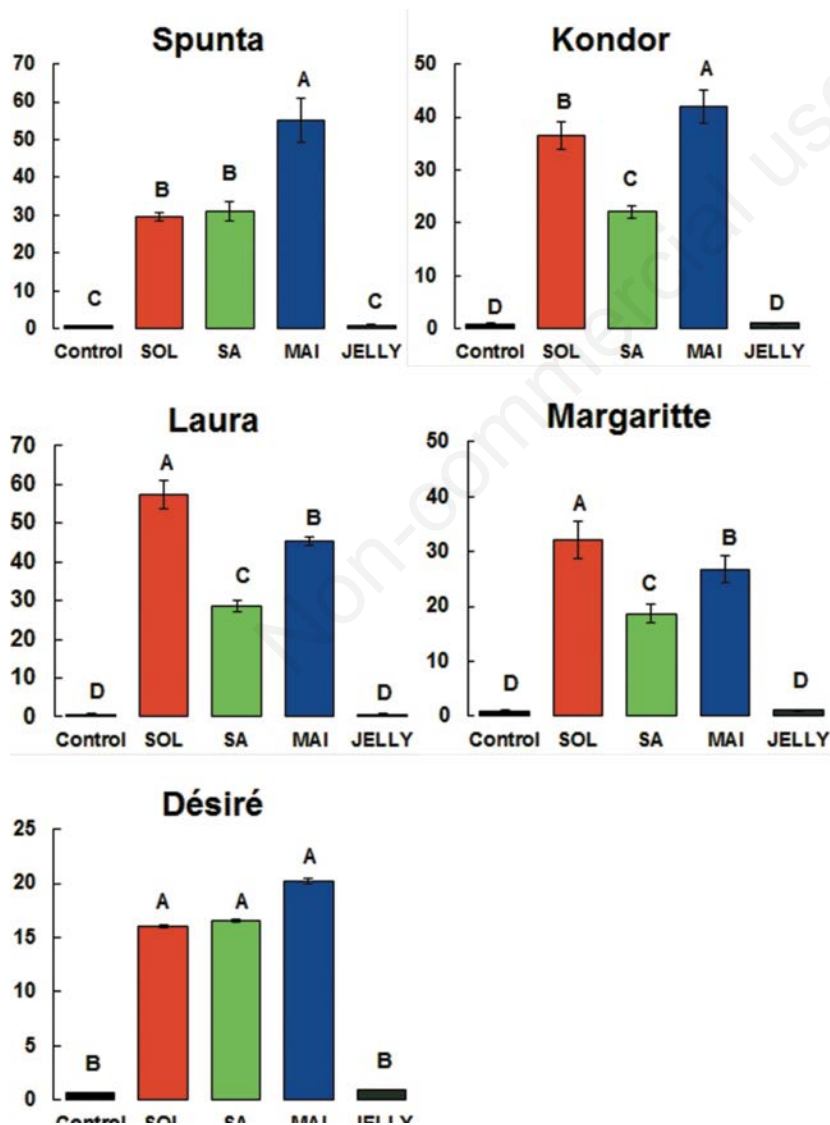


Figure 4. Aggressiveness of strains *E. carotovora*, measured as a weight loss of tubers (%).

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