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A METHOD FOR THE RAPID DETECTION AND IDENTIFICATION OF HALO BLIGHT PATHOGEN ON COMMON BEAN

TATJANA POPOVIĆ[1,*], JELICA BALAŽ[2] and SLAVIŠA STANKOVIĆ[3]

[1] *Institute for Plant Protection and Environment,* 11000 Belgrade, Serbia
[2] *University of Novi Sad, Faculty of Agriculture,* Novi Sad, Serbia
[3] *Faculty of Biology, University of Belgrade,* 11000 Belgrade, Serbia

*Corresponding author: tanjaizbis@gmail.com

Abstract - A diagnostic method based on nested-PCR, followed by ELISA and conventional bacteriology tests, for the rapid and reliable detection of halo blight pathogen *Pseudomonas savastanoi* pv. *phaseolicola* (*Psp*) collected from infected bean leaves and seeds is described. *Psp* formed white, small and flat colonies on nutrient agar medium, creamy white, flat and circular on Milk-Tween agar medium and light yellow, convex and shiny on modified sucrose peptone agar medium. Eighteen Gram-negative, catalase-positive and oxidase-negative strains were subjected to nested PCR with primers P 5.1/P 3.1 and P 5.2/P 3.2, which directed the amplification of the 450 bp target DNA fragment in all tested strains. According to the results of DAS- and PTA-ELISA with respect to reactivity to specific antibodies, all analyzed strains belonged to *Psp* bacterium. Pathogenicity was tested on bean pods and cotyledon leaves, on which greasy spots were formed. *Psp* did not cause hypersensitive reaction on the leaves of tobacco and geranium. Strains produced levan, fluorescent pigment, oxidative metabolism of glucose, did not reduce nitrate, did not produce indole and H_2S, did not hydrolyze starch, gelatin and esculin; they produced acid from glucose, mannose, sucrose and glycerol, and did not produce acid from maltose, starch, esculin, dulcite, sorbitol, inositol and erythritol.

Key words: *Pseudomonas savastanoi* pv. *phaseolicola*; halo blight; bean; identification

INTRODUCTION

Pseudomonas savastanoi pv. *phaseolicola* (Burkholder 1926) Gardan et al. 1992 (*Psp*), the causal agent of halo blight, is an economically important bacterium of beans all over the world (Bradbury, 1986; Franc, 1998) including Serbia (Balaž, 1985; Popović, 2008; Popović et al., 2012). The bacterium gained economic significance in the 1960-70s in the USA and some European countries, mainly due to the development of susceptible beans cultivars and intensive commercial exchange of seeds over long distances. Yield reductions up to 43% were recorded in the UK and the USA (CPC, 2004). In response to the epiphytotic outbreak of the disease on beans, intensive selection work was started in the USA, which included the transfer of resistance genes from different bean genotypes (Zaumeyer and Meiners, 1975).

Halo blight was registered in Serbia during 1970-80s, when American and German varieties predominated in the domestic bean production (Balaž, 1985). In years with a cool and rainy spring, cases of complete destruction of string bean crops were recorded in the Vojvodina Province (Balaž, 1989). In recent years, however, this bacterium ceased being a major problem in bean production in our country, mainly because of favorable weather conditions (warm and dry weather during string bean emergence and initial stages of development) and growing of bean cultivars resistant to *Psp*.

Psp infects beans during their emergence, intensive growth, flowering and pod maturation. It may occur on bean leaves, stems, growing points, pods and seeds (Schwartz, 1989; Franc, 1998). Intensive chlorosis, the typical symptom of halo blight, is the result of the action of phaseolotoxin, a toxin containing *N-phosphosulfamylornithine* as the major functional component (Schwartz, 1989).

Psp can be easily isolated from halo spots on the leaves, stems or pods using a standard isolation medium (Schaad, 1988; Popović, 2008). As *Psp* is a seedborne pathogen, several semiselective media can be used for isolation from seed (Mohan and Schaad, 1987; Moser et al., 1994; Gozczynska and Serfontein, 1998; ISF, 2006; Kurowski and Remeeus, 2008). The aim of this study was to elaborate a rapid diagnosting method for the detection and identification of *Psp* strains from infected bean leaves and seeds that can be recommended for routine testing of this bacteria.

MATERIALS AND METHODS

Bacterial isolation

Isolation from leaves with halo blight symptoms was carried out on three samples of infected beans (varieties Oplenac, Zlatko and Slavonski žutozeleni), using macerated plant fragments obtained from the transition zone between healthy and diseased tissues plated on NA (nutrient agar). Isolation from seed was carried out on extracts obtained from two infected bean samples (varieties Dvadesetica and Oplenac), using two semiselective media, MT (Milk-Tween agar) described by Goszczynska and Serfontein (1998) and MSA (modified sucrose peptone agar) described by Mohan and Schaad (1987). Extraction was performed from whole bean seeds in a sterile extraction solution in the proportion of 1:2 (1 g of seed in 2 ml of solution) for 24 h at 5°C) (ISF, 2006; Kurowski and Remeeus, 2008). A pure culture of *Psp* reference strain (Ps12) was also plated on used media. The Petri dishes were incubated at 28-30°C for five days. After this, the sample plates were visually assessed for the presence of colonies with typical *Psp* morphology by comparison with the reference strain. Suspected colonies, as well as the reference strain, were transferred onto a King's B agar and incubated for 3-4 days at 27°C. Eighteen representative strains were selected for further work (Table 1). Throughout the study, 24- to 48-hour-old strains grown on NA were used.

Pathogenicity

Pathogenicity of the tested strains was checked by: a) injection of a bacterial suspension (in the concentration of 10^7 CFU ml^{-1}) into young string bean pods with a hypodermic syringe (Balaž et al., 1995; Popović, 2008), and b) toothpick wounding of cotyledons (ISF, 2006; Kurowski and Remeeus, 2008; Popović, 2008).

Hypersensitivity reaction was tested on tobacco and geranium leaves by injecting the bacterial suspension (in the concentration of 10^7-10^8 CFU ml^{-1}) with a hypodermic syringe (Klement et al., 1990).

Nested-PCR

Nested PCR (polymerase chain reaction) was conducted with DNA extracted from pure bacterial cultures (Schaad et al., 2001). Cells from 0.5 mL of bacterial suspension (3×10^8 CFU mL^{-1}) were used for DNA extraction. The modified method for the detection of phaseolotoxin genes was given by Güven et al. (2004). 0.5 mL of cell suspension were boiled for 15 min. The cell debris was removed by centrifugation for 10 min at 11 000 rpm. 2 µL of supernatant were used for amplification. For the first PCR primers P 5.1: 5'-AGCTTCTCCTCAAAACACCT-GC- 3' and P 3.1: 5'-TGTTCGCCAGAGGCAGT-CATG-3' were used. Primers P 5.1 and P 3.1 directed the amplification of the 500-bp DNA fragment. For the second PCR, primers P 5.2: 5'-TCGAACAT-

Table 1. Isolation of *Pseudomonas savastanoi* pv. *phaseolicola.*

Strains	Bean variety	Isolation from	Medium
TP5	Oplenac	Leaf	NA
TP6	Oplenac	Leaf	NA
TP11	Slavonski žutozeleni	Leaf	NA
TP12	Slavonski žutozeleni	Leaf	NA
TP16	Zlatko	Leaf	NA
TP17	Zlatko	Leaf	NA
TP106	Dvadesetica	Seed	MSP
TP108	Dvadesetica	Seed	MSP
TP127	Dvadesetica	Seed	MSP
TP114	Dvadesetica	Seed	MT
TP117	Dvadesetica	Seed	MT
TP118	Dvadesetica	Seed	MT
TP232	Oplenac	Seed	MSP
TP233	Oplenac	Seed	MSP
TP234	Oplenac	Seed	MSP
TP229	Oplenac	Seed	MT
TP230	Oplenac	Seed	MT
TP231	Oplenac	Seed	MT

CAATCTGCCAGCCA-3' and P 3.2: 5'-GCCTTT-TATTATTGCCGTGGGC-3' were used. Primers P 5.2 and P 3.2 directed the amplification of the 450-bp DNA fragment. A reference strain of *Psp* (Ps12) was used as a positive control and a reference strain of *Erwinia amylovora* NCPPB 595 was used as the negative control.

The PCR amplification assay was performed in a 25-µL reaction mixture containing Taq DNA polymerase 1.25 U, 50 mM KCl, 30 mM Tris-HCl, 1.5 mM Mg^{2+}, 0.1 % igepal-CA630, 200 µM dNTP, 0.4 µM of primers and 1 µL of DNA. A Mastercycler ep gradient S (Eppendorf, Germany) was used for PCR with the following profile amplifications: an initial 3 min incubation at 94°C, a manual "hot start" step at 80°C, 25 cycles (1 min at 94°C, 1 min at 58°C and 1 min at 72°C), and a final extension step of 10 min at 72°C. After the first PCR, products were diluted 10-fold and 2 µL were used for the second PCR. The amplified DNA fragments were electrophoresed in 2 % agarose gels in 1xTBE buffer and visualized with ultraviolet light after ethidium bromide staining.

PTA- and DAS-ELISA

Double-antibody sandwich (DAS)-ELISAs and plate trapped antigen (PTA)-ELISAs were performed with commercial kits by Loewe Biochemica GmbH, Germany and ADGEN Phytodiagnostics, Neogen Europe Ltd., Scotland, U.K., respectively. The assays followed the manufacturer's instructions. An ELISA reader (BIO-TEK ELx800UV), at a wavelength of 405 nm, was used for reading the results. Results for each tested strain were calculated as an average of two replications (two wells). Samples were considered positive if their absorption value was two or more times greater than that of the negative control.

Phenotypic characterization

All strains of *Pseudomonas syringae* pv. *syringae* van

Fig. 1. *Pseudomonas savastanoi* pv. *phaseolicola*, view of bacteria colonies on different media, NA (left), MT (middle), MSP (right).

Hall (*Pss*) were characterized by the following tests: KOH test, oxidase activity, catalase activity, oxidative/fermentative (O/F) metabolism of glucose, levan production on NA with 5% sucrose - NSA, nitrate reduction, gelatin, starch and esculin hydrolysis, H_2S production, indole production and acid production from carbohydrates (Goszczynska et al., 2000). *Psp* and *Pss* were distinguished by the following differential tests: esculin hydrolysis, gelatin hydrolysis and use of sorbitol, inositol and erythritol as carbon sources (Goszczynska et al., 2000). Reference strain of the bacterium *Pss* GSPB 1142 was used as control in these tests.

RESULTS AND DISCUSSION

Bacterial isolation

Psp was successfully isolated from infected bean leaves on NA and from infected seeds on semiselective media MT and MSP (Fig. 1). After three days of growth on NA, *Psp* colonies were white, small and flat, about 2 mm in diameter. After four days of incubation on MT medium, the colonies were creamy white, flat and circular unequal in size (3-5 mm in diameter). According to ISF (2006), *Psp* colonies on this medium could contain light blue fluorescent pigment. After four days of growing on MSP medium, the colonies were light yellow, convex and shiny, about 2-3 mm in diameter. Many authors recommended the semiselective medium MT and MSP for *Psp* isolation (Jansing and Rudolph, 1996; NSHS, 2002; ISF, 2006; Kurowski and Remeeus, 2008; Popović et al., 2012). *Psp* isolation from bean seeds was also performed with King B medium, KBC medium and LPGA medium (NSHS, 2002).

All tested strains were Gram-negative; they produced levan and green fluorescent pigment. These characteristics corresponded to those reported for *Psp* by Schaad et al. (2001) and CPC (2004).

Pathogenicity

On bean pods, wet and oily spots could be seen on the inoculated tissue after three days. A whitish bacterial exudate formed several days after inoculation, and the edge of the spots became reddish in color. Lelliott and Stead (1987) reported inoculation of immature bean pods for testing the pathogenicity of isolates of *Psp*, reporting the occurrence of wet spots as a typical positive reaction. To test the pathogenicity of *Psp* isolates, Balaž (1985) used a method of wounding the pod pericarp before the the grain filling stage with a syringe needle and a method of spraying of young bean pods. The latter method showed that the youngest pods were most sensitive.

Bean cotyledons showed disease symptoms five days after the inoculation. These were dark green, greasy spots around the wounded place. Ten days after inoculation, entire cotyledons were covered with small oily spots. According to the ISF (2006), chlorotic halo may develop on first true leaves of inoculated bean plants because of the activity of phaseolotoxin. Chlorotic halos were not observed in our study, which may be due to elevated temperatures in the climate chamber (20°C). According to Nüske and Fritsche (1989), the optimum temperature for the development of phaseolotoxin is 18°C.

The examined strains caused hypersensitive reaction on tobacco and geranium leaves, which developed within 24 h after inoculation. This was consistent with the results of other authors (Balaž, 1985; Van Vuurde and Van den Bovenkamp, 1989).

Nested-PCR

The results of the nested-PCR assay showed that using primer pairs P5.1/P 3.1 and P 5.2/P 3.2 directed the amplification of the 450-bp target DNA fragment (Fig. 2). The advantages of identification of *Psp* strains by PCR were also mentioned by Rico et al. (2003). According to Audy et al. (1996), there was a possibility of using PCR to detect *Psp* directly from bean seeds. The method involved rapid DNA extraction, a shorter period of seed wetting with a solution of sodium hydroxide, and the application of specific primers. With this method, it is possible to detect a single or several infected seeds in a sample of 10 000, so it can be used for easy and direct detection of *Psp* in commercial seed lots of beans (Audy et al., 1996).

PTA- and DAS-ELISA

The PTA- and DAS-ELISA tests showed that all of the examined strains reacted with antibodies specific for *Psp*. Using the ELISA test for *Psp* identification, Wyatt et al. (1989) r detected 10^4 cells mL^{-1} and pointed out that *Psp* isolates exhibit serological uniformity in contrast to other plant pathogenic bacteria. Van Vuurde and Van den Bovenkamp (1989) used immunofluorescence (IF) for *Psp* determina-

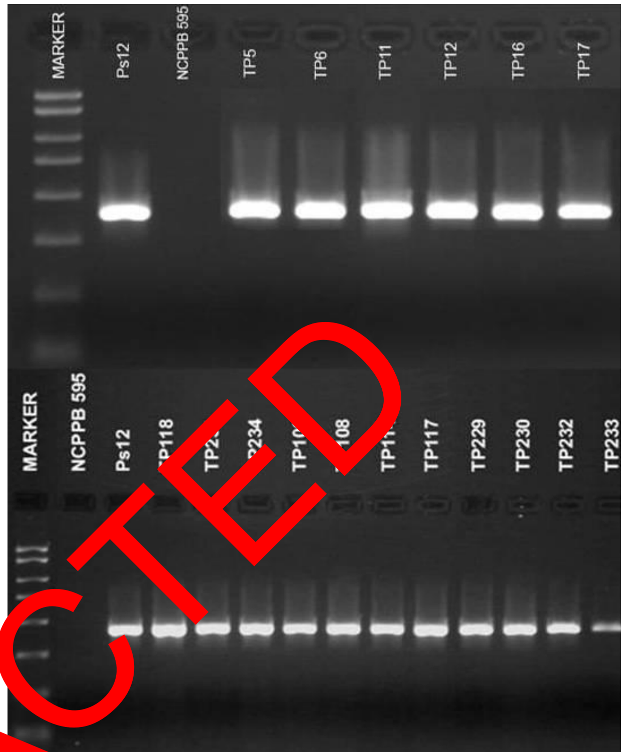

Fig. 2. Amplification of a 450 bp DNA fragment from the phaseolotoxin gene using P 5.1/P 3.1 and P 5.2/P 3.2 primers

tion in bean seeds. According to these authors, the tested method was fast, simple and inexpensive, but the reliability of test results depended on antiserum solution because IF allows the detection of 10^3 cells mL^{-1}, whereas other authors claimed to have detected 80 cells ml^{-1} in seed extract (Trigalet et al., 1978) or 10^2 (Bazzi and Calzolari, 1982). In addition, other serological methods of Psp identification can be found in the literature, such as the agglutination test (Guthrie et al., 1965) and the agar diffusion test (Guthrie et al., 1965). Various serological tests for *Psp* identification (for pure cultures or plant material) are presently available on the market (Express, Identikit, Fluorescan-IF, ELISA).

Phenotypic characterization

All strains were Gram-negative, aerobic, catalase-positive and oxidase-negative bacterium. Strains produced levan, did not reduce nitrates, did not produce indole or H_2S, did not hydrolyze gelatin, starch or esculin. All strains produced acid from glucose, mannose, sucrose, and glycerol, and did not produce acid from maltose, starch, esculin, dulcit, sorbitol, inositol and erythritol. The results suggest that the strains appear phenotypically similar. The obtained results were in agreement with those published for *Psp* by Balaž (1985, 1989), Lelliott and Stead (1987), Van Vuurde and Van den Bovenkamp (1989) and Schaad et al. (2001). Based on the responses to the differential tests described by Lelliott and Stead (1987), it was concluded that the examined strains belonged to *Psp* because they did not hydrolyze esculin or gelatin, and did not use sorbitol, inositol and erythritol as carbon sources, while the control strain of *Pss* GSPB 1142 hydrolyzed esculin and gelatin and used sorbitol, erythritol, and inositol as carbon sources.

This study describes a rapid, reliable and practical method for the routine detection and identification of *Psp* from infected bean plant and seed material.

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