USE OF THE rep-PCR TECHNIQUE FOR DIFFERENTIATING ISOLATES OF RHIZOBACTERIA

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A B S T R A C T

In this study, the rep-PCR technique was used to differentiate isolates of bacteria belonging to genus *Pseudomonas* and phosphate-dissolving bacteria collected from the root vicinity of apple and sour cherry trees. DNA amplification was carried out with complementary primers for repetitive sequences: REP (repetitive extragenic palindromic sequence), ERIC (enterobacterial repetitive intergenic consensus) and the BOX element. The most differentiated DNA profiles were observed when using REP1R-I and REP2-I primers, in reactions with which 25 different DNA patterns were obtained for 28 isolates. In reactions with the primers ERIC1R and ERIC2 or BOXA1R, 24 and 22 patterns were obtained, respectively. Following the use of all the primers, no differences were found in the DNA profiles of two isolates of *Pseudomonas* bacteria and three isolates of phosphate-dissolving bacteria. This result suggests that the isolates in which no DNA polymorphism was observed belong to the same bacterial strain.

Key words: strains of beneficial rhizobacteria, genomic fingerprint, repetitive sequences

INTRODUCTION

In the rhizosphere soil, bacteria can be found that colonize the roots and stimulate the growth and development of fruit plants (PGPR – Plant Growth-Promoting Rhizobacteria). Bacterial strains with a beneficial

effect on plants belong to various genera, for example, *Pseudomonas, Bacillus, Azotobacter* or *Rhizobium*. The beneficial action of these bacteria consists in producing and delivering growth-promoting substances to plants, facilitating the uptake of minerals from the soil, limiting the negative influence of toxic heavy metals, exerting an antagonistic action against pathogens and increasing plant resistance to abiotic stresses (Joseph et al., 2007). Isolation and selection of the beneficial strains of bacteria will make possible practical use of them in microbiological preparations for organic fruit production. The selection of beneficial bacterial strains prior to carrying out tests to determine their effect on plants requires them to be positively identified. Distinguishing bacterial isolates on the basis of physiological, biochemical and biological tests is not always successful (Olczak-Woltman et al., 2007). For example, it has been found that it is not possible to distinguish 215 of the 257 strains of *Xanthomonas campestris* pv. *poae* and pv. *graminis* by analyzing the profiles of fatty acids (Louws et al., 1994). For that reason, in order to be able to differentiate bacterial isolates, molecular biology techniques based on the PCR reaction have begun to be used.

In the genome of bacteria there are recurring DNA sequences whose arrangement, unique for each bacterial strain, forms the basis of a technique that makes it possible to differentiate isolates of bacteria (DNA fingerprint). The repetitive sequences that have been identified so far are described as REP (repetitive extragenic palindromic sequence), ERIC (enterobacterial repetitive intergenic consensus) and BOXA, a subunit of the BOX element (De Bruijn, 1992; Martin et al., 1992). Amplification of DNA fragments by means of primers complementary to repetitive sequences is called the rep-PCR technique (repetitive sequences PCR) (Louws et al., 1994).

The rep-PCR technique has been used to determine DNA profiles of the symbiotic bacteria *Rhizobium meliloti* (De Bruijn 1992), pathogenic bacteria *Pseudomonas syringae*, *Xanthomonas campestris* and *Xanthomonas oryzae* (Louws et al., 1994), *Xanthomonas campestris* (Louws et al., 1995), *Xanthomonas fragariae* (Pooler et al., 1996), thermophilic bacteria of the genera *Geobacillus*, *Anoxybacillus* and *Bacillus* (Adiguzel et al., 2009), and the entomopathogen *Bacillus thuringiensis* (Reyes-Ramirez and Ibarra, 2005).

The aim of this study was to use the rep-PCR technique to differentiate the strains of bacteria collected from the root vicinity of apple and sour cherry trees.

MATERIAL AND METHODS

Preparation of the isolates of rhizobacteria

The experimental material comprised 28 isolates of bacteria obtained from soil samples of the root vicinity of apple and sour cherry trees growing in the Ecological Orchard of the Institute of Horticulture in Nowy Dwór. Isolates of the bacte-

ria of the genus *Pseudomonas* and of the phosphate-dissolving bacteria were obtained by culturing soil solutions on Gould's substrate (Hankin et al., 1971) and Pikovska's substrate (Husen, 2003), respectively. The cultures were set up with the bacterial inoculum at a dilution of 10^{-3}. Each substrate-containing dish received 100 µl of the suspension. The bacteria were isolated from those dishes in which the number of colonies did not exceed 50. The isolates were selected on the basis of a fluorescent dye produced on the Gould's substrate (*Pseudomonas* bacteria), or a change in the colour of the Pikovska's substrate from white to transparent (phosphate-dissolving bacteria). The obtained strains were purified and then tested for pectinolytic enzyme production, in order to exclude potential pathogens, on a minimal salts medium with the addition of 5 g/l of pectin (Gould et al., 1985). The strains that did not show any signs of growth on the minimal salts medium with pectin were set aside for further studies.

DNA isolation

Extraction of DNA from bacterial colonies was carried out by means of a commercial GeneMatrix Bacterial & Yeast Genomic DNA Purification Kit (EURx) for isolating DNA from bacteria and yeast. DNA concentration in the samples was measured with a spectrophotometer at a wavelength of 260 nm. For further analyses they were diluted to final concentration of 12.5 ng DNA/µl.

PCR conditions

Amplification of DNA fragments of the selected isolates was carried out with the use of primers complementary to repetitive sequences in the bacterial genome: REP1R-I (5`-IIIICGICGICATCIGGC-3`) and REP2-I (5`-ICGICTTATCIGGCCTAC-3`), ERIC1R (5`-ATGTAAGCTCCTGGGGATTCAC-3`) and ERIC2 (5`-AAGTAAGTGACTGGGGTGAGCG-3`), and BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3`).

The reactions were carried out in Engine Dyad (Bio-Rad) DNA thermocycler. The reaction mixture (20 µl) consisted of 1x buffer for PCR, 0.2 mM $MgCl_2$, 0.25 mM of each nucleotide, 0.75 µM of each primer, 1.5 U of DreamTaq™ polymerase (Fermentas) and 25 ng DNA.

The following thermal profiles of the reactions were used:

- REP-PCR – 42 cycles (94 °C x 1 min, 52 °C x 1 min, 65 °C x 8 min);
- ERIC-PCR – 42 cycles (94 °C x 1 min, 52 °C x 1.5 min, 65 °C x 8 min);
- BOX-PCR – 37 cycles (94 °C x 1 min, 40 °C x 2 min, 72 °C x 2 min).

The products of the reactions were separated in a 2% agarose gel, stained with ethidium bromide and visualized under UV light. Each reaction was repeated three times on the same DNA matrix. Only informative and reproducible products of the reactions were analyzed.

RESULTS AND DISCUSSION

As a result of the analysis of DNA polymorphism generated in the reactions with the REP primers, the presence of 3 to 9 reaction products, from 250 to 4700 bp in size, were determined for each sample. No differences in the obtained DNA products were found between two isolates of *Pseudomonas* and between three isolates of phosphate-dissolving bacteria (Fig. 1). The reactions carried out with the ERIC primers produced for each isolate from 3 to 8 reaction products, 150 to 3500 bp in size. No differences were observed in the obtained DNA fragments between two isolates of *Pseudomonas* bacteria and within two groups of phosphate-dissolving bacteria consisting of two and three isolates, respectively (Fig. 2). After using the BOX primer, from 5 to 13 reaction products, ranging in size from 300 to 3300 bp, were obtained for each matrix. The same DNA patterns were found in two isolates of the bacteria *Pseudomonas*. A lack of differences between isolates was also found within two groups of phosphate-dissolving bacteria. One of them contained three, and the other four, isolates (Fig. 3).

The rep-PCR technique had been useful in differentiating 31 strains of *Xanthomonas* and 12 strains of *Pseudomonas* when the reactions produced from 5 to 20 differentiating PCR products in the size range of 100 to 5000 bp (Louws et al., 1994). While differentiating 15 strains of *Bacillus* and *Geobacillus* thermophilic bacteria, from 3 to 9 DNA fragments were obtained for a single strain (Adiguzel et al., 2009). The rep-PCR technique was also effective in differentiating 33 strains of soil bacteria belonging to the genera *Rhizobium*, *Agrobacterium*, *Pseudomonas* and *Escherichia.* As a result of the reactions, DNA fragments were obtained whose size ranged from 300 to 5000 bp (REP primers) or from 100 to 3500 bp (ERIC primers), on the basis of which the tested isolates could be distinguished (De Bruijn, 1992). As a result of the rep-PCR reactions carried out to analyze strains of *Bacillus thuringiensis*, from 5 to 13 DNA fragments were obtained, ranging in size from 200 to 3800 bp (Reyes-Ramirez and Ibarra 2005).

In this study, the most differentiated DNA patterns were obtained by using the REP primers. Less differentiated patterns were obtained following the use of the primers ERIC and BOX. After using all the primers, no differences in the DNA profiles were found between two isolates of *Pseudomonas* (Fig. 1, 2, 3). The use of the BOX primer helped to distinguish two groups of isolates of phosphate-dissolving bacteria, one consisting of three and the other of four isolates that did not differ within their respective groups (Fig. 1). After using the ERIC primers, two DNA patterns were observed in each of these groups of isolates (Fig. 2). In the reaction with REP primers, two DNA profiles were found for one of the groups of isolates, like in the ERIC-PCR reaction. The other group of isolates was differentiated as three separate bacterial strains (Fig. 3).

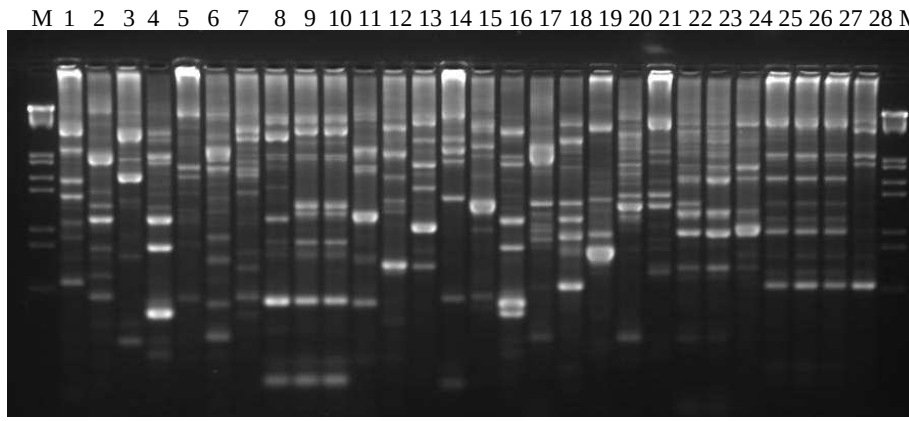

Figure 1. DNA patterns of rhizobacterial isolates generated in the reactions with REP1R-I and REP2-I primers. Lanes 1-12: isolates of *Pseudomonas,* lanes 13-28: isolates of the phosphate-dissolving bacteria M – Marker λ DNA EcoRI/Hind III

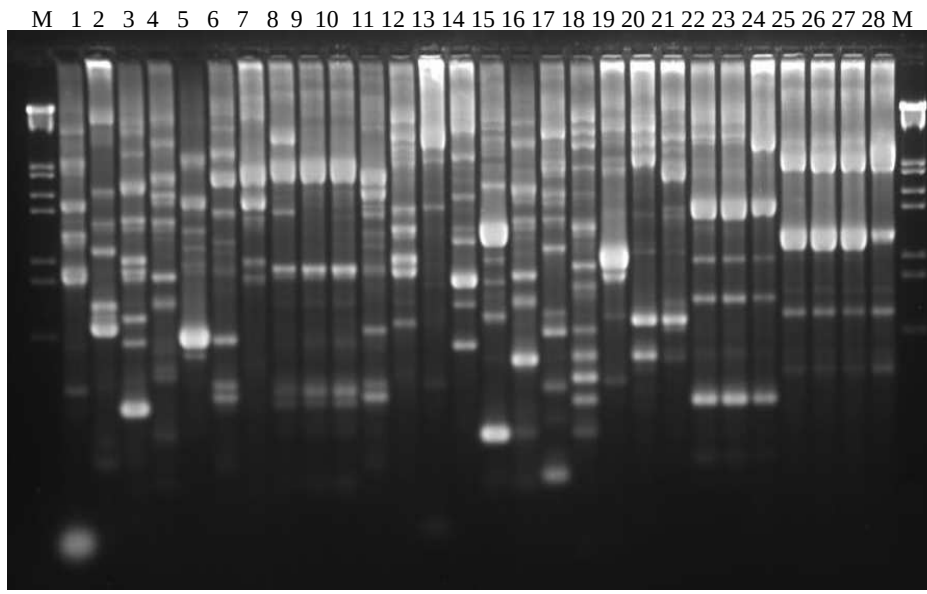

Figure 2. DNA patterns of rhizobacterial isolates generated in the reactions with ERIC1R and ERIC2 primers. Lanes 1-12 isolates of *Pseudomonas,* lanes 13-28 isolates of the phosphate-dissolving bacteria. M - Marker λ DNA EcoRI/Hind III

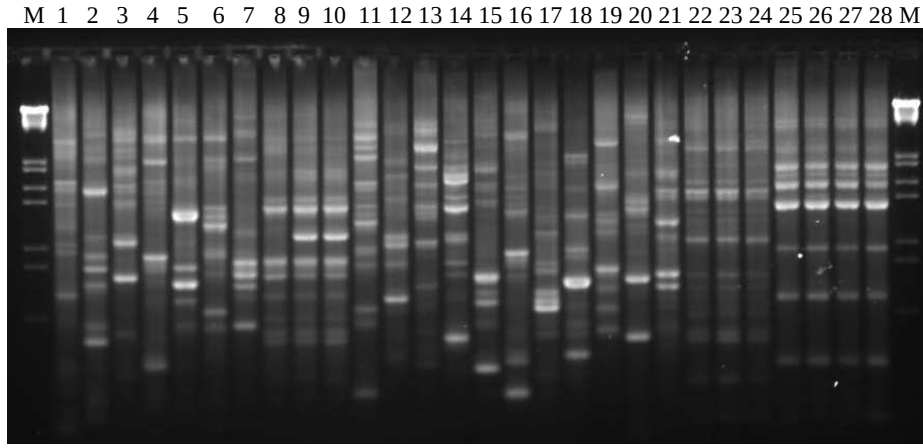

Figure 3. DNA patterns of rhizobacterial isolates generated in the reactions with the BOXA1R primer. Lanes 1-12 isolates of *Pseudomonas*, lanes 13-28 isolates of the phosphate-dissolving bacteria M – Marker λ DNA EcoRI/Hind III

The reaction with the BOX primer resulted in the largest number of DNA fragments obtained; however, no polymorphism could be demonstrated within the group of the tested isolates, which could be distinguished by means of the other primers. This outcome indicates greater usefulness of ERIC and REP primers for differentiating isolates of bacteria collected from the root vicinity of apple and cherry trees. REP primers had proved to be more useful than the BOX primer for distinguishing *Geobacillus* strains (Meintanis et al., 2008). A different result was obtained in differentiating *Geobacillus* and *Bacillus* strains, where the BOX primer was found to be of greater usefulness for differentiating these bacterial strains than REP and ERIC primers (Adiguzel et al., 2009). Louws et al. (1995) found that ERIC and BOX primers were more useful than REP primers for differentiating the strains of *Xanthomonas*.

The DNA patterns obtained in this study were all different for the isolates originating from different samples of the soil, which indicates wide diversity among the isolates of *Pseudomonas* and phosphate-dissolving bacteria. A similar result had been obtained with the rep-PCR technique, where no fragments of the same size were found for 7 strains of *Pseudomonas*, which is an indication of high diversification within this genus (De Bruijn, 1992).

The reactions that were carried out in this study did not make it possible to differentiate two isolates of the bacteria *Pseudomonas* and three isolates of the phosphate-dissolving bacteria. This outcome suggests that the isolates in which no polymorphism was observed belong to the same bacterial strain. The tests confirmed the usefulness of the rep-PCR

technique for differentiating isolates of rhizobacteria. The results of this study will find application in the selection of bacterial strains which are to be used in tests to determine their effect on the growth and development of fruit plants.

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ZASTOSOWANIE TECHNIKI rep-PCR DO ODRÓŻNIANIA IZOLATÓW BAKTERII RIZOSFEROWYCH

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S T R E S Z C Z E N I E

W pracy zastosowano technikę rep-PCR do odróżnienia izolatów bakterii *Pseudomonas* oraz bakterii rozpuszczających związki fosforu, pozyskanych z gleby ze strefy korzeniowej drzew jabłoni i wiśni. Amplifikację DNA wykonano z użyciem starterów komplementarnych do sekwencji powtarzalnych: REP (repetitive extragenic palindromic sequence), ERIC (enterobacterial repetitive intergenic consensus) oraz elementu BOX. Najbardziej zróżnicowane profile DNA obserwowano używając starterów REP1R-I i REP2-I, w reakcjach z którymi uzyskano 25 wzorów DNA dla 28 izolatów. W reakcjach ze starterami ERIC1R i ERIC2 (24) oraz BOXA1R (22) otrzymano odpowiednio 24 i 22 wzory. Po zastosowaniu wszystkich starterów nie stwierdzono różnic w profilach DNA dwóch izolatów bakterii z rodzaju *Pseudomonas* oraz trzech izolatów bakterii rozpuszczających związki fosforu. Wynik ten sugeruje, że izolaty, u których nie obserwowano polimorfizmu DNA, należą do tego samego szczepu bakterii.

Słowa kluczowe: szczepy bakterii rizosferowych, markery molekularne, sekwencje powtarzalne