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Full Length Research Paper

Bacillus pumilus, a new pathogen on potato tubers in storage in Mali

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Soft rot occurred severely in potato tubers stored in traditional and ameliored storehouses at Sikasso, Mali. 17 infective bacterial isolates were isolated from potato rot tissues (*Solanum tuberosum* L var. Odessa). Out of all, the isolate Od23 was found pathogenic and was characterized as rod-shaped, Gram positive, endo-spore formers and yellow pigment producers. This isolate which was found to be the principal organism responsible for potato rot in storage in Sikasso, grew at a temperature range from 5 to 45°C, with optimum temperature of 30 - 35°C. However, it showed strong pathogenicity to potato tubers at 30°C at 3 days. Furthermore, the 16S DNA analysis confirmed that the obtained isolate was *Bacillus pumilus.* All Potato varieties cultivated in Mali responded to infection with *B. pumilus*. Potato var. Sahel was the most susceptible, while Pamina appeared the most resistant potato variety from Mali. According to literature review, this is the first report on the occurrence of *B. pumilus* as a causal agent of potato soft rot in storage in the region of Sikasso, Mali.

Key words: Potato, storage, *Bacillus pumilus,* soft rot, Mali.

INTRODUCTION

Potato (*Solanum tuberosum* L.) has been widely cultivated in Sikasso (Mali) as an important vegetable crop. Since most potatoes are harvested intensively from mid May to late June, they are stored under high temperature condition of about 30 - 35°C for a long period. However, potato harvesting often coincides with the rainy season in Mali, thus, adequate ventilation to maintain adequate humidity in the improved storehouse is not readily achieved. In addition, potato tubers are easily wounded and spoiled by pathogenic and even by saprophytic fungi inhabiting the surface of the tubers because of their soft and weak tissues (Kasmire and Cantwell, 1992). Potatoes stored under traditional and improved storage conditions have been known to be attacked mainly by *Penicillium* sp., and *Fusarium* sp. (Heilmann et al., 2006; Johnson et al., 1997; Salas et al., 2003). Unfortunately, potato suffers from several diseases at all stages of its life. All the parts of the plant particularly tubers are attacked by a number of, pathogens including fungi and bacteria. They cause several kinds of rot, anthracnose, scab, necrosis, spot and mildew (Messiha et al., 2007; Lee et al., 2002; Chuang et al., 1989). In Mali, the commercial viability of potato has been threatened by the frequent occurrence of bacterial soft rot in storage, a disease of potato which is generally reported to be caused by the phytopathogenic bacterium *Erwinia carotovora* var. *carotovora* (Bradbury, 1986; Beaulieu et al., 2008). Symptoms include water-soaking or yellowish-brown rot of tubers. It causes severe economic losses in traditional storehouses, where the incidence of this disease reached up to 30%. In Mali, we observed a resistance of this disease to all the treatments traditionally used against potato soft rot due to Erwinia species. Preliminary isolation trials indicated the presence of a pathogen, other than *E. carotovora* var. *carotovora*, associated with the disease.

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Thus, the aims of this work are to isolate and identify the causal agent(s), describe pathological properties and test the varietal sensitivity.

MATERIALS AND METHODS

Isolation of bacteria from soft rot potatoes

Bacteria were isolated from rot potato tubers collected at Sikasso, Mali. Potato samples were obtained from random storage chambers in the sites. A total of 323 rot potatoes were sampled. Potato tubers showing water-soaking or yellowish- brown rot under low temperature storage condition were used for isolation. These were washed with tap water and cut longitudinally. The diseased potato tissues were cut into 5 mm cubes by using sterilized surgical blade. 3 pieces of potato tissues were ground in 1 ml of distilled water using a mortar and pestle. The suspension was streaked onto potato dextrose agar (PDA). Plates were incubated at 28°C for 48 h. Bacterial colonies were picked at random from the plates, checked for purity and grouped according to colony colour and morphology, cell shape, growth rate and Gram reaction.

Culture and maintenance of isolates

Bacteria were cultured on Tryptic soy agar. Gram-negative and Gram-positive isolates were subsequently studied considering that soft-rot symptoms of potato were generally associated to Gram negative bacteria. The bacterial isolates were cultured in Tryptic soy broth (TSB) and for maintenance, in nutrient broth–glycerol (8: 2 (v/v) and frozen at −70°C) and French agar (nutrient broth, 8 g l^{-1}; thiamine, 80 mg l^{-1} and agar, 7.6 g l^{-1}).

Characterisation of the pathogen

Bacteriological characteristics of the isolates were examined by using the methods described by Lelliott and Stead (1987) and Palleroni (1984) (Bergy's Manual). Briefly, bacterial cultures (18 h old) were streaked on Tryptic soy agar (TSA) medium and incubated for 24 h at 28°C for colony characterisation, cell morphology, gram stain, LOPAT test (levan type colonies), oxidase reaction, potato rot, arginine dihydrolase, and fluorescent pigment on King's medium B (proteose peptone #3, 20 g; K_2 $HPO_4 \cdot 3H_2O$, 2.5 g; MgSO4·7H2O, 6 g; glycerol, 15 ml; agar, 15 g; and distilled water, 1 L). Potato rot and pectate degradation tests were conducted according to the method described by Lelliott and Stead (1987). Biochemical characteristics of the isolates such as oxidation reaction, arginine hydrolysis activity and nitrate reduction were tested by using the methods described by Goto and Takikawa (1984a, 1984b). Utilization of carbohydrates was also investigated by using the Microlog GN2 microplates system (Lee et al., 1997; Wilson et al., 1999; Duncan et al., 2002; Gilho et al., 2002). The bacterial isolates were grown on Biolog universal growth medium (BUGM) (Biolog Inc., Hayward, CA) and incubated at 28°C for 18 h.

Antibiotic resistance

To screen for antibiotic resistance to differentiate between closely related micro-organisms, isolates were spread over tryptic soy agar (TSA) medium for 18 – 20 h at room temperature as described previously (Maniatis et al., 1989). The antibiotics tested were penicillin, enoxacin, ceftriaxone, erythromycin, cephalotin, chloramphenicol, Kanamycin and gentamycin.

Pathogenicity test

Healthy potato tubers were used for the pathogenicity test. With the use of a toothpick, wounds were made on a potato tuber slice to inoculate the causal agent. The bacterial suspension obtained from 48 h old cultures on PDA agar was collected in distilled water and adjusted to 1.0 x 10^7 cfu/ml. The causal agent was inoculated at the level of 100 µl (10^7 cfu/ml) on the wounds. Potato tubers inoculated were incubated at 25°C in a moist chamber for 3 days. Control samples were similarly tested with sterile distilled water only and kept at the same conditions. The inoculated tubers were examined for potato soft rot. In order to confirm pathogenicity of the casual agent in storage conditions in improved storehouses, potato tubers inoculated with the bacterial suspension as described above were incubated at 5°C for 2 months. Pathogenicity test against potato was similarly conducted using sliced samples.

To investigate growth pattern of Od23 isolate which showed the strongest pathogenicity, growth rates of the pathogen at different temperatures (5, 15, 20, 25, 30, 35, 40, and 45°C) were investigated by measuring turbidity.

Ribosomal sequences (16S rDNA)

DNA was extracted from the pathogenic isolates (Maniatis et al., 1989). Polymerase chain reaction (PCR) amplification of 16S rDNA was made using universal primers (Widmer et al., 1998). The fragment amplified was subsequently cloned into the PCR 2.1 TOPO vector (Invitrogen Corporation Carlsbad, CA, USA) for sequencing. DNA sequencing was done in the laboratory of Microbiology, department of Biology, at the University of Sherbrooke, Canada.

Response of potato varieties to infection: tuber slices of different varieties of potato cultivated in Mali were inoculated with 10 µl of bacterial suspension placed in a hole dug in the centre of each slice. Control potato slices were treated similarly with 10 µl of bacterial-free solution. Inoculated slices were placed in a plastic bag for 48 h at 30 - 35°C in the laboratory and daily examined. Rot severity was assayed using an arbitrary 0 - 5 scale where 0= no symptoms, 1 = 1 - 25% of rotten potato cells, 2 = 26 - 50% of rotten potato cells, 3 = 51 - 75 % of rotten potato cells and 5 = 76% - completely rotted potato slice cells. Disease severity index (DSI) was calculated 15 days after inoculation, according to the Methods of Vakalounakis (1990) as follows: DSI= d/(d max × n) × 100. Where, d is the disease rating possible and n is the total number of potato slices examined in each replicate.

RESULTS

Isolation of bacteria from soft rot potatoes and pathogenicity test

From sampled rot potatoes, 210 bacteria were isolated. Out of the isolated bacteria, 17 were potato pathogenic. (Table 1A) Isolates showed various virulence effects which depended on the inoculation methods (Table 1B), incubation conditions (temperature) and the isolates tested. Isolate Od23 gave the highest virulence effect (75% rot severity) under wound inoculation methods.

Table 1A. Potato pathogenic bacteria isolated from different potato cultivars.

Cultivars	Total bacteria	Potato athogenic bacteria
Claustar (Cl)	4	0 (0)*
Spunta (Sp)	4	1 (25)
Odessa (Od)	9	1 (11)
Total	17	2 (33)

*Percentage of potato soft rot pathogenic bacteria compared to the total number of potato pathogen Isolated

Table 1B. Soft-rot severity of potato var. Sahel induced by *B. pumilus* Od23 as influenced by different inoculation methods.

Bacterial isolate	Soft rot severity (%) incited		
	Wounding	Puncturing	Spraying
B.pumilus Od223	75	60	0

Under spray inoculation, Od23 isolate failed to incite symptoms.

Characterisation and identification of the pathogens

The morphological, physiological and biochemical characters of Od23, indicated that the isolate was rod–shaped, gram positive, endo-spore former and yellow pigment producer (Table 2). The catalase, methyl red reaction, aerobiosis and H_2S production tests were positive. The isolate utilized galactose, glucose, inositol, mannitol, trehalose, xylose andd weakly utilized glycerol and had a negative reaction for starch hydrolysis, nitrate reduction, V.P. test and indole formation. It did not utilize arabinose, lactose and maltose. It is also able to grow in the presence of 50 g erythromycin but sensitive to 50 g penicillin and streptomycin. The results suggest that Od23 is *Bacillus pumilus.* The 16S DNA sequencing results, indicate 99% similarity between the bacterial isolate Od23 and *B. pumilus* and confirmed the morphological, physiological and biochemical identification results.

Reaction of potato varieties to B. pumilus Od23

Significant variances were obtained between potato varieties in response to infection with *B. pumilus* Od23 isolate (Figure 1A and Table 3). Potato var. Sahel was the most susceptible to *B. pumilus* Od23 infection (50% severity) followed by var. Odessa (35%), Claustar (30%) and Pamina that showed (20%) soft rot severity. A moderate reaction to *B. pumilus* Od23 infection was recorded with Yukon Gold (15% soft rot severity) (Figure 1B and Table 3). Potato var. Russet and Norland showed a sufficient resistance against *B. pumilus* Od23 infection since only 9% soft rot severity was exhibited. Yukon Gold, Russet and Norland are potato varieties from Canada selected for their resistance to *Erwinia* species.

Table 2. Morphological, physiological and biochemical characters of the pathogenic bacterial isolate Od23 obtained from naturally infected potato tubers.

Character	Bacterial isolate Od23
Shape of cell	Rod
Motility	+
Gram Reaction	+
Spore forming	+
Catalase production	+
Yellow pigment	+
Starch hydrolysis	-
Nitrate reduction	-
V.P test	-
Methyl red reaction	+
Indole formation	-
Potato soft rot	+
Aerobiosis	+
H_2S production	±
Carbon source utilisation	
Arabinose	-
Galactose	+
Glucose	+
Glycerol	±
Lactose	-
Maltose	-
Mannitol	+
Sensitivity to antibiotics	
Erythromycin 50 g	R
Penicillin 50 g	S
Streptomycin 50 g	S

(+) = Positive reaction; (-) = Negative reaction; (±) = Weekly reaction; (R) = resistant and (S) = Sensitive.

DISCUSSION

In West Africa, many soft-rot diseases have been associated with *E. carotovora.* This pathogen is characterised by the production of extracellular enzymes such as pectate lyases (Valenzuela-Zapata, 1994; Daniels et al., 1988). Potato soft-rot symptoms were reproducible under laboratory conditions and were similar to those observed in storehouse during storage. In order to make an accurate diagnosis, it was important to

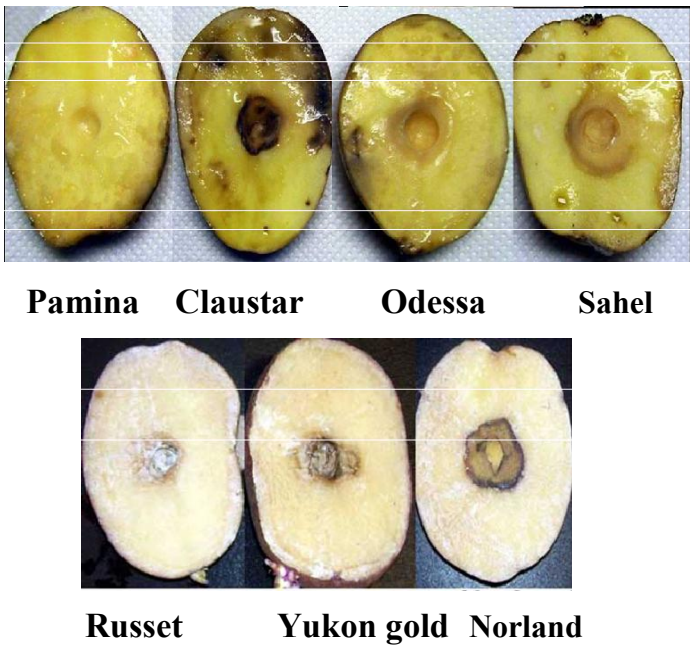

Figure 1. Soft rot severity of potato cultivars induced by *B. pumilus* Od23.

Table 3. Soft rot severity (%) to tubers of various potato varieties caused by inoculation with the isolate *B. pumilus* Od23.

Potato varieties	Provenance	Soft rot severity (%) caused by the bacterial isolate *Bacillus pumilus* Od23
Sahel	Mali	50
Odessa	Mali	35
Pamina	Mali	20
Claustar	Mali	30
Yukon Gold	Canada	15
Russet	Canada	9
Norland	Canada	9

identify and characterize the causal agents of the disease. The results show that Od23 is gram positive while *E. carotovora* is gram negative suggesting that the causal agent of patato soft-rot in storage in Mali is different from *E. carotovora*. Koch's postulates and extensive biochemical characterisation employing carbon source utilisation profiles, showed a 87% similarity between the bacterial isolate Od23 and *Cellulomonas hominis* CDC-A-3 (Bathily, 2007). Based on morphologic and physiologic properties, the isolate Od23 appeared to be different from *E. carotovora* (Bathily, 2007a, 2007b). The bacterial isolate Od23 appeared to be resistant to 50 g erythromycin but sensitive to 50 g penicillin and streptomycin, and did not utilize arabinose, lactose and maltose. These identification trials suggested that the isolate Od23 is *B. pumilus* as reported previously (Schaad, 1980; Gaber and Gazar, 1983). The last result was confirmed by the 16S DNA sequences showing a 99% similarity with Od23 and *B. pumilus*. Out of all the isolate bacteria, only the bacterial isolate Od23 appear to cause symptoms similar to sot-rot symptoms. Under spray inoculation, *B. pumilus* Od23 failed to incite soft-rot symptoms on potato tubers, indicating that this pathogen needs wound to infect potato tubers. These data are consistent with those reported by Gabr and Gazar (1983) and Saleh et al. (1997). *Bacillus* species are common in soil and some may be involved in post harvest diseases.

The bacterial isolate *B. pumilus* Od23*, Pseudomonas fluorescens, Corynobacterium, E. carotovora* subsp *atroseptica* and other *bacillus* spp. are known to cause bacterial soft rot to storage crops (Ciampi et al. 1976 and Ciampi and Huguelet, 1979) . *B. pumilus, B. subtilis* and *B. polymyxa* are reported to cause post harvest soft rot of vegetables (Chiu et al., 1964). Gaber and Gazar

(1983) reported that the bacteria (*B. pumilus and B. polymyxa*) were causal pathogens of head rot of cabbage. *B. pumilus, B. subtilis, B. coagulans* and *B. polymyxa* have recently been reported to be the main causal agent of garlic cloves post-harvest decay (Saleh, 1995; Galal et al., 2002). Also, *B. pumilus* causes brown spots on fruit and leaves of Balady peach (Saleh et al., 1997).

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