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Prevalence of the Pectolytic Enterobacterial Diseases in the Major Potato Producing Regions in Morocco

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Abstract: *The main objective of this study is monitoring the prevalence of blackleg and tuber rot caused by pectolytic enterobacteria in potato crop of moroccan regions known by differences in climatic conditions. During the years 2014 to 2016, 107 potato fields were surveyed. Samples were collected from tubers and plants suffering from soft rot and blackleg, respectively. In the fields surveyed, prevalence varies from 0 to 14%. In the same region, prevalence could vary from 0 to 9% (Haouz). In the fields where certified seed was used, the average of prevalence didn't reach 12% while it reaches more than 28% in regions where non-certified seed was used. The biochemical and molecular studies of the strains isolated from the samples collected show a high biodiversity between the strains of the collection. This study shows that in Morocco, there is not a correlation between the climatic conditions and the degree of severity of the diseases.*

Keywords: *blackleg, climatic conditions, prevalence, potato, soft rot*

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I. INTRODUCTION

Potato (*Solanum tuberosum*) is the only vegetable listed among the five principal world food crops [1-2-3]. Currently, more than a billion people eat potatoes regularly [4]. Although potatoes have great potential to produce high yields per unit area [5-6], they are prone to a wide range of diseases that drastically reduce yield and quality [7-8]. Of particular importance are pectolytic enterobacteria which cause stem and tuber rot in potato [8]. What makes the disease more difficult to control, is the fact that the symptoms developed on the plant are not strain specific but are dependent on the climatic conditions prevailing at the infection stage [9]. The pathogens infect the crop in the field, in transit and in storage. Seed piece decay, blackleg, and aerial stem rot occur in the field while soft rot affects the crop in the field, in transit and in storage [10-11-12]. The type of bacteria that causes these symptoms is divided into several species and subspecies on the basis of molecular, biochemical and host range differences [13-14-15]. The most common pathogens which affect potato are *Pectobacterium atrosepticum* (Pa), *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) and *Dickeya* spp. [16-17]. Among these species, Pa causes tuber and stem rot of potatoes and exclusively infects potato [18]. The other pathogens are considered broad host range pathogens for two reasons. Firstly, they have been isolated from many different plant species and secondly, single strains can be pathogenic to numerous plant species under experimental conditions [19].

The potato is the third most grown crop in Morocco [3-20-21]. Pa is particularly damaging potato crops and thus, this pathogen is economically considered as one of the most important pathogen of potatoes [22]. Different aspects of tuber and stem rot and the causal pathogens have been reviewed. Attention has been focused on the identification and characterization methods [20-23-24], genetic diversity and molecular typing in Morocco [21-25]. However, there have been no studies monitoring the prevalence of these diseases in the different regions of Morocco based on differences in climatic conditions. Several epidemiological studies assure that there is a high correlation between virulence and environmental conditions [18-26-27-28].

Morocco is divided into five main types of climates, ranging from humid to per-arid (Fig. 1). The per-humid bioclimatic stage is not significant and is limited to the crest of Rif mountains (<5km^2) [29]. In the present study the objectives are: (i) Assess the prevalence of *Pectobacterium* sp. isolated from potato in farms in different moroccan regions (Saïs, Loukkos, Doukkala, Haute Moulouya, Haouz, Moyen Atlas, Gharb, Chaouia, Oriental, Sous) and (ii) characterize moroccan Pectobacterium strains on the basis of microbiological, biochemical and molecular methods.

II. Material And Methods

II.1. Potato diseases survey in Morocco

To assess the importance of potato diseases (soft rot and blackleg), specific surveys were conducted in the major potato producing areas in Morocco. During our survey, 107 fields were randomly selected at intervals of about at least five km to record i) the severity of bacterial diseases of all potato fields of the visited regions by scoring the incidence of plants showing the characteristic symptoms of the diseases and ii) the occurrence and the prevalence of soft rot and blackleg diseases depending upon the seeds used were certified or not. The survey was conducted during the main rainy seasons 2014 to 2016 between the months of October and April when most fields had a well established potato crop.

II.2. Sample handling and isolate collection

During the years 2014 to 2016, a survey was conducted in three of the five natural regions in Morocco (arid, semi-arid and humid/sub-humid). Potato production is not concentrated in the other two regions (per-humid and hyper-arid). The survey was carried out in 10 potato growing areas, namely, Saïs, Loukkos, Doukkala, Haute Moulouya, Haouz, Moyen Atlas, Gharb, Chaouia, Oriental and Sous (Fig. 1). Overall, 107 potato fields were surveyed and 375 samples of tubers and stems were collected from plants suffering from blackleg or soft rot. Tubers and stems suspected to be infected by Pectobacterium were taken and 2–3 cm long segments were cut off from the edge of the lesions. The segments were rinsed in sterile water to eliminate the rotted tissue and then squeezed into a tube containing 2 ml sterile deionized water. After shaking for 30 s, one drop of the resulting suspension was spread on LPGA medium in Petri dishes and incubated at 26°C for 24h. After incubation, the colonies showing the morphology of Pectobacterium or Dickeya were isolated and grown in pure cultures [21]. All strains isolated were tested on slices of potato for a test of pathogenicity. A total of 102 Pectobacterium strains were isolated from samples collected during visits. All strains were isolated on LPGA medium.

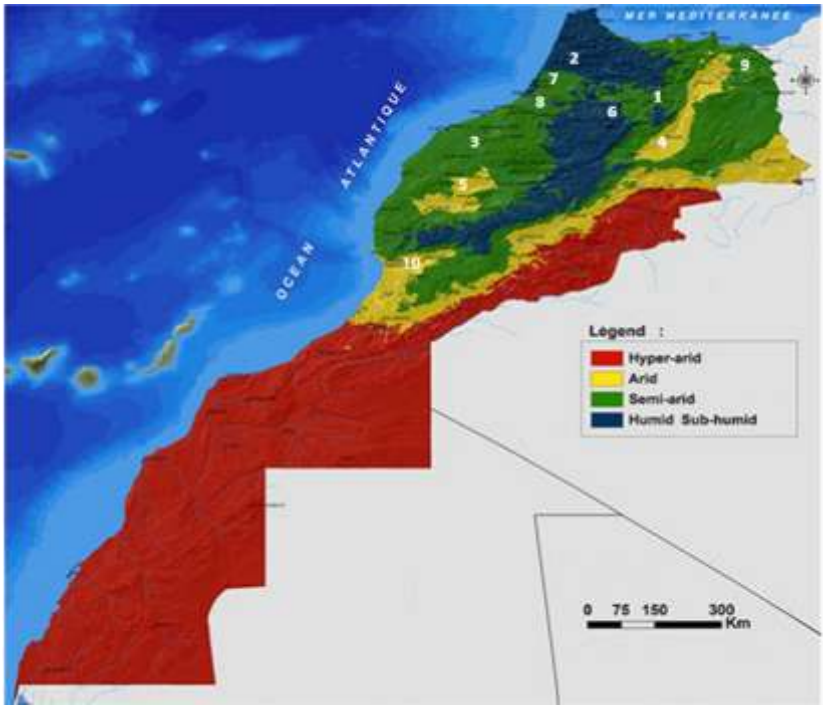

Figure 1. The natural regions of Morocco and the sampling areas (1 to 10). (Adapted from Aridity index of De Martonne)

II.3. Biochemical and physiological tests

To identify Pectobacterium and Dickeya, all strains were tested for Gram reaction (using the KOH test), oxidase reaction, catalase reaction, fermentative reaction, reductase activity of nitrate, and King B medium. All confirmed strains were assigned to species and subspecies on the basis of tests for acid production from lactose, arabitol, sorbitol, melibiose, trehalose and α-methyl-d-glucoside, production of indole from tryptophan, lecithinase activity and reducing substances from sucrose. Ability to grow on 37°C and 39°C and at 5% or 6% NaCl was also tested [14-30-31-32-33].

The control isolates B 1158^T (*P. carotovorum*) and $B1156^T$ (*D. chrysanthemi*) were obtained from the CNRST (Centre National pour la Recherche Scientifique et Technique) of Morocco.

II.4. DNA extraction

Bacterial suspensions in sterile distilled water were prepared from 24h cultures on LPGA medium, and their concentration adjusted to 10^{10} cells ml^{-1} after measuring absorbency at 350 nm on a spectrophotometer (UNICO). Genomic DNA from bacterial strains was extracted using the bacterial genomic DNA extraction kit Promega (Promega, Madison, USA).

II.5. Detection and identification by PCR

Conventional PCR reactions were carried out with a specific PCR assay for primers Y1 (5´- TTA CCG GAC GCC GAG CTG TGG CGT-3') and Y2 (5'-CAG GAA GAT GTC GTT ATC GCG AGT-3') selected from the pectate lyase-encoding *pel* gene sequences of *P. carotovorum* [34]. A specific PCR assay for Pa was performed using primers ECA1f (5'-CGG CAT CAT AAA AAC ACG-3') and ECA2r (5'-GCA CAC TTC ATC CAG CGA-3') following the protocol of [35], with slight modifications. Briefly, PCR reaction mix was carried out in 25 µl containing 2.5 µl DNA, 0.5 U Taq DNA polymerase, 2.5 µl 10 × PCR buffer, 100 µM of each dNTPs, 2 mM MgCl2, 0.5 µM of each primer. DNA amplification was performed on a icycler (BIO-RAD) under the following conditions: 5 min at 95ºC for initial denaturation, 40 cycles of 30 s at 94ºC, 45 s at 62ºC and 45 s at 72ºC, followed by a final elongation step of 8 min at 72ºC. PCR products (6 µl) were separated by gel electrophoresis in 1.5% agarose gels in TBE buffer. Following staining with ethidium bromide, the gels were viewed and photographed under UV transilluminator.

II.6. Data analysis

Data analyses were carried out using the program of R Core Team [36]. The correlation between the prevalence of soft rot and blackleg of potatoes and the climatic conditions was analyzed by one-way analysis of variance (ANOVA). ANOVA was also used to examine the correlation between the prevalence of the two diseases (soft rot and blackleg) and the nature of seeds used (certified or not). The chosen level of significance was $p < 0.05$.

III. RESULTS

III.1. Bacterial diseases of potato crop

The 107 fields surveyed have shown that soft rot, blackleg and wilting occur sporadically in potato production areas. Soft rot and blackleg were particularly present. Infected plant stems show black appearance at the base of the stem that quickly girdles the stem as the symptom expands upward (Fig. 2.a). Tuber soft rot on infected potato exhibits cream to tan and water-soaked surface. This decay can expand rapidly, resulting in rotting tissue that is slimy and water-soaked (Fig. 2.b).

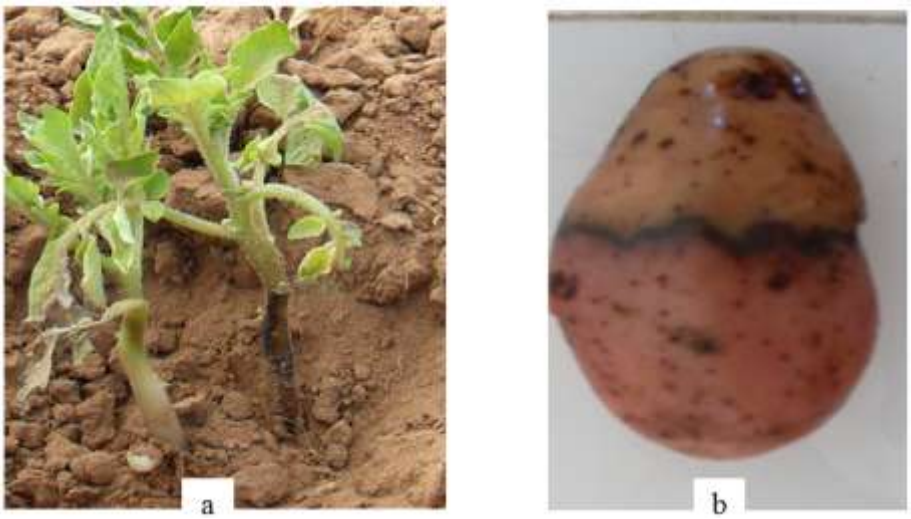

Figure 2. Blackleg of potato plant (a) and soft rot potato tuber (b) collected on the field

III.2. Sample handling and isolate collection

The prevalence percentages of soft rot and blackleg in the different fields visited during the years 2014 to 2016 are shown in figure 3. The results show that regardless of the sampling area, climatic conditions and the

time of collection, soft rot and blackleg diseases are present in all the fields surveyed. Overall, the prevalence of these diseases was most common in Saïs and Chaouia (Semi-arid), Loukkous (Sub-humid) and Sous (Arid). Looking at trends of the diseases in the sampling area, the percentage of prevalence varies from 0% (Haouz, which falls in arid region) to 14% recorded in both Saïs and Loukkous. These two areas fall in semi-arid and sub-humid regions, respectively. The average of the percentage of prevalence in all the areas was 8.2%. However, statistical analysis of the data indicated that there is no correlation between the prevalence of soft rot and blackleg and the climatic conditions in the regions of the survey ($p = 0.1117$).

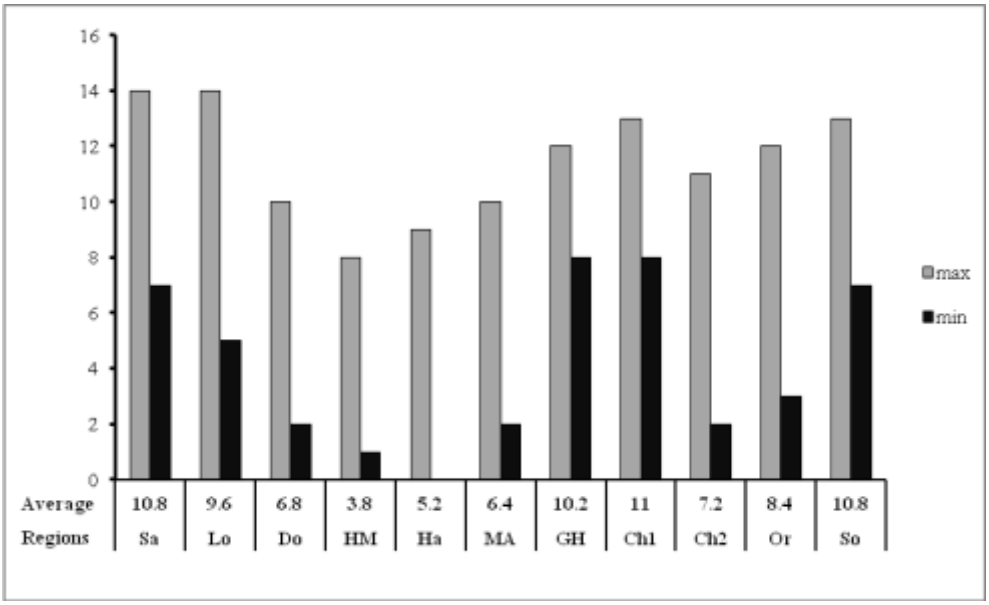

Figure 3. Maximum and minimum prevalence of blackleg and soft rot in the various collection areas. Sa: Saïs, Lo: Loukkos, Do: Doukkala, HM: Haute Moulouya, Ha: Haouz, MA: Moyen Atlas, GH: Gharb, Ch1,Ch2: Chaouia, Or: Oriental, So: Sous

III.3. Prevalence of soft rot and blackleg diseases depending on the seeds used

Out of the 107 fields surveyed, 32 fields belonging to the same region (Saïs) were found to use certified and non certified seeds. Table 1 shows the prevalence of soft rot and blackleg diseases depending upon the seeds used were certified or not. When the nature of seeds was used as the criterion for measuring the prevalence of the diseases on potato, it became obvious that there is a significant ($p = 0.002$) difference in prevalence between areas depending upon the seeds were certified or not. The severity of the two diseases was highest when non certified seeds were used. The minimum of the prevalence was 14% (Ait oulal 3) when non certified seeds were used, it reached 100 % in Taoujtate 5. However, in fields where certified seeds were used, the percentage of prevalence was lower not exceeding 14%. The average of the incidence of the soft rot and blackleg was greater than 28% in fields where non certified seeds were used while it didn't reach 12% in the fields when certified seeds were used.

Table 1. Percentage of blackleg and soft rot prevalence in fields using certified and non certified seed

Field	**% Prevalence (Certified seed)**	**Field**	**% Prevalence (Non-certified seed)**
Ait oulal 1	7	Ait oulal 3	14
Ait oulal 2	8.5	Ait oulal 4	15
Haj kaddour 1	10	Haj kaddour 3	17
Haj kaddour 2	10	Haj kaddour 4	28
Taoujtate 1	10.5	Taoujtate 5	100
Taoujtate 2	13.5	Taoujtate 6	66
Taoujtate 3	14	Taoujtate 7	38
Taoujtate 4	14	Taoujtate 8	46
Meknes 1	11	Meknes 3	16
Meknes 2	12	Meknes 4	15
Bouderbala 1	14	Bouderbala 3	16
Bouderbala 2	14	Bouderbala 4	15
Agouray 1	14	Agouray 3	16
Agouray 2	8	Agouray 4	16
Adarouch 1	8.5	Adarouch 3	16
Adarouch 2	12	Adarouch 4	16

Average	**11.66**	**Average**	**28.12**

III.4. Biochemical and physiological tests

Table 2 shows the biochemical differentiation characteristics of *Pectobacterium carotovorum* subspecies. Among the 102 Pectobacterium strains isolated and on the basis of biochemical profiles, 68.6% and 14.7% strains were identified as Pcc and Pa, respectively. All of them were gram negative, facultatively anaerobic, oxidase negative, catalase positive, non-fluorescent on King's B medium (KB) and showed soft rot symptoms on slices of potato. All strains grew at 37°C and in 5% NaCl and were lecithinase negative. They could reduce nitrate and could produce acid from trehalose, glucose, lactose and are indole negative. Among the strains, 16.6% were able to reduce substances from sucrose and 3% were able to tolerate 6% NaCl. Then, 39.4% of strains could produce acid from α-methyl-D-glucoside and all strains could produce acid from melibiose. Among all the strains, only one strain was inuline positive in our collection. Results showed a high variability among strains. Results also revealed that Pcc was the main pathogen (68.6% of all strains) and *D. chrysanthemi* was absent from the strains tested. Finally, 16.7% of the isolates were atypical of either Pcc or Pa.

Strains were characterized further by biochemical tests. The delineating biochemical test helped distinguish species of Pectobacterium from Dickeya. However, there were a number of atypical strains that had one or more tests that were not consistent with the species description.

Table 2. Biochemical differentiation characteristics of *Pectobacterium carotovorum* subspecies

Number of strains	α-methylglucoside	Melibiose	Utilization of citrate	Growth at 37°C	inuline	Succrose reducing substances
15	+	+	+	+	-	+
1	+	+	-	+	+	+
70	-	+	+	+	-	-
1	-	+	+	+	-	+
15	+	+	+	+	-	-

III.5. Identification and detection with specific primers

Among the 102 Pectobacterium isolates characterized with physiological and biochemical tests, 78 strains yielded a 434-pb DNA fragment by PCR with the Y1 and Y2 primers (Fig. 4) which shows that these strains are Pcc. Of all the isolates, only 24 strains yielded an amplification product of 439 pb using the specific primers Eca1 and Eca2 (Fig. 5), which indicates that the strains are Pa.

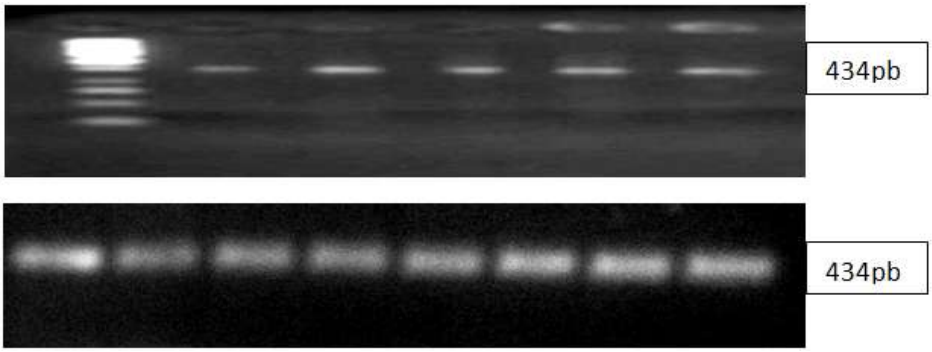

Figure 4. Agarose gel of PCR amplification of *P. carotovorum* DNA by primers Y1 and Y2

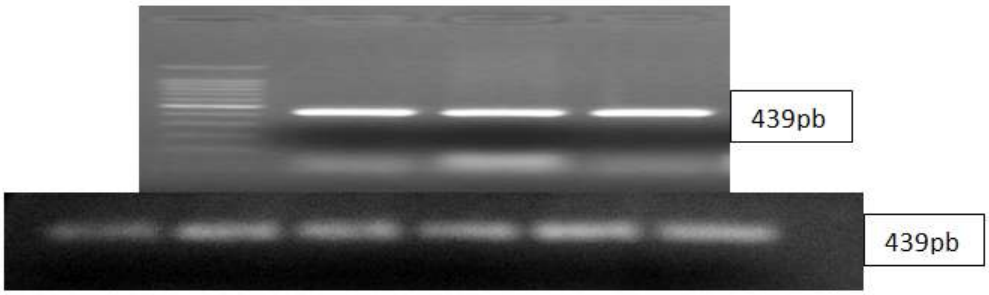

Figure 5. Agarose gel of PCR amplification of *P. atrosepticum* DNA by primers Eca1 and Eca2

IV. DISCUSSION

Many studies have been done in Morocco focusing on the methods of detection and molecular biodiversity of Pectobacterium. However, no study has evaluated the percentage of prevalence of the soft rot or blackleg, which can give much more information about these diseases in Morocco. Thus, we made an assessment of the importance of these diseases in the different regions known by a major potato production. The assessment has been conducted by determining the percentage of prevalence in these regions characterized by differences in climatic conditions, as many epidemiological studies of Pectobacterium have linked virulence to environmental conditions.

The study revealed that the fields surveyed show the presence of various diseases particularly soft rot and blackleg. According to the results obtained in our study, the highest prevalence due to blackleg and soft rot was recorded in Saïs and Loukkous followed by Chaouia 1 and Souss. Saïs and Chaouia fall in semi-arid zone while Loukkous falls in humid/subhumid zone. Chaouia 2, an area present in the same region with Chaouia 1, recorded lower disease prevalence. Blackleg and soft rot prevalence was also recorded to show different results in areas with the same climatic conditions; Souss and Haute Moulouya, characterized by the same climatic conditions (arid) recorded an average maximum prevalence of 13% and 9%, respectively. Our results show that there are no significant differences between the percentages of prevalence of soft rot and blackleg in Pectobacteriums in the different areas of our survey.

The disease incidence and the severity of blackleg and soft rot were reported to depend on temperature and free water [18-22], and optimal conditions for the two diseases development are between 15 and 25°C with prevailing wet conditions [37]. In other studies, the environmental factors, especially temperature, have long been correlated with the epidemiology of soft rot pathogens [10], and *Pectobacterium* spp. were reported to be characterized according to the climatic conditions under which they thrive and infect their hosts [38]. However, fairly recently, there have been new updates in respect to the occurrence of blackleg and soft rot diseases across the globe. [39] reported strains of Pectobacterium detected on potato with symptoms of blackleg in Canada. [40] published a report of Pectobacterium strains causing blackleg and soft rot on potato in New Zealand. According to [9], Pcc has been established as a true causal agent of blackleg in temperate climates in Scotland and Canada. These reports show that Pectobacterium strains are geographically well distributed regardless of climatic conditions.

The prevalence of the soft rot and blackleg diseases measured in the survey was substantially higher when non-certified seeds were used, in which more than 28 % of the average of prevalence was recorded. The results showed in table 1 also revealed that in some fields where non-certified seeds were used, the percentage of soft rot and blackleg can reach 100 %. This is most likely related to a very low seed potato quality where all seeds used were totally infected. The same survey revealed less than 12 % of prevalence of soft rot and blackleg when certified seeds were used, which demonstrate that certified seeds used have no efficient level of resistance to these diseases. The incidence of the diseases recorded even when certified seeds were used can be explained by a latent infection as it was showed that the bacteria may be found in all tissues, stems, roots, leaves and (progeny) tubers [12-38]. It can also be explained by an excessive crop of the plant and/or the irrigation water considered as a source of contamination and dissemination [26-27]. From these results, it is evident that the yield losses caused by blackleg and soft rot will not be reduced until the latent infections can be detected in seed lots.

The results found demonstrate that in Morocco the percentage of prevalence of these diseases may probably depend on the origin of the seed and is unlikely influenced by the climatic differences between the regions. It is known that the percentage of prevalence increases during periods of post-harvest storage, which gives considerable damage especially if the distribution has been delayed in a hot and humid climate.

Finally, our biochemical and physiological studies confirmed a high biodiversity in the population of Pectobacterium and an absence of Dickeya which is very normal due to the ecological requirement of the bacterium that are absent in Morocco. Pcc represents 76.5% in our collection of strains whereas Pa represents only 23.5%. Our collection contains 16.6% atypical strains with biochemical and physiological characteristics of the two sub-species.

V. Conclusion

The results demonstrate that regardless of the climatic conditions, potato grown in all the regions surveyed is prone to blackleg and soft rot. There is also no correlation between the degree of severity of these diseases and the climatic conditions. On the other hand, even certified seeds used have not an effective level of resistance to soft rot and blackleg.

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