BIOLOGICAL AND MOLECULAR CHARACTERISTICS OF IRANIAN BIOVARS 2A AND 2T OF *RALSTONIA SOLANACEARUM*

V. Hasabi[1] and S.M. Taghavi[2]

[1] *Department of Plant Protection, Isfahan University of Technology, Isfahan, Iran*
[2] *Department of Plant Protection, College of Agriculture, Shiraz University, Shiraz, Iran*

SUMMARY

The biological and molecular characteristics of 8 strains of biovars 2A of *Ralstonia solanacearum* from the Iranian province of Isfahan and of 7 isolates of biovar 2T from the province of Khuzestan were investigated morphologically, physiologically, biochemically, and using pathogenicity tests, BOX-PCR, ERIC-PCR and RFLP. Biovar 2A strains showed irregular, creamy-white and highly fluid colonies with pink center on TZC medium, slow spreading necrosis in hypersensivite reaction (HR) after 48 h, produced melanin in tyrosin test, were less pectolytic and did not utilize ribose and tryptophan. By contrast, biovar 2T strains had small size, less fluid, round and deep red colonies with a thin white margin, did not produce melanin, had a higher pectolytic activity, rapid HR on tobacco leaves after 24 h and utilized ribose and tryptophan. Comparative analysis of the BOX-PCR and ERIC-PCR fingerprint of the 15 strains at the 45% similarity level, revealed two main clusters. comprising all biovar 2A strains (cluster 1) or all biovar 2T strains (cluster 2). RFLP profiles of both biovers did not show any polymorphism. Typically, biovar 2A isolates were more pathogenic and bacterial density was higher in the xylem of all hosts tested. There was no significant difference in biovar 2A and 2T survival in loamy-clay soil, which was up to 186 days under controlled conditions.

Key words: Biovars 2A and 2T, rep-PCR, *Ralstonia solanacearum.*

INTRODUCTION

Ralstonia solanacearum (E.F. Smith) Yabuuchi *et al.* (1995), is the causal agent of bacterial wilt, a devastating disease in most tropical and subtropical and some warm temperate areas (Grimault *et al.*, 1994; Hayward 1991) which can also occur in cool temperate areas (French and Martin, 1985; Janse, 1996). The disease was recorded in several hundred plant species distributed in more than 50 families (Hayward, 1991). Many economically important crops such as potatoes, tomatoes, and bananas are affected.

R. solanacearum is a complex taxonomic unit whose strains display a great diversity at different levels, i.e. physiological, serological, genetic, and in host range (Buddenhagen *et al.*, 1962; Hayward 1991). To describe this intraspecific variability, several classification systems have been proposed. Thus, the species was subdivided into five races according to the host range (Buddenhagen *et al.*, 1962; He *et al.*, 1983) and into six biovars based on the utilization of three disaccharides (maltose, lactose, and cellobiose) and three hexose alcohols (mannitol, sorbitol, and dulcitol) (Hayward, 1964; Hayward *et al.*, 1992). Although there is no general correlation between races and biovars, strains of biovar 2 strains almost always belong to race 3.

RFLP has been used to study the relationship of *R. solanacearum* strains representing three races and five biovars, separating this species into two major groups, the "Asiaticum" and the "Americanum" divisions, comprising strains from Asia and America, respectively (Cook *et al.*, 1989, 1991). Further investigations comparing sequences of 16S rRNA and of the *hrp* gene region (Taghavi *et al.*, 1996; Fegan and Prior, 2005) and AFLP (Poussier *et al.*, 2000) supported the separation according to 51 geographic origin. More recently, Fegan and Prior (2005) proposed a new classification system based on sequence analysis of the 16S-23S internal transcribed spacer (ITS), endonuclease gene and *hrp* genes and divided the *R. solanacearum* complex into four phylotypes.

Hayward *et al.* (1992) identified a tropical variant of biovar 2 (N2 or 2T), now referred to as the sixth biovar. Biovar 2A/race 3 is the causal bacterial wilt of potato in highlands and cool climates and has a wide distribution (Hayward, 1994; Janse, 1996). Biovar 2T is found in tropical lowland like the Brazilian and Peruvian belt of South America, Africa, Japan and Indonesia (Hayward, 1994; Marin and El-Nashaar, 1993) and has also been reported from Iran (Nouri *et al.*, 2006; Irandoust *et al.,* 2008). The purpose of this study was to compare the biological and

Corresponding author: V. Hasabi
Fax: +98.2188309358
E-mail: Vahideh_Hasabi@yahoo.com

molecular characteristics *R. solanacearum.*biovars 2A and 2T from Iran.

MATERIALS AND METHODS

Bacterial strains. A total of 15 previously isolated and identified strains of biovars 2A and 2T of *R. solanacearum* (Nouri *et al*., 2006) were used in this study (Table 1). Strains of biovars 2A were collected from the potato-growing areas of Isfahan province and strains of biovars 2T were isolated from the main potato and tomato-growing areas of Khouzestan province.

Morphological, physiological and biochemical tests. Each bacteria strain was streaked on triphenyl tetrazolium chloride (TZC) medium (Kelman, 1954) and incubated at 28ºC for 48 h. Physiological and biochemical tests including Gram stain, oxidase, catalase, nitrate reduction, Tween 80, starch and gelatin hydrolysation, tyrosine, levan production, pectinase production and carbohydrate oxidation were conducted according to the methods described by Schaad *et al.* (2001) and Hayward (1964).

Hypersensivity reaction. The ability of strains to induce hypersensivite reaction (HR) on tobacco leaves (*Nicotiana tabacum* cv. White Burley), was tested according to Lozano and Sequeira (1970). The fully expanded leaves were infiltrated by injecting a bacterial suspension (OD_{600} nm = 0.5 corresponding to 10^6-10^8 CFU ml^{-1}) into the intercellular spaces with a hypodermic syringe fitted with a fine needle. Reactions were recorded after 8, 12, 24, 48 and 72 h.

Pathogenicity tests. *Hosts.* For pathogenicity tests potato (cvs. Agria, Arinda, Labida, Sante, Marfona and Ramus), tomato (cv. Early urbana VF), eggplant (cv. Depressum), geranium (*Pelargonium x hortorum*) and petunia (*Petunia hybrida*) plants were used. Seedlings were transplanted in plastic pots (8 cm diameter) containing a soil-vermiculite mixture, and grown in a greenhouse under two different temperature conditions, i.e. 25-31°C and 39-45°C. Relative humidity (RH) was 70%.

Inoculation. Bacterial strains were grown on TZC agar for 2 days at 28°C, suspended in sterile distilled water (OD_{600} nm = 0.5 corresponding to 10^6-10^8 CFU ml^{-1}). Plants at the fourth to fifth true-leaf stage were inoculated by puncturing the basal part of the stem with a needle dipped in inoculum. Four plants of each host were inoculated with each strain and, together with controls inoculated with water, were placed in a greenhouse at 25-31°C and 39-45°C, under natural light conditions and 70% RH. Pots were arranged in a completely randomized way and the plants were evaluated every second day, up till the 28th day post inoculation scoring the percentage of wilted leaves with an arbitrary scale where: 0 = no leaves wilted, 1 = 1-25% wilted, 2 = 26-50% wilted, 3 = 51-75% wilted and 4 = all leaves wilted or dead (Swanson *et al*., 2005). To determine bacterial density in the xylem, a piece of stem 2-3 cm long was cut from the base and placed in a glass container with 9 ml sterile water. Within 18 h, 10-fold serial dilutions were made, streaked on TZC plates in triplicate and incubated at 28°C for 48 h. Typical colonies of *R. solanacearum* were counted and total CFUs were calculated per gram of fresh matter and were log-transformed (Grimault *et al.*, 1994).

Statistical analysis. The Statistic Analysis System program (SAS/IML, version 6.10) was used for analysis of variance (ANOVA) followed by LSD test at 5% for mean separation.

DNA extraction. Bacterial strains were grown on TZC medium, single colonies of the strains were transferred to CPG broth and grown overnight on a rotary shaker at 150 rpm and 28ºC. One ml of the culture (OD_{600}=0.4) was used for genomic DNA extraction according to the method of Mury and Tampson (1980).

PCR. Strain identification was confirmed by PCR using the *R. solanacearum*- specific primer pair PS759f (GTCGCCGTCAACTCACTTCC) and PS750r (GTC GCCGTCAGCAATGCGGAATCG) (Ito *et al.*, 1998) and primers set RS1F (ACTAACGAAGCAGAGATG-CATTA) and RS1R (CCCAGTCACGCAGAGACT) (Pastrik *et al.*, 2000). PCR reactions were done as described by Ito *et al.* (1998) and Pastrik *et al.* (2000), respectively.

Rep-PCR. *PCR analysis.* The genetic diversity among *R. solanacearum* strains was assessed by rep-PCR using primer sets ERIC1R (5'-ATGTAAGCTCCTGGGGAT TCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGT-gACg-3)' and BOXA1R (5'-CTACGGCAAGGC-GACGCTGACG-3') (Versalovic *et al.,* 1991). PCR amplification was conducted in a final volume of 15 µl, consisting of 1X PCR reaction buffer, 0.4 mM $MgCl_2$, 0.5 mM of each dNTP, 2 pmol of each primer, 1 U of *Taq* DNA polymerase (CinnaGen, Iran) and 2 µl of extracted DNA. The thermocycler (Techne-TC-512) was programmed as follows: an initial denaturation of 95ºC for 5 min, followed by 35 cycles of 95ºC for 30 sec, 58ºC for 38 sec and 72ºC for 45 sec, with a final extension at 65ºC for 16 min, followed by hold time at 4ºC until samples were collected.

Amplified products (10 µl) were resolved by 2% agarose gel electrophoresis in 1X TBE buffer, pH 8, at 120 V. The gel was stained with ethidium bromide (0.5 mg ml^{-1}) and DNA amplicons were detected with a UV trans-illuminator and photographed under UV light (Louws *et al.*, 1994; Sander *et al.,* 1998).

Data analysis. rep-PCR fingerprint profiles were used

to determine the genetic similarity among strains. Each band with different electrophoretic mobility was assigned a position number and scored as either 1 or 0 based on its presence or absence in this position. Similarity coefficients for possible pairs of strains based on fingerprint groups were estimated by Dice (1945) method and dendrograms were constructed from the similarity coefficient data by NTSYS-pc (version 2).

RFLP analysis. Extracted DNA was digested with the restriction enzymes *Alu*I, *Rsa*I and *Taq*I (Promega, USA). Digested DNA samples (6 µl) were run on a 6% polyacrylamide gel in 0.5X Tris-borate-EDTA buffer (89 mM Tris-HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA) and electrophoresed at 200 V for 5 h.

Determination of biovar 2A and 2T survival in the soil. To determine the survival of biovars 2A and 2T, strains AD4 (biovar 2T) and Ps3 (biovar 2A) were selected. For introduction into soil (loamy clay type) after 48 h, bacterial cultures on TZC medium, were suspended in distilled water and the bacterial density was adjusted spectrophotometrically (OD_{600}= 0.5 equal to 10^6-10^8 CFU ml^{-1}).

Soil samples were kept in sterile Petri dishes at 20ºC and 50% RH. Survival was checked 0,2,4,8,16,32,64, 128 and 256 days after inoculation. For bacterial detection, 1 g of soil was suspended in 9 ml of sterile CPG broth and grown overnight on a rotary shaker at 150 rpm and 28ºC. Then, 10-fold serial dilutions of the suspension were made and used in PCR (with primer set RS1F and RS1R) and for plating on modified TZC (containing 1 ml chloramphenicol, 4.5 ml polymixin B and 0.24 ml crystal violet) in triplicate and incubated at 28°C for 48 h (Messiha, 2006).

RESULTS AND DISCUSSION

Morphological, physiological and biochemical characteristics. All isolates identified as biovar 2A strains, produced irregular, creamy-white and highly fluidal colonies with pink center, which turned the medium brown after 48 h on TZC medium, but all strains of biovar 2T produced less fluidal, circular, deep red colonies with a thin white margin.

All strains were Gram, starch, gelatin and levan negative and oxidase, catalase, nitrate and Tween 80 positive**.** All biovar 2A and 2T strains produced acid from glucose, maltose, arabinose, trehalose, sucrose, cellobiose, fructose, galactose, lactose, mannose, tartaric acid, alanin, inositol, propanol and glycerol, but they did not utilize raffinose, rhamnose, dulcitol, lysine or mannitol even after 28 days. Biovar 2T strains produced acid from ribose and utilized tryptophan while biovar 2A strains did not oxidize ribose and tryptophan even after five weeks of incubation. All of the biovar 2T isolates were also more pectolytic than biovar 2A. In the tyrosin test biovar 2A strains produced melanin and turned brown while biovar 2T did not.

Hypersensivity reaction. Biovar 2T strains induced a rapid HR on tobacco leaves after 24 h and the lesion be-

Table 1. Strains of *R. solanacearum* used in this study and classifications established according to biovar.

Code no.	Strain	Host	Geographical origin	Biovar
1	PS1	Potato	Isfahan	2A
2	PS3	Potato	Isfahan	2A
3	PS5	Potato	Isfahan	2A
4	PS6	Potato	Isfahan	2A
5	PS7	Potato	Isfahan	2A
6	PS8	Potato	Isfahan	2A
7	PS9	Potato	Isfahan	2A
8	PS10	Potato	Isfahan	2A
9	KOB1	Tomato	Khuzestan	2T
10	KOB2	Tomato	Khuzestan	2T
11	SH12	Potato	Khuzestan	2T
12	SH16	Potato	Khuzestan	2T
13	DO24	Tomato	Khuzestan	2T
14	AD4	Potato	Khuzestan	2T
15	AD5	Potato	Khuzestan	2T

Table 2. ANOVA results for effect of potato cultivar, biovar and temperature on disease progress and disease severity (21 and 28 days post inoculation with biovars 2A and 2T of *R. solanacearum*) in 6 cultivars of potato.

Source of variation	Mean squares			
	df	Symptom start day	Disease severity (21 days)	Disease severity (28 days)
Cultivar	5	9.30**	921.84**	2196.53**
Biovar	2	3422.3**	18422.59**	23902.67**
Temperature	1	249.03**	3755.82**	4462.80**
Cultivar × Biovar	10	7.70**	465.43**	719.63**
Cultivar × Temperature	5	4.77**	208.48**	112.93**
Biovar × Temperature	2	67.70**	2245.23**	2277.31**
Cultivar × Temperature × Biovar	10	4.23**	220.47**	180.09**
Error	72	0.037**	3.19**	0.084**

** Denote significant difference between treatments at P<0.01.

Table 3. ANOVA results for effect of biovar on disease progress and disease severity (21 and 28 days post inoculation with biovars 2A and 2T of *R. solanacearum*) in tomato, petunia, geranium and eggplant.

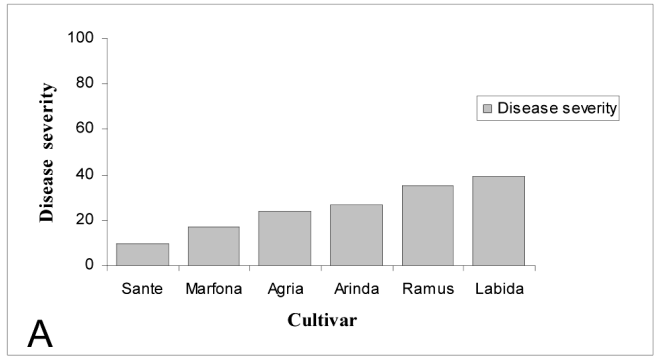
Source of variation		Mean squares			
		df	Symptom start day	Disease severity (21 days)	Disease severity (28 days)
Biovar	Tomato	2	45.50**	5498.48**	5570,37**
	Petunia	2	28.16**	4644.32**	5447.20**
	Geranium	2	31.50**	2834.28**	2835.12**
	Eggplant	2	28.16**	4754.15**	5002.28**
Error	Tomato	3	0.16	0.48	0.37
	Petunia	3	0.16	0/096	0.0066
	Geranium	3	0.16	0/54	0.54
	Eggplant	3	0.16	4/87	0.37

came progressively darker and visible after 48 h while biovar 2A strains elicited a yellowish discoloration of infiltrated tissues after 24 h and caused slow spreading necrosis after 48 h. Tobacco leaves infiltrated with sterile water were unaffected.

Pathogenicity tests. Onset of wilting elicited by biovars 2A and 2T was significantly different (Table 2 and 3). Symptoms usually consisted of wilting of the leaves in potato, tomato, eggplant, geranium and petunia. Infection induced a considerable ooze and decay of the pith surrounding the point of inoculation after which the plants wilted and died.

According to ANOVA (Table 2), there was significant three-way interaction between biovar, temperature and cultivar in wilt disease progress (disease start day), disease severity (21 and 28 days post inoculation) and bacterial density in xylem on the susceptibility of potato plants to biovars 2A and 2T strains. Furthermore, there was significant difference in disease index in potato cultivars (Table 2). Accordingly, the disease index of cv. Sante was scored as 1 (tolerant), cv. Marfona 2 (semi tolerant), cvs Agria and Arinda 3 (susceptible) and cvs Ramus and Labida 4 (very susceptible) to both biovars (Fig. 1A). As compared with biovar 2T strains, those of biovar 2A were more pathogenic and aggressive to all hosts, (Fig. 2B), the bacterial density in xylem was higher (Fig. 2A, B) at both temperature regimes, and symptom appeared in a shorter time after inoculation. Disease development was significantly reduced and started much later in plants inoculated with biovars 2A and 2T under cool conditions (Fig. 1B).

Susceptibility of six potato cvs (Agria, Arinda, Marfona, Labida, Ramus and Sante), tomato, eggplant and petunia plants was determined showing significant differences in aggressiveness among biovar 2A and 2T strains over a four weeks period. Isolates of biovar 2T caused a milder disease in all species and cultivars inoculated, compared with isolates of biovar 2A, at both temperature regimes. Bacterial density in inoculated plants xylem was generally positively correlated with the severity of wilting. The pathogenicity experiment showed that potato cultivars were significantly different from each other in susceptibility to biovars 2A and 2T.



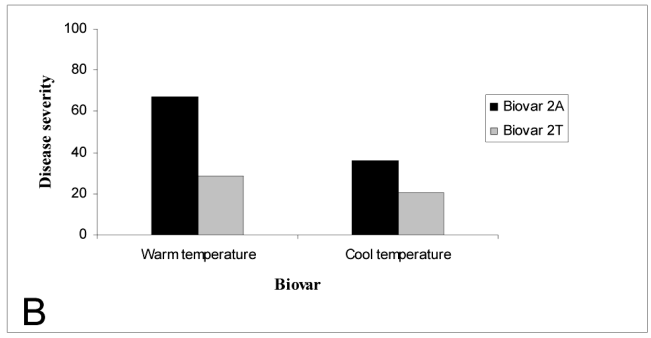


Fig. 1. Disease severity of biovars 2A and 2T in (a) potato cultivars (b) warm (39-45°C) and cool (25-31°C) conditions. Values with different letters are significantly different according to LSD ($p = 0.05$).

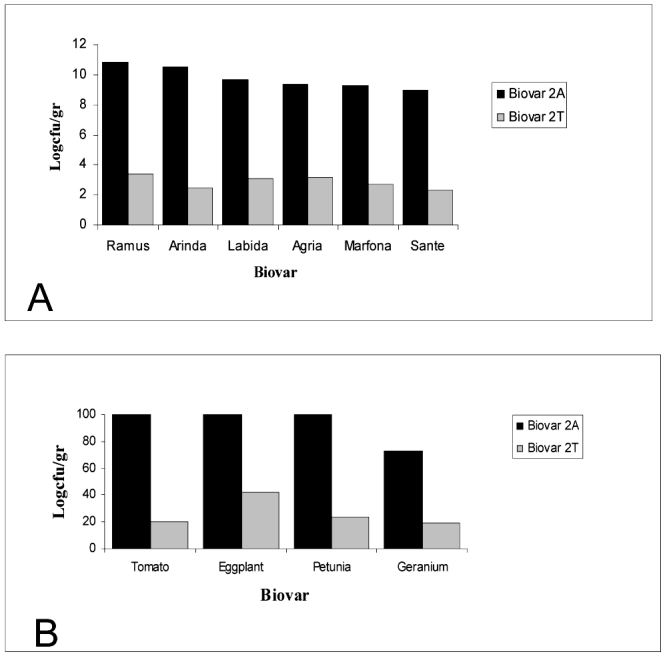


Fig. 2. Bacterial density of biovars 2A and 2T in (a) potato cultivars (b) tomato, eggplant, petunia and geranium. Values with different letters are significantly different according to LSD ($p = 0.05$).

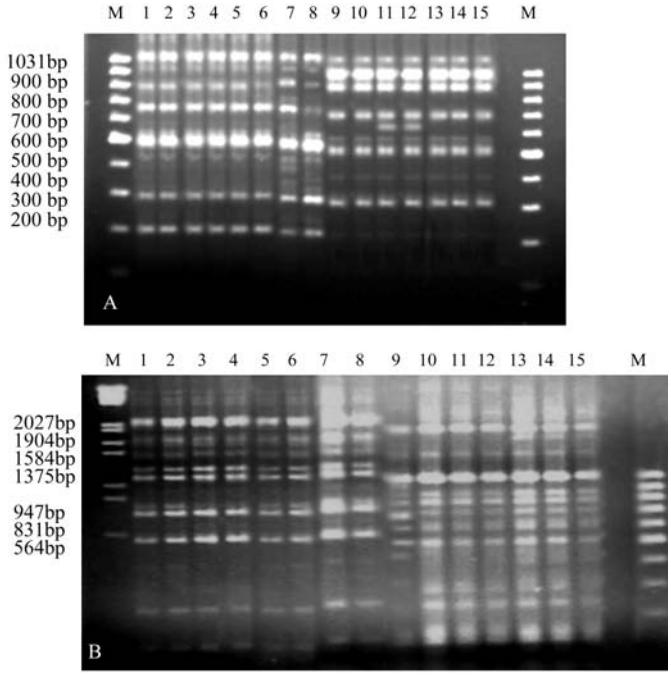


Fig. 3. Agarose gel showing representative patterns of Iranian strains generated by repetitive sequence-based polymerase chain reaction with (a) BOX (b) ERIC: 1) ps1, 2) ps3, 3) ps5, 4) ps6, 5) ps7, 6) ps8, 7) ps9, 8) ps10, 9) Kob1, 10) Kob2,11) Sh12, 12) Sh16, 13) Do24, 14) AD4, 15) AD5, 16) H2O, M(a), M(b) 100bp ladder DNA, III DNA.

For example, cv. Sante was reasonably tolerant (disease index =1) to both biovars in cool and warm conditions, whereas cvs Ramus and Labida were highly susceptible. Among the remaining cultivars, Marfona was moderately tolerant, but Agria and Arinda were most susceptible.

All the strains subjected to PCR yielded a single 281 bp fragment when amplified with PS759f and PS760r, and a single 718 bp fragment with primer set RS1F and RS1R. The results confirmed that all 15 strains belong to the *R. solanacearum* species complex.

Rep-PCR. Rep-PCR genomic fingerprints of the 15 Iranian strains were generated by BOX and ERIC primer sets. Both BOX primer and ERIC primer sets gave clear genomic PCR profiles that were highly reproducible. The BOX primer produced 10 bands per strain, and only a single polymorphic band of *ca*. 600 bp was observed in strains Sh12 and Sh16 (Fig. 3a). ERIC primers generated 10 to 12 bands per strain within a range of *ca.* 300 to 5000 bp showing no polymorphism (Fig. 3b). The strains were comprised in two groups

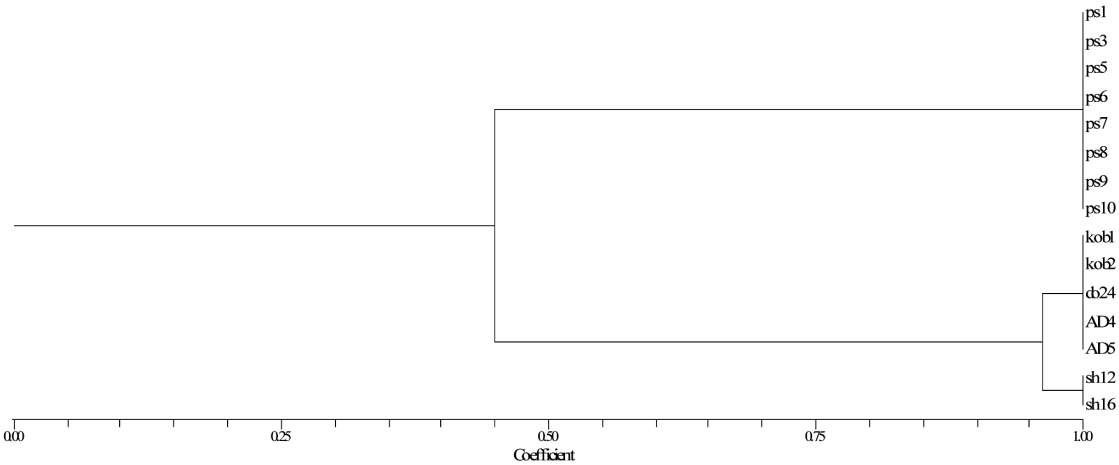


Fig. 4. Genetic diversity of 15 strains of biovars 2A and 2T of *R. solanacearum* on the basis of Box and ERIC 1) biovar 2A strains (ps1, ps3, ps5, ps6, ps7, ps8, ps9, ps10) 2) biovar 2T strains: a (Kob1,Kob2, Do24, AD4, AD5), b (Sh12, Sh16).

based on duplicate analyses of both BOX-PCR and ERIC-PCR (Fig. 4) at 45% similarity level. Group 1 contained all biovar 2A strains with 100% similarity and group 2 consisted of all biovar 2T strains. At a similarity level of 97%, group 2 strains were distributed in two sub groups: 2a (Kob1, Kob2, Do24, AD4 and AD5) and 2b (Sh12 and Sh16).

RFLP analysis. All strains generated 5 bands in the range of 150, 200, 300, 400 and 500 bp by *AluI* and 2 bands within a range of 300 and 400 bp by *TaqI* and *RsaI*. No polymorphism was detected in biovars 2A and 2T strains.

Determination of biovar 2A and 2T survival in the soil. Strain PS3 (biovar 2A) and AD4 (biovar 2T) survived in loamy clay soil *in vitro*, up to 186 days, and there was no significant difference in survival of biovars 2A and 2T. The detection of bacteria from each soil sample was carried out periodically by PCR, using species-specific primers, and by cultivation in TZC. It was found that the strains are viable up to 186 days, a long enough time to guarantee inoculum survival for next year potato cropping.

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