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EXPERIMENTAL ARTICLES

Phenol Oxidase Activity of *Azospirillum brasilense* Sp245 Mutants with Modified Motility and *Azospirillum brasilense* Sp7 Phase Variants with Different Plasmid Composition

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Abstract—Herein, we reveal the alteration in phenol oxidase enzymes complex production from *Azospirillum brasilense* Sp245 omegon mutants with polar and lateral flagella dysfunction and from *A. brasilense* Sp7 phase variants with different plasmid composition. The enzymatic activities for various laccases, tyrosinases, Mn-peroxidases, and lignin peroxidases as well as the isomorphic composition of intracellular laccases and tyrosinases were estimated for the studied variants and the parent strains. It was noted that various genetic events correlating with phenotypic heterogeneity in *A. brasilense* populations affect their phenol oxidase activity level.

Keywords: Azospirillum, plasmid rearrangements, omegon mutants, phenol oxidase activity, laccase, tyrosinase, Mn-peroxidase, lignin peroxidase

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Diazotrophic bacteria of the genus *Azospirillum* are intensively studied objects in the field of plant-microbe interaction owing to their potentially high nitrogen-fixing activity and the ability to produce plant hormones and other physiologically active substances (Saikia et al., 2012). Azospirilla are able to maintain the physiologically active condition in the rhizosphere, rhizoplane, and in internal parts of the root of the host plant. It is known that phenolic compounds are among the limiting factors in the process of rhizobacterial survival (Dutta and Podile, 2010). Substances of aromatic nature are specific signals that induce chemotaxis of rhizobacteria (Somers et al., 2004; Shaw et al., 2006; Kumar et al., 2007). However, aromatic compounds are also the main toxicants since in the formation of symbiotic relationship the protective mechanism of the macrohost is activated similar to the plant–pathogen reaction (Narula et al., 2009). To overcome the protective barrier of plant polyphenols, azospirilla require the ability to synthesize specific enzymes involved in detoxification of these compounds.

A group of enzymes that are able to oxidize aromatic compounds is commonly termed phenol oxidases (Rabinovich et al., 2004; Sinsabaugh, 2010). For the first time the presence of the polyphenol oxidase activity in azospirilla has been reported in the literature for the species *A. lipoferum* indicating the presence of intracellular laccases activity only in the variants with changed motility (Givaudan et al., 1993; Faure et al., 1994; Diamantidis et al., 2000). Later we discovered a number of strains of this genus with phenol oxidase complex that includes, in addition to laccase, tyrosinase, manganese peroxidase, and lignin peroxidases (Nikitina et al., 2010). In our view, it is interesting to investigate whether *Azospirillum* mutants with modified motility can produce phenol oxidase enzymes.

Constructive metabolism of *Azospirillum*, adapting them to the dynamic conditions of the rhizosphere, is determined by a large genome, which, apart from the chromosome, contains numerous plasmids (Katsy, 2011). In the works of Petrova et al. (2005, 2010), by the example of the strain *A. brasilense* Sp7 and its derivatives it was shown that spontaneous changes in plasmid composition had a negative impact on biofilm formation and also affected the level of bacterial resistance to ampicillin and surfactants. The authors suggested that coordinated expression of the complete set of plasmid genes, that affect a wide range of cell structures and functions, is important for more rapid adaptation to a new environment (Petrova et al., 2010). In this regard, it was appropriate to study phenol oxidase activity of these variants.

Thus, the goal of the present work was to analyze the changes in the activity of the enzymes of phenol

oxidase complex of omegon mutants of *A. brasilense* Sp245 with altered motility and in intrapopulation variants of the strain *A. brasilense* Sp7.

MATERIALS AND METHODS

Organisms and cultivation conditions. Bacterial strains *A. brasilense* Sp245 and *A. brasilense* Sp7 from the collection of rhizosphere microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Science (IBPPM RAS) were used in the work and their mutants were obtained by the staff of the laboratory of genetics, IBPPM RAS. Omegon mutants of *A. brasilense* Sp245: *A. brasilense* SK039 and SK454, and spontaneous phase variants *A. brasilense* Sp7: *A. brasilense* Sp7.1, Sp7.4 (Shelud'ko et al., 1998; Petrova et al., 2005; Kovtunov et al., 2013).

Cultivation of bacteria was carried out in Erlenmeyer flasks (100 mL) in liquid malate-salt medium of the following composition (g/L): KH_2PO_4, 0.1; K_2HPO_4, 0.4; NaCl, 0.1; $Na_2MoO_4 \cdot 7H_2O$, 0.002; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.02; malic acid, 5.0; NaOH, 1.7; NH_4Cl, 1.0; $CaCl_2$, 0.02 (pH 6.8). The medium was sterilized for 30 min at 121°C. As inductors, 1 mM $MnSO_4 \cdot 5H_2O$; 0.1 mM $CuSO_4$, and guaiacol (Acros Organics, United States) at the final concentration of 0.1 mM were added into the medium. The inoculum was taken from one-day culture grown in the medium of the same composition. Bacteria for the study were cultivated in an incubator at 37°C for 48 h.

Determination of enzymatic activity. The cells were precipitated by centrifugation; the supernatant was used to determine extracellular enzymatic activity. The cells were washed from the medium with phosphate buffered saline (pH 7), homogenized by ultrasound for 3–5 min, centrifuged, and the supernatant was used for determination of the activity of intracellular enzymes. Enzyme activity was detected spectrophotometrically on a Specord M 40 (Carl Zeiss, Germany).

The activity of the Mn-peroxidase was determined by the rate of oxidation of 2,6-dimethoxyphenol (Acros Organics, United States, $\varepsilon = 30.5\ mM^{-1}\ cm^{-1}$) at 30°C (Paszczynski et al., 1988). The reaction mixture contained: 50 mM sodium tartrate buffer (pH 4.5), 1 mM 2,6-dimethoxyphenol, 1 mM $MnSO_4 \cdot 5H_2O$, and a sample aliquot; the reaction was started by adding 100 μL of 1 mM H_2O_2.

The activity of lignin peroxidase was determined by the rate of oxidation of veratric alcohol (Acros Organics, United States) to veratraldehyde at the wavelength of 310 nm at 30°C ($\varepsilon = 9.3\ mM^{-1}\ cm^{-1}$) (Orth et al., 1993). The reaction mixture contained: 0.1 mM sodium tartrate buffer (pH 3.0), 2 mM veratric alcohol, and a sample aliquot; the reaction was started by adding 200 μL of 0.4 mM H_2O_2.

The activity of laccase was determined by the rate of oxidation of 2,6-dimethoxyphenol to a stable cation radical at the wavelength of 468 nm at 30°C (Edens et al., 1999). The reaction mixture contained: 50 mM sodium tartrate buffer (pH 4.5), 0.2% 2,6-dimethoxyphenol, and a sample aliquot.

The activity of tyrosinase was determined by the rate of oxidation of L-3-(3,4-dihydroxyphenyl)alanine (L-DOPA) (Acros Organics, United States, $\varepsilon = 3.7\ mM^{-1}\ cm^{-1}$) at 475 nm at 30°C (Horowitz et al., 1970). The reaction mixture contained: 50 mM Tris-HCl buffer (pH 7.5), 2 mM L-DOPA, and a sample aliquot.

The amount of the enzyme catalyzing conversion of 1 μM substrate in 1 min was taken as a unit of activity. Specific activity was expressed in units per 1 mg of protein. Protein concentration was assayed by the Bradford method (Bradford, 1976).

Native (nondenaturing) electrophoresis in 7.5% polyacrylamide gel was carried out on the VE-4M device for vertical electrophoresis (Helicon, Russia) according to the Laemmli method (Laemmli, 1970) but without SDS, β-mercaptoethanol, and subsequent boiling. Visualization of the protein bands with enzymatic activity was performed using specific chromogenic substrates: L-DOPA for tyrosinase and 2,6-dimethoxyphenol for laccase activities.

Statistical processing of the results. All experiments were performed in at least three replicates in three independent experiments. In the evaluation of the obtained results the method of calculating the standard deviation of the mean with the program Microsoft Office Excel 2010 was used; data have corresponding confidence intervals at the confidence level 0.95.

RESULTS AND DISCUSSION

Ability to produce laccase, tyrosinase, manganese and lignin peroxidases in *A. brasilense* Sp245 and its omegon mutants deficient in the production of flagella. The strain *A. brasilense* Sp245 has a mixed type of flagellation; it produces the polar flagellum in media of different density and numerous lateral flagella in the media with concentration of agar ≥0.4%. Omegon insertion mutagenesis of *A. brasilense* Sp245 was used to obtain the strains with altered motility and flagellation (Shelud'ko et al., 1998). SK039 is mutant for the putative gene of 3-hydroxy isobutyrate dehydrogenase (*mmsB1*) and has defects in the formation of polar flagella (Kovtunov et al., 2013). Inactivation of the putative gene of lipid metabolism *mmsB1* also influenced some characteristics of the bacterial cell surface including changes in the relative content of some fatty acids in lipopolysaccharide preparations, relative hydrophobicity, and dynamics of the aggregation of planktonic cells (Shumilova et al., 2016). The mutant

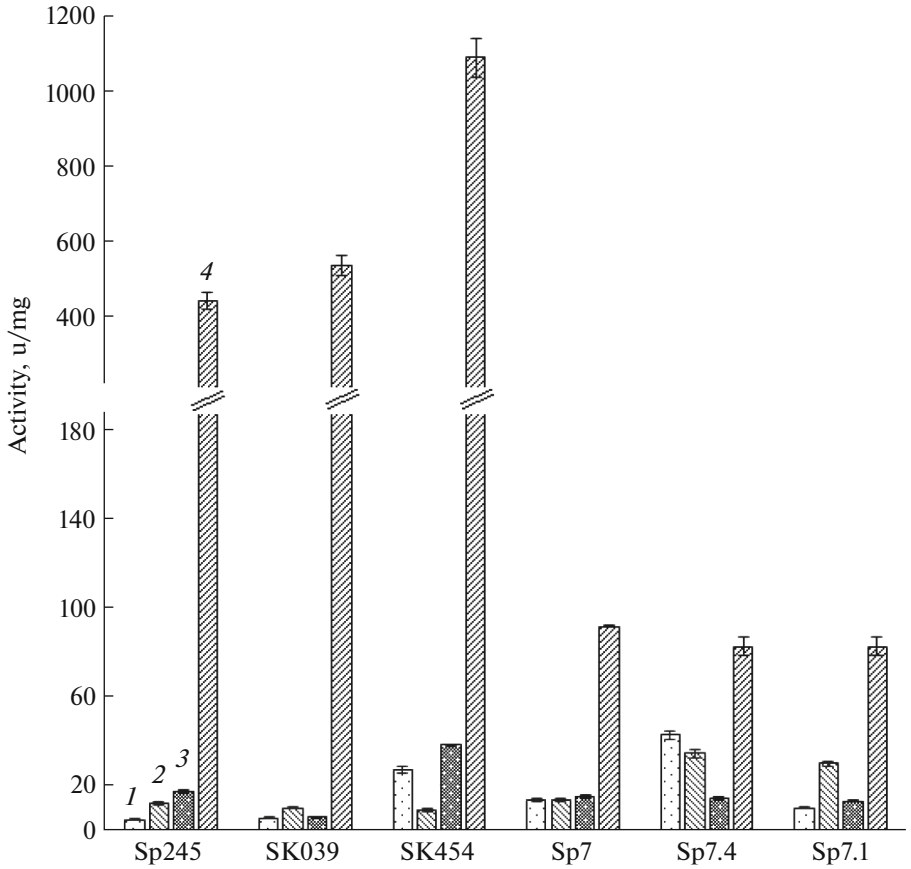

Fig. 1. Enzymatic activity in culture fluid and intracellular extracts of *A. brasilense*: (*1*) intracellular laccase activity; (*2*) extracellular laccase activity; (*3*) intracellular tyrosinase activity; (*4*) extracellular tyrosinase activity.

SK454 is deprived of the ability to form polar flagellum; however, in dense media a small portion of the cells in the population have lateral flagella. For SK454 a decline in the capacity for adsorption on the roots of wheat compared with the wild type was showed but significant changes in the cell surface of this strain were not detected (Shelud'ko et al., 2010). The parent strain *A. brasillense* Sp245 and the mutants had the same plasmid composition. The loss of ability to move was common for these omegon mutants, namely, the lack of motility when cultured in liquid medium and the inability to form swarming rings by means of flagellar motility in semisolid media (Shelud'ko et al., 1998; Shelud'ko and Katsy, 2001; Kovtunov et al., 2013; Shumilova et al., 2016).

In the motile strain of *A. brasilense* Sp245 and its omegon mutants defective in motility the ability to produce phenol oxidase enzymes including both the extracellular and intracellular forms of laccases, tyrosinases, and manganese and lignin peroxidases, was detected using specific substrates (Figs. 1, 2). In the study of the activity of oxidases in the culture liquid and in cell extracts, approximately the same level of production of extracellular and intracellular laccases and tyrosinases was revealed in the parent and mutant strains. However, for the strain SK454 doubling of the extracellular tyrosinase activity and a more than five-fold increase in intracellular laccase activity (Fig. 1) compared to the wild type were found. SK454 is the most nonmotile of the studied strains since its cells are deprived of the ability to form a polar flagellum both in liquid and on solid media (Shelud'ko et al., 1998; Shelud'ko and Katsy, 2001).

Using the method of electrophoretic separation of proteins in polyacrylamide gel (PAGE) and specific staining with chromogenic substrates made it possible to visualize the protein bands with tyrosinase and laccase activity in cell extracts of *A. brasilense* strains Sp245, SK039, and SK454 (Figs. 3, 4). As can be seen from the presented zymograms, *A. brasilense* Sp245 and SK454 produced one form of laccase with approximately the same molecular weight. In the case of SK039 two protein bands with laccase activity were visualized, one of which differed in weight from the laccases of Sp245 and SK039 (Fig. 3). Specific L-DOPA staining of the gel plate revealed the presence of two forms of extracellular tyrosinases in *A. brasilense*

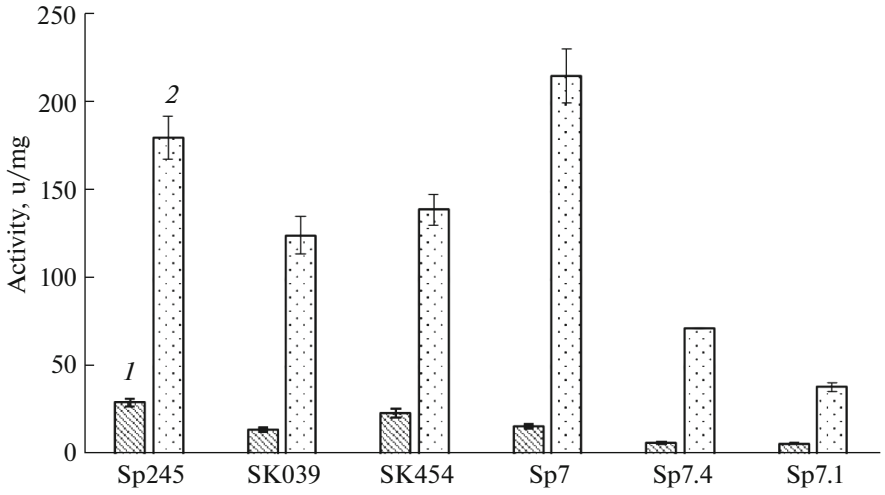

Fig. 2. Extracellular lignin peroxidase (*1*) and Mn-peroxidase (*2*) activity of *A. brasilense* strains.

Sp245 and the loss of the isoform with higher molecular weight in its mutants (Fig. 4).

In 1993–1994 a group of scientists in the study of *A. lipoferum* mutants with modified motility showed that nonmotile *A. lipoferum* 4T and *A. lipoferum* 4T and 4 Bp (spontaneous variant of the strain *A. lipoferum* 4B) had laccase activity in contrast to the motile *A. lipoferum* 4B (Givaudan et al., 1993; Faure et al., 1994). In our study, it was found that the motile strain *A. brasilense* Sp245 and its omegon mutants defective in the formation of polar and/or lateral flagella were able to synthesize extracellular and intracellular laccases and tyrosinases. However, the strains with a mutation associated with the loss of motility had altered production of phenol oxidases, as was evidenced by the increase of enzymatic activity (SK454) and isomorphic composition (SK039) of phenol oxidases.

Previously we discovered the ability of azospirilla to decompose lignin-like compounds mediated by the action of lignin and Mn-peroxidases (Kupryashina et al., 2015); we also hypothesized that these enzymes along with laccases and tyrosinases performed a protective function in the relationship of the bacteria with the host plant during formation of the symbiotic relationships (Kupryashina et al., 2015). The enzymatic activity of peroxidases in the phenol oxidase complex of *A. brasilense* Sp245 and its omegon mutants is shown on Fig. 2. The obtained data indicate a decrease in the activity of lignin- and Mn-peroxidases in the mutant strains. It is probable that the production of these enzymes may be reduced in the strains with altered motility, and, consequently, with the changed strategy of interaction with a macropartner.

Ability to produce laccase, tyrosinase, manganese and lignin peroxidases in *A. brasilense* Sp7 and its spontaneous variants. Bacteria of the species *A. brasilense* have a multicomponent genome often undergoing spontaneous rearrangements, in particular, leading to changes in the plasmid profiles of the strains (Petrova et al., 2005; Katsy et al., 2011). The type strain *A. brasilense* Sp7 has plasmids with molecular weight of 90 MDa (pRhico), 115 MDa (p115) and over 300 MDa. Sequenced and annotated 90 MDa plasmid pRhico (also termed p90) from *A. brasilense* Sp7 contains the genes whose products are involved in chemotaxis, motility, as well as synthesis and export of polysaccharides, and in the interaction of bacteria with plant roots (Vanbleu et al., 2004).

Variants of the type strain *A. brasilense* Sp7 (Sp7.1 and Sp7.4), which lost the 115 MDa plasmid and differed from the wild type by the apparent molecular weight of the pRhico plasmids, (124 and 131 MDa, respectively), were previously selected (Petrova et al., 2005). Spontaneous plasmid rearrangements in the variants of the strain Sp7 were accompanied by variations in morphology, colony color, intensity of pigment formation, and changes in the activity of biofilm formation (Petrova et al., 2005, 2010). It was shown that while the mutants of Sp7.1 and Sp7.4 less actively colonized plant roots during the first hours of interaction, their flagellation and motility in liquid media did not differ from those of the strain *A. brasilense* Sp7 (Petrova et al., 2010). Comparative hybridization and PCR analysis of the DNA from the strain *A. brasilense* Sp7 and its variants using a collection of primers to pRhico and ΦAb-Cd prophage demonstrated the diversity of spontaneous genetic rearrangements, which in some cases affected not only the different loci

ot pRhico, p115, but also the ~376-MDa megaplasmid, in which ΦAb-Cd prophage is localized (Petrova et al., 2005; Katsy and Petrova, 2015). The existence of plasmid rearrangements in bacteria as a form of intrapopulation variability determines emergence of the individuals and clones different from the original type by a number of characteristics, particularly, increased resistance of bacteria to various biotic and abiotic factors.

The results of our experiments showed that the activity of lignin- and Mn-peroxidases of the strains with altered plasmid composition was significantly lower the corresponding enzymatic activity of the parent strain. The production of extracellular lignin- and Mn-peroxidases from Sp7.4 and Sp7.1 was more than two times lower compared to *A. brasilense* Sp7 (Fig. 2).

When growing bacteria on solid agar media we noted the appearance of a brown pigmentation of colonies of *A. brasilense* Sp7.4 while colonies of the parent strain as well as of the Sp7.1 variant at the same culture conditions had a creamy white color (Fig. 5). Dark brown color of the colonies of Sp7 was also noted in the work of Katsy and Petrova (2015). In almost all cases pigmentation of fungi and bacteria is associated with tyrosinase activity (Claus and Decker, 2006). According to reports tyrosinase is involved in melanin synthesis, which is included in the mechanisms of resistance to the radiation of visible light and ultraviolet (Halaouli et al., 2006). However, we did not record significant differences in the enzymatic activity of extracellular and intracellular tyrosinases in *A. brasilense* Sp7 and its spontaneous variants (Fig. 1). Data of native electrophoresis and specific staining for tyrosinase indicate that there was a loss of one of the isoforms of extracellular tyrosinases in the strains Sp 7.1, Sp 7.1 (Fig. 4).

At the same time laccase activity both in the culture fluid and in the cell extracts in the Sp7.4 was by more than two times higher than the enzymatic activity of the Sp7 (Fig. 1). For Sp7.1 the increased activity of extracellular laccase and the reduced activity of this enzyme in the cell extracts was noted in comparison with the parent strain. Our findings are consistent with the work of Faure et al. where for *A. lipoferum* strain 4T the correlation between intracellular laccase activity and the level of bacterial melanization was noted (1994). Specific staining of PAGE for the laccase activity made it possible to detect the difference in the production of intracellular laccases from Sp7, Sp7.4, and Sp7.1 (Fig. 3). In *A. brasilense* Sp7 one of the isoforms of the enzyme was visualized. For variants with altered plasmid profile the presence of new laccase isoform was observed; the variant Sp7.4 also retained the isoform of the enzyme of the parent strain and Sp7.1 lost it.

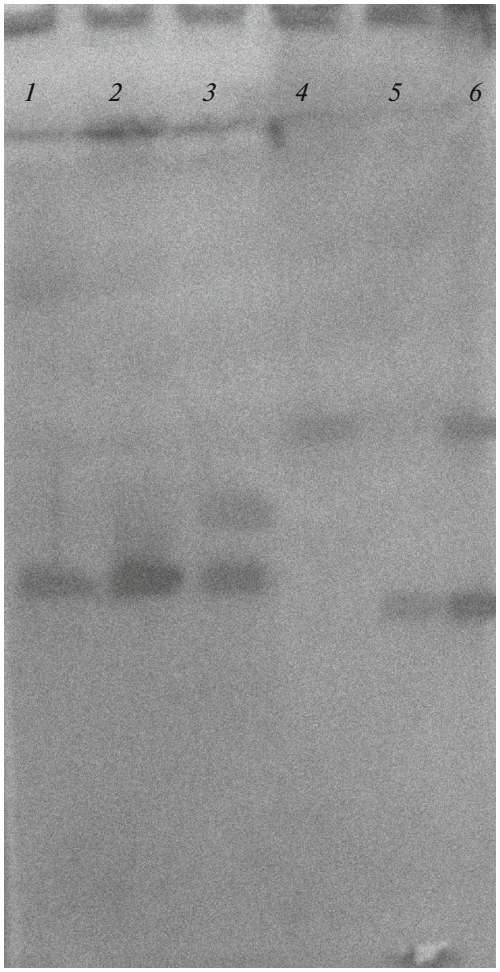

Fig. 3. PAGE of intracellular extracts from different strains of *A. brasilense* and specific staining for laccase activity: (*1*) Sp245; (*2*) SK454; (*3*) SK039, (*4*) Sp7; (*5*) Sp7.1; (*6*) Sp 7.4.

Thus, in the study the data on the ability to produce laccases, tyrosinases, lignin- and Mn-peroxidases in strain *A. brasilense* Sp245 and its omegon mutants SK454, SK039, as well as in the model strain *A. brasilense* Sp7 and its intrapopulation variants Sp7.1 and Sp7.4 with a modified plasmid profile were obtained. It was noted that various genetic events correlating with phenotypic manifestation of the heterogeneity of populations of *A. brasilense* and affecting the motility of the cells, social behavior, namely, the biofilm formation and colonization of plants (Petrova et al., 2005, 2010; Shelud'ko et al., 2010) also affect the level phenol oxidase activity of the bacteria. It can be assumed that the strategies of survival and interaction with the host plant of *Azospirillum*, wild strains and their variants with different phenotypic changes, in one way or another are connected with the action phenol oxidase enzymes, owing to which the mechanisms

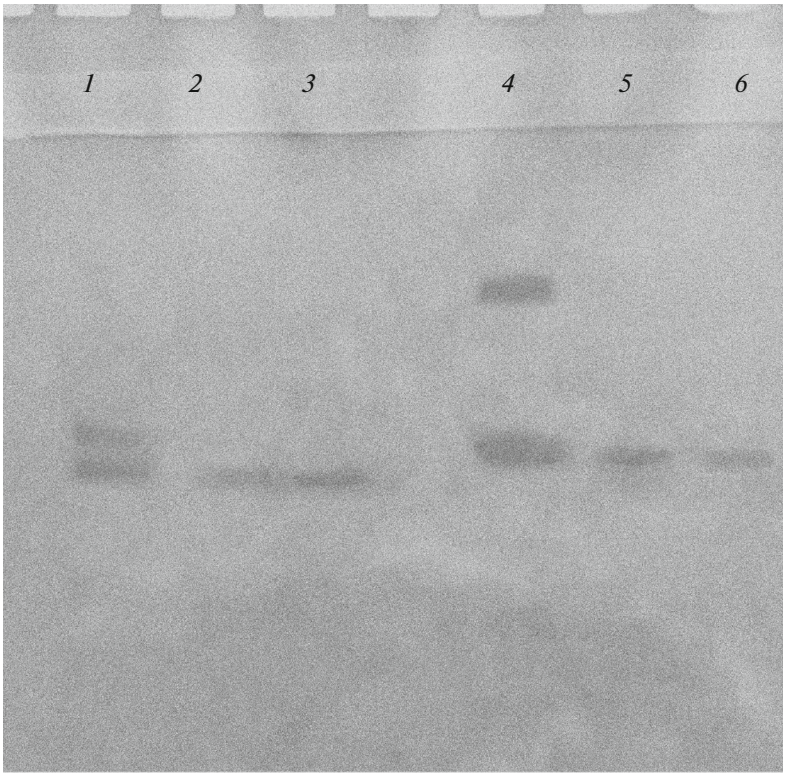

Fig. 4. PAGE of intracellular extracts from different strains of *A. brasilense* and specific staining for tyrosinase activity: (*1*) Sp245; (*2*) SK454; (*3*) SK039, (*4*) Sp7; (*5*) Sp7.1; (*6*) Sp7.4.

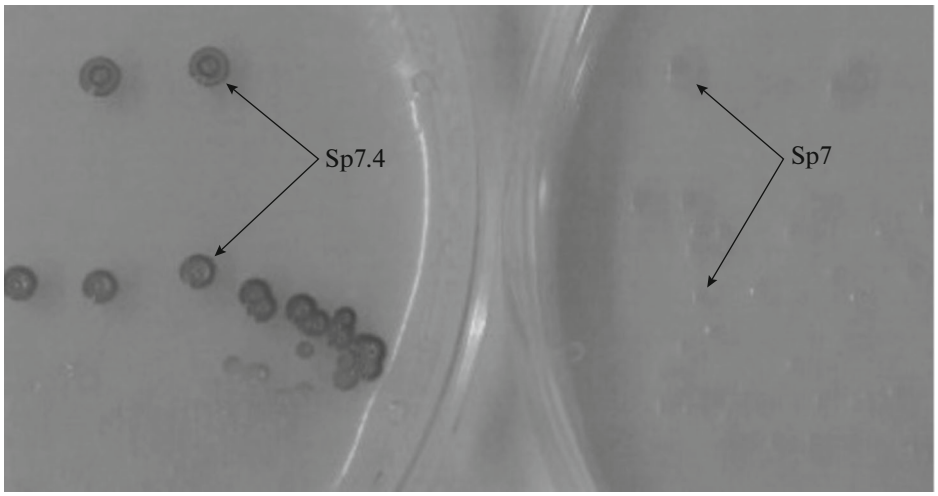

Fig. 5. Colonies of *A. brasilense* Sp7 and Sp7.4 on the solid nutrient medium.

of resistance are realized whether it is an overcome of the soil phenolic barrier, the toxic effect of secondary metabolites of the host plant or limiting biotic and abiotic factors.

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