METHOD FOR DIFFERENTIATING *BURKHOLDERIA GLADIOLI* PATHOVARS

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ABSTRACT

Burkholderia gladioli was first described as a phytopathogen and later associated with certain pulmonary infections. Plant strains were divided into pathovars (*alliicola* and *gladioli*) according to plant host and nutrition differences were established. This paper represents an easy-to-perform molecular method for differentiating plant pathovars of *B. gladioli*. According to our results, both pathovars cause natural infections of gladiolus and onion, and have similar utilization properties. The pathogens can be distinguished by RAPD. Primer CUGEA1 gives one strong constant product of ~340 bp for *B. gladioli* pv. *alliicola* and ~550 bp for *B. gladioli* pv. *gladioli*, and is suitable for differentiation.

***Key words**: Burkholderia gladioli, pathovars, phytopathogens, differentiating*

INTRODUCTION

The representatives of genus *Burkholderia* are versatile, wide spread microorganisms, which inhabit a wide range of ecological niches including water, soil, rizosphere, industrial regions, plants, mushrooms, and insects (Govan et al. 2000; Coenye and Vandamme 2003; Kikuchi et al. 2005). Most *Burkholderia* species are not closely specialized bacteria being of major interest for their pathogenicity in both plants and people.

Burkholderia gladioli was considered as a homogenous species and a typical plant pathogen which is divided into two pathovars according to their specialization to the host plant. *B. gladioli* pv. *alliicola* causes „slippery skin" on onion bulbs and dry necrotic spots on onion leaves (Bradbury, 1986). In Bulgaria the disease was known as "mealy soft rot" and caused great damage in the 1960s (Vitanov 1967, 1970). *B. gladioli* pv. *gladioli* infests onion, rice, gladiolus, crocus, and other ornamental plants (Bradbury, 1986; Uchida, 1995; Keith et al., 2005; Ura et al., 2006). Gladiolus bulbs develop pale yellow round spots, which darken to brown, sink and cover with a sleek film (Hristova et al., 1966), other plants develop soft rot (Bradbury, 1986). *B. gladioli* is a known phytopathogen in six continents (Bradbury, 1986; Lee et al., 2005).

As a human pathogen *B. gladioli* was established in the last years. The bacterium is a common agent in nosocomial infections and CF patients (Graves et al., 1997). Cases of keratitis and osteomyelitis have also been described (Ritterband et al., 2002; Boyanton et al., 2005 Lestin et al., 2008). *B. gladioli* was found to be susceptible to complement-mediated lysis of pooled human serum *in vitro*, implying that healthy individuals should be immune to infection. However, one case of infection was in a non-immunocompromised patient (Graves et al., 1997). Some *B. gladioli* strains are responsible for cases of food poisoning (Jiao *et al*., 2003). It is not known if one or the two of the pathovars have a role in human infections or the patients carry strains with different pathogenicity.

Commercial systems were frequently unable to identify *B. gladioli* correctly (Graves et al., 1997) and investigations have been devoted on methods for differentiation of *B. gladioli* from the closely related *B. cepacia* in clinical samples (Baurenfeind et al. 1998; Brisse et al. 2000). Works on distinction between the pathovars of *B. gladioli* almost lack. One investigation on the basis of RFLP-analysis of 16S rRNA gene for differentiation among *B. cepacia* genomovars I to V and also found differences between the reference strains of *B. gladioli* pathovars (Segonds et al. 1999). However, plant pathogenic and saprophytic isolates by the means of molecular techniques have not

been studied. This paper represents a new easy-to-perform molecular method for differentiating *B. gladioli* pathovars isolated from bulb plant samples.

MATERIALS AND METHODS

Strains. The 15 *B. gladioli* strains included in this study originated from diseased scales of onion bulbs (*Allium cepa*) and internal tissues of gladiolus corms. Pathogenicity of the strains was previously confirmed by an artificial inoculation of onion. Species identification was carried out by the BIOLOG^{TM} system (Bogatzevska et al., 2010) and PCR with species-specific primers CMG 16-1 and G 16-2, described by Bauernfeind et al. (1998) (Stoyanova et al., 2011).

Two type strains - *Burkholderia cepacia* NBIMCC 8566 (LMG 1222, ATCC 25416), and *Burkholderia gladioli* pv. *gladioli* NBIMCC 8569 (ATCC 10248, LMG 2216) and one reference strain - *B. gladioli* pv. *alliicola* NBIMCC 8516 (ICMP 3950a) were used as controls.

DNA extraction was performed after cultivation of the strains in Luria-Bertrani Broth at 28°C and 200 rpm for 24 h. Cell density of the suspensions was measured on a spectrophotometer (GeneQuant Pro, Amersham Technologies, UK) at 600nm. A quantity of each strain suspension corresponding to $2x10^9$cells/ml was taken for centrifugation and further processing. Genome DNA was extracted by DNeasy Blood & Tissue Purification Kit (QIAGEN GmbH, Germany) according to the manufacturer's instructions. Control of yield and purity of obtained DNA was performed by measuring on a spectrophotometer (GeneQuant Pro, Amersham Technologies, UK) at 230 nm, 260 nm, 280 nm, and 320 nm. Final volumes with DNA concentration of 70-100 μg/ml were stored at -20°C until further use.

RAPD-PCR. A total of six oligonucleotide primers (CUGEA1-6) were used for initial experiments with the type and reference cultures. The RAPD-RCR was performed according to the procedure described by Momol et al. (1997). The PCR mixture contained 1x PCR buffer, 2,5 mmol/l $MgCl_2$, 50 *p*mol each primer, 0.1 mmol/l dNTP mix, 0.5U *Taq* DNA polymerase, and 100 ng of DNA. Amplification was performed with initial denaturation step at 94 ^oC for 4 min; followed by 35 cycles of denaturation at 94 ^oC for 1 min, annealing at 42 ^oC for 1 min, extension at 72 ^oC for 1 min'30 s; and a final extension step at 72 ^oC for 5 min. The amplified products were electrophoretically separated in 1.5% (w/v) agarose gel at 100V for 1h in 1x TBE buffer, stained with ethidium bromide and visualized under UV light. Subsequently, two of the primers (CUGEA1 and CUGEA2) were chosen for PCR amplification for the Bulgarian *Burkholderia* isolates.

RESULTS

Preliminary experiments were carried out with six CUGEA primers. Four of them (CUGEA3, 4, 5, and 6) gave from several to many products some of which varied in the separate repetitions of the experiment, therefore, and were unsuitable for comparison and analysis. Two primers (CUGEA 1 and 2) were selected for subsequent investigations and RAPD-analysis with these primers was repeated 5 times.

CUGEA2 gave one distinct product of about 700 bp for *B. cepacia* and one distinct product for *B. gladioli* (both pathovars) with approximate length of 630 bp (Fig. 1). These bands were constantly received at all repeats of the experiment. Some weaker additional products were observed in some of the PCR-repeats but their amplification was not constant. All fifteen isolates amplified the product of ~630 bp which was characteristic for *B. gladioli* (Fig. 1) in confirmation with the results from the PCR with species-specific primers (Stoyanova et al., 2011). RAPD-analysis with CUGEA2 differentiated *B. gladioli* from *B. cepacia* but did not differentiate *B. gladioli* pathovars.

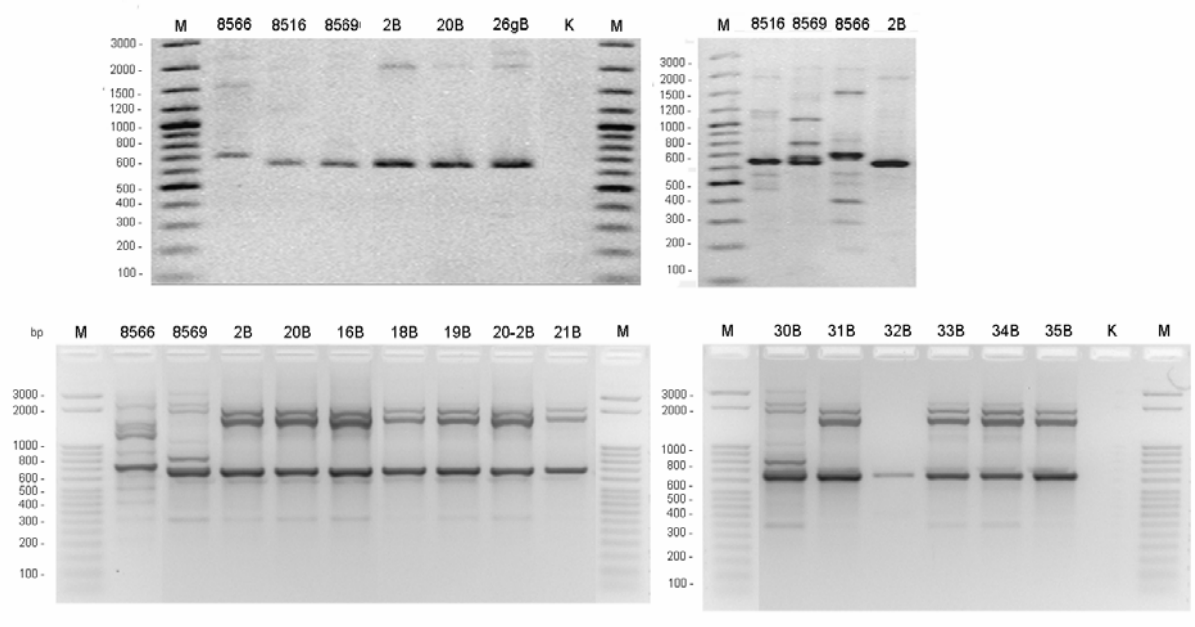

Fig. 1. RAPD patterns with PCR-amplification with CUGEA2

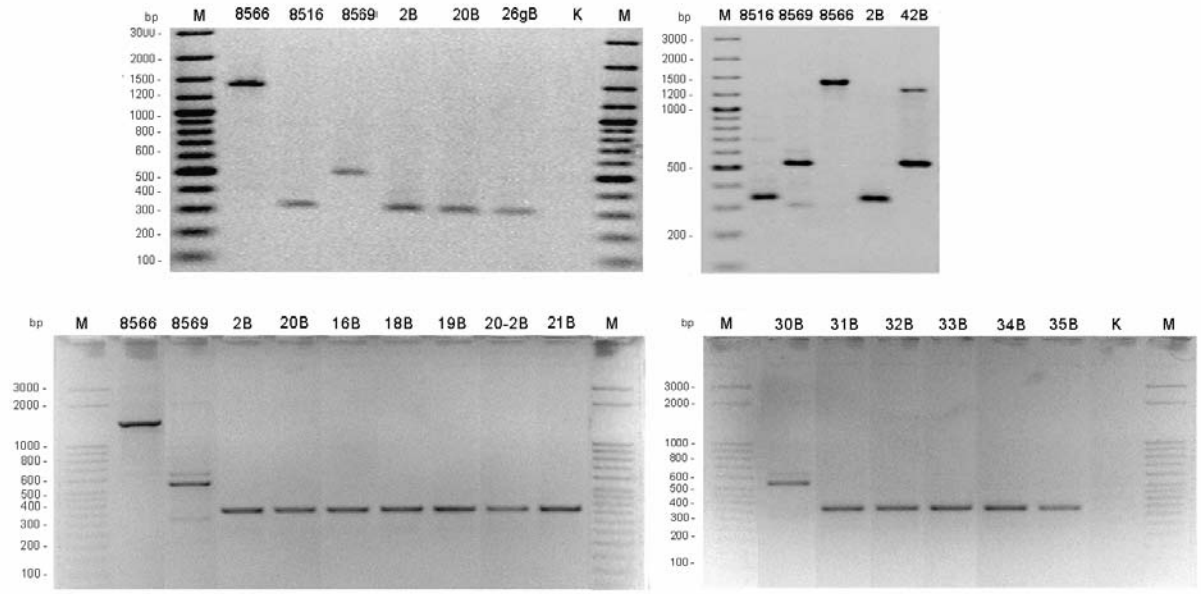

Fig. 2. RAPD patterns with PCR-amplification with CUGEA1

RAPD with CUGEA1 also gave one constant product with different length for each of the type strains tested at all PCR-repeats. The amplified product of *B. cepacia* had approximate length of 1400 bp, the product of *B. gladioli* pv. *alliicola* was about 340 bp, and the one of *B. gladioli* pv. *gladioli* - 550 bp. Some minor products could also be observed in addition to the main one. Thirteen of our isolates showed amplified products with same length as *B. gladioli* pv. *alliicola* and two – same length as *B. gladioli* pv. *gladioli* (Fig. 2).

On the basis of the PCR analysis it can be concluded that 13 strains isolated from onion and gladiolus belong to *B. gladioli* pv. *alliicola* and 2 strains belong to *B. gladioli* pv. *gladioli*.

DISCUSSION

The strains established as the two pathovars of *B. gladioli* were isolated from onion and gladiolus. To date *B. gladioli* pv. *alliicola* was known to attack only onion as opposed to *B. gladioli* pv. *gladioli*, which was a known pathogen of onion, gladiolus, crocus and other plants. Differentiation of *B. gladioli* pathovars was made only on the basis of host, biochemical and physiological properties (Bradbury, 1986; Uchida, 1995; Keith et al., 2005; Ura *et al.*, 2006). According to our results both pathovars can cause natural infection in onion and gladiolus, and gladiolus is a common host in addition to onion. The strains also could not be distinguished on the basis of biochemical properties. According to a study based on the data from BIOLOG^{TM} system (Stoyanova et al., 2011), *B. gladioli* strains form two separate clusters in the cluster analysis at 80% similarity. However, strains from both pathovars are included in the two clusters, the differences being not in relation to the pathovar division.

Impossibility to differentiate *B. gladioli* pathovars could be explained by the pathovar system being obsolete. Nevertheless, the pathogens could still be clearly distinguished by RAPD with CUGEA1. The stated molecular method also allows the fast distinction between *B. cepacia* and *B. gladioli* in absence of species-specific primers in combination with basic biochemical characterization for genus *Burkholderia*.

CONCLUSIONS

Onion and gladiolus are common hosts for both pathovars *B. gladioli* pv. *alliicola* and pv. *gladioli*.

B. gladioli pv. *alliicola* and *B. gladioli* pv. *gladioli* can be clearly differentiated by the use of RAPD with primer CUGEA1.

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