SHORT COMMUNICATION

ACIDOVORAX CATTLEYAE-THE CAUSAL AGENT OF BACTERIAL BROWN SPOT OF *PHALAENOPSIS LUEDDEMANNIANA* IN POLAND

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SUMMARY

In September 2009, in commercial greenhouse production of phalaenopsis orchid (*Phalaenopsis lueddemanniana*) in the Lodz region (Poland), atypical leaf spot symptoms were observed on about 15% of the plants. On 7-month-old leaves large, brown, sunken spots surrounded by yellow, water-soaked tissue, were observed. Gram-negative bacteria causing hypersensitivity reaction on tobacco, but not possessing pectolytic properties, were isolated from the brown spots. Sequence analysis of the 16S rDNA gene fragment, selected phenotypic characteristics and a pathogenicity tests on healthy phalaenopsis plants led to the conclusion that *Acidovorax cattleyae* is the causal agent of orchid brown spot in Poland. Analysis of genetic diversity of tested isolates by PCR-MP technique revealed only small differences between the studied isolates.

Key words: 16S rRNA gene, biochemical features, diversity, PCR-MP.

In Poland, interest in commercial cultivation of orchids has increased in the last decade. Due to the fact that, in most cases, these plants are imported as young seedlings from western Europe and Asia, several new and previously unlisted pathogens are observed. Among the fungal diseases, up to now the presence of *Phytophthora* rot (*Phytophthora palmivora*), *Phytium* root rot (*Pythium splendens*), Fusarium stem base rot (*Fusarium oxysporum*), anthracnose (*Colletotrichum gloeosporioides*) and gray mould (*Botrytis cinerea*) was observed. In addition, *Pectobacterium carotovorum*, a bacterial pathogen causing soft rot was recorded (Orlikowski and Szkuta, 2006; Orlikowski and Ptaszek, 2009). In 2009, in phalaenopsis (*Phalaenopsis lueddemanniana*) pot-grown in greenhouses of the Lodz region (Poland) brown necrotic leaf spots were observed on about 15% of the plants. Initially, small water-soaked spots occurred on seven-month-old leaves which, over time, became larger and brownish and surrounded by a yellow halo. In the final stage, total collapse and death of the whole plant was observed. Symptoms resembled those caused by *Acidovorax cattleyae* (Janse, 2006). The aim of our study was to determine the etiology of the observed lesions and to characterize their causal agent.

Five diseased phalaenopsis plants were analyzed. A fragment of plant tissue at the border between symptomatic and healthy tissue was excised with a sterile scalpel and homogenized in a Petri dish with a small amount of sterile distilled water. The resulting suspension was transferred with a bacterial loop on the surface of KB medium (King *et al*., 1954) and nutrient agar with 5% sucrose (NAS). After three days of growth on KB medium, colonies were 1 mm in diameter, flat and white with regular edges. From the isolation plates, five colonies were purified on NAS and selected for further study. Selected isolates (A01 to A05) were subjected to Gram reaction (3% KOH) according to Schaad *et al*. (2001) and their hypersensitivity on tobacco cv. Samsun (Klement, 1963) was tested. Furthermore, the pectolytic activity of all the bacterial isolates was tested on slices of potato tubers. The tubers were disinfected with 50% ethanol, dried and cut into slices of about 5 mm thick and the slices were placed on moist filter paper in sterile Petri dishes. Approximately two loops of each bacterial isolate grown on NAS were uniformly spread onto the surface of the slices. Rot development was examined 24-48 h post inoculation with incubation at 25°C. Arginine dihydrolase activity and reduction of nitrate to nitrite was determined according to Schaad *et al.* (2001) at 28°C. Gelatine hydrolysis was tested as described by Fahy and Persley (1983). Acid production and utilization of carbohydrates were determined on the minimal medium according to Hugh and Leifson (1953) containing bromothymol blue and a carbon source at 1% final concentration.

Results were scored after three days of incubation at 26°C. Action on litmus milk was determined on the BBL™ litmus milk medium (Becton, Dickinson and Co., France). Growth at 41°C was determined on liquid NB medium with 1% glucose for three days. In all tests,

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a type strain of *Acidovorax cattleyae* LMG 2364 was included. The 16S rRNA gene was amplified with primers fD1 and rP2 (Weisburg *et al*., 1991). Then, its 800 bp long 5' fragment was sequenced using primers fD1 and 800r (Weisburg *et al*., 1991; Drancourt *et al*., 1997). The resulting sequence was compared to sequences deposited in GenBank using the BlastN algorithm in order to find the highest similarity. Pathogenicity tests were carried out on healthy phalaenopsis plants. Suspensions at the concentration of 10^7 CFU ml^{-1} of the bacterium under test and of the reference strain LMG 2364 were injected into leaf mesophyll with a hypodermic syringe. Each strain was tested on a separate plant. Sterile distilled water was used as negative control. Inoculated plants were incubated at 28°C at a relative humidity of 60-80% and 800 lux light. To fulfil Koch's postulates, bacteria were re-isolated from the border between diseased and healthy tissue on NAS medium and identified based on colony morphology and 16S rRNA gene sequence. To determine the genetic diversity of isolated *A. cattleyae* strains, a PCR melting profile (PCR-MP) protocol was used (Masny and Plucienniczak, 2003). For this purpose, DNA was isolated according to Aljanabi and Martinez (1997) and three sets of restriction enzymes, adaptors and primers were used: *Hin*dIII (Masny and Plucienniczak, 2003), *Apa*I (Grady *et al*., 1999) and *Pst*I (Waugh *et al*., 1997). The digestion of 100 ng DNA with each of the endonucleases *Apa*I, *Hin*dIII (Fermentas Thermo Scientific, Lithuania) and *Pst*I (Roche, Germany), was carried out according to manufacturers' instructions. Digested DNA was ligated with an appropriate adaptor: *Hin*dIII (Masny and Plucienniczak, 2003), *Apa*I (Grady *et al*., 1999) and *Pst*I (Waugh *et al*., 1997). PCR amplifications were performed with specific primers: (i) for *Hin*dIII-digested DNA, POWAGCTT [5'-CTCACTCTCACCAACGTCGACAGCTT-3', Masny and Plucienniczak (2003)]; (ii) for *Apa*I-digested DNA, *Apa*I [5'-GACTGCGTACAGGCCC-3', Grady *et al*. (1999)]; (iii) for *Pst*I-digested DNA, PstI+0 (5'-GACTGCGTACATGCAG-3'). PCR mastermix and amplification conditions were as described by Kaluzna *et al*. (2010) but a denaturation temperature 91°C was used. PCR products were separated in 1.5% agarose gels in TBE buffer, which were stained with ethidium bromide (0.5 mg l^{-1}) and visualized under a UV transilluminator.

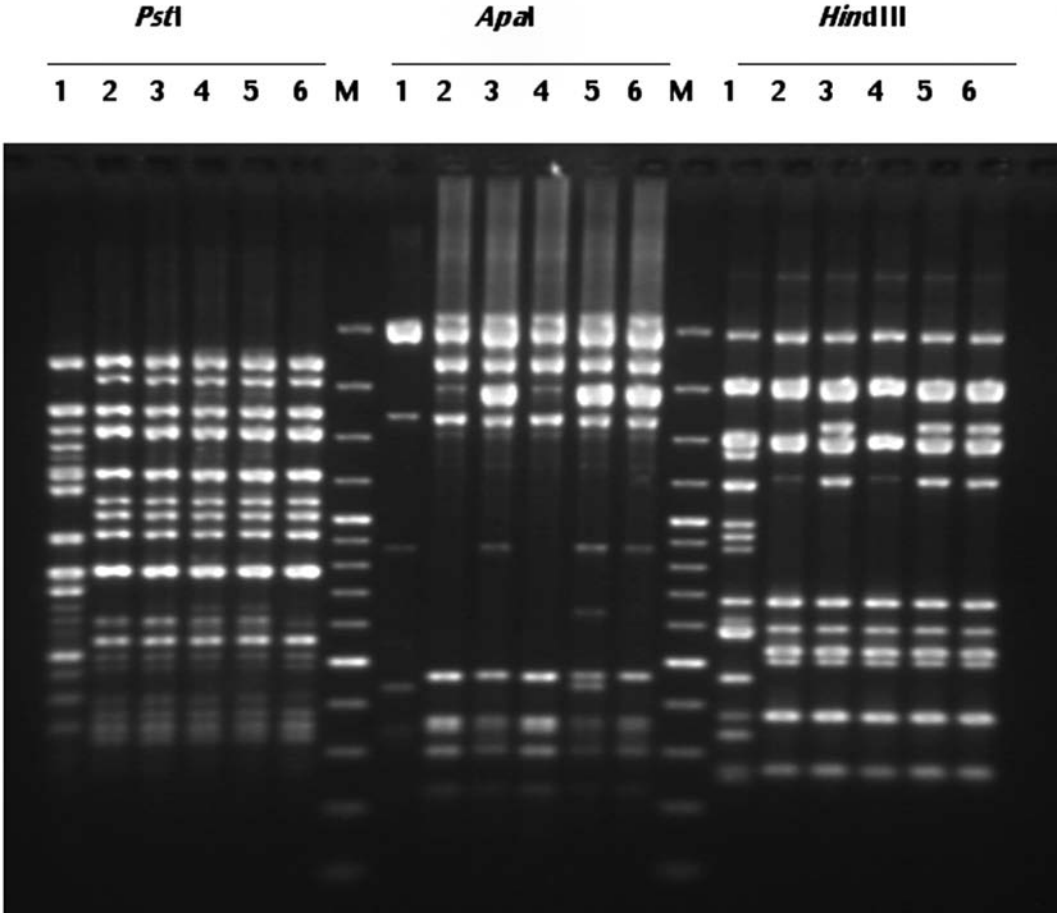

Fig. 1. Electrophoretic patterns obtained after PCR MP with three enzymes: *Pst*I, *Apa*I, *Hin*dIII. Lane 1, LMG 2364; lane 2, A01; lane 3, A02; lane 4, A03; lane 5, A04; lane 6, A05; lane M, marker 100 bp Ladder (Thermo Scientific Fermentas).

Table 1. Phenotypic characteristics of bacteria isolated in Poland from phalaenopsis with symptoms of brown spot.

Characteristic	Isolate/strain	
	Acidovorax isolates tested A01, A02, A03, A04, A05	LMG2364^T
Utilization of:		
D-glucose	+	+
D-xylose	+	+
D-sorbitol	+	+
Ethanol	-/+	+
D-mannitol	+	+
Sodium citrate	+	+
D-fructose	+	+
D-mannose	+	+
Maltose	+	+
Nitrate reduction	-	-
Gelatine liquefaction	-	-
Arginine dihydrolase	+	+
Litmus milk	ALK	ALK
Growth at 41°C	-	-
Hypersensitive response on tobacco	+	+
Pectolytic activity on potato	-	-

Five bacterial isolates, labelled A01 to A05, used for further study, were Gram-negative, caused a hypersensitivity reaction on tobacco, and did not show pectolytic activity on potato slices. Most of the tested features of all isolates corresponded to those reported by Schaad *et al*. (2008) and Willems *et al*. (1992). However, some characteristics such as: nitrate reduction, arginine dihydrolase, growth at 41°C and the utilization of some carbon compounds (D-fructose and maltose) differed from those reported by Schaad *et al*. (2008) for *A. cattleyae*. The tested isolates were able to utilize of D-xylose in agreement with Schaad *et al*. (2008) but in disagreement with the results by Willems *et al*. (1992). Although these features differed from those reported earlier, they were identical to the characteristics of the type strain *A. cattleyae* LMG 2364 used in our analyses (Table 1).

BlastN analysis of the 16S rDNA sequences of the tested isolates showed identity with those of *A. cattleyae* (GU339087.1), *A. citrulli* (CP000512.1) and *A. avenae* (EF418616.1). This is not surprising because it is known (Schaad *et al*., 2008) that these three bacterial species, although pathogens of different host (orchid, melon and maize, respectively) are closely related, and their 16S rDNA sequences are almost identical. Biochemical, physiological and pathogenicity tests, however, can readily discriminate these species (Schaad *et al*., 2008).

In pathogenicity tests, the first symptoms were observed 24 h post inoculation, when the leaves infiltrated with bacteria showed water-soaked spots that, with time, increased steadily in size and became necrotic. Two weeks post inoculation, the leaves became yellow, lost turgor and wilted. Generally our isolates showed greater virulence than the reference strain LMG 2364. No symptoms were observed on control plants. From plants with symptoms, bacteria with identical colony morphology to those used for inoculation were isolated and their identity was confirmed by 16S rRNA gene sequencing.

Analysis of genetic diversity revealed only small differences in amplification profiles between Polish strains. Three slightly different pattern types were obtained for reactions with *Apa*I and two for *Hin*dIII. Amplification patterns of the type strain of *A. cattleyae* were more distinct, but this strain shared many common bands with our isolates (Fig. 1). It is difficult to state if such a diversity is common for *A. cattleyae* strains because up to now apparently there are no studies on this subject recorded in the literature.

The present investigation shows that *A. cattleyae* is the causal agent of a brown spot disease of phalaenopsis in Poland. This pathogen was first described Italy (Pavarino, 1911) as *Pseudomonas cattleyae*, then re-classified as *A. avenae* subsp. *cattleyae* (Willems *et al*., 1992), finally as *A. cattleyae* (Schaad *et al*., 2008). Infections by this bacterium have been recorded from Australia, Italy, the Netherlands, Philippines, Portugal, Taiwan and the USA (Stovold *et al*., 2001; Scortichini *et al*., 2005; Janse, 2006). Based on the present study, Poland can be now added to this list.

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