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Molecular Markers Associated with Some Resistance Potato Cultivars to Bacterial Wilt Disease

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Abstract: Bacterial wilt (B.W) disease caused by *Ralstonia solanacearum* in potato. Among six of commonly cultivated potato cultivars (*Solanum tuberosum*), Nicola and Lady rosetta cultivars were high susceptible to bacterial wilt disease, but Santana and Valor cultivars were moderately susceptible and/or resistance to the disease. Also, Diamont and Lady balfor cultivars appeared resistance to bacterial wilt disease. Proteomic profiles were very different for protein band density in comparison between healthy and infected potato cultivars. Genetically, seven RAPD primers gave polymorphism with the studied genotypes using RAPD-PCR technique. Only three primers exhibited molecular markers among these genotypes. Where, three molecular markers were detected in primer (C20); first was positive marker linked with susceptible (S) cultivars, meanwhile, second was detected positively with moderate and/or susceptible (M/S) cultivars to (B.W) and negatively with resistance (R) cultivars. Only one positive marker was detected in primer (A14) liked with R cultivars. Two molecular markers were detected in Primer (C11) positively liked with R cultivars. Genetically, these cultivars were divided to three cluster groups, Diamont and Lady blfor in the first cluster as R cultivars and Santana and Valor in the second cluster as M/S cultivars, Nicola and Lady rosetta were in the third cluster as S cultivars. Using one SRAP1 primer for sequence related amplification polymorphism in studied potato cultivars by SRAP-PCR technique gave polymorphism and showed positive and negative markers explained the interaction between different exons for susceptibility or resistance of potato cultivars with B.W.

Key words: Potato · Bacterial wilt Resistance · SDS Electrophoresis · RAPD · SRAP

INTRODUCTION

Potato (*Solanum tuberosum* L.*)* is one of the most important crops grown in the world today; it is the fourth main food crop after wheat, maize and rice [1]. Potato plants are affected by many biotic and abiotic factors, including pathogens and environmental stresses [2, 3]. Bacterial wilt is a serious economic problem in several countries where potatoes are cultivated over large areas and economical exportation crop like Egypt. *Ralstonia solanacearum* is the most devastating bacterial pathogens which infect more than 50 botanical families. The bacterium is found in worldwide, mainly in tropical and subtropical areas, but also in warm-temperate countries and even in some cool-temperate regions [4]. The natural resistance of plants to diseases is based not only on preformed defenses, but also on induced mechanisms. The induced mechanisms are associated with local changes at the site of pathogen infection, such as the hypersensitive response (HR), which is one of the most efficient forms of plant defenses. The HR also leads to an increase in the activity of enzymes. Proteomics a new science dealing with studying proteins and peptide (secretome) was vigorously introduced in the field of plant pathology [5, 6], but its role in disease incidence and pathogenecity still unclear.

Recent advances in plant pathology clearly indicate that enzymes and other proteins secreted from the parasite alls are main determinants of the outcome of interaction processes. The secretome, which includes

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secreted proteins and outer-membrane bound proteins, plays an important role in sensing the environment, molecular signaling and communications and negotiation with the host and in uptake nutrients. Using MS/MS to identify proteins secreted by *Fusarium graminearum* during growth on 13 media *in vitro* and *in planta* during infection of wheat heads [6] identified a total of 289 proteins (229 *in vitro* and 120 *in planta*). They found that 49 of the *in planta* proteins were not found in any of the *in vitro* conditions. The presence of these proteins in the *in planta* but not *in vitro* secretome might indicate the significant fungal lysis occurs during pathogenesis. Identified virulence factors involved in bacterial with disease caused by the phytopathogenic *Ralstonia solanacearum* [7]. They found that this pathogen secretes 70 proteins and this secretome may be novel bacterial with virulence factors. The application of random amplified polymorphic DNA (RAPD) analysis [8] has proven useful in the development of DNA markers that are linked to disease and insect resistance in several crops. Bulked segregant analysis [9] is an efficient method of rapidly identifying molecular markers linked to a specific gene and has been used to find resistance genes in several crop species.

Therefore, we decided to use bulked segregant analysis to identify RAPD markers linked to the gene controlling bacterial wilt resistance. The major disadvantage of the RAPD technique is its low reproducibility [10]. Further, the RAPD technique is highly sensitive to reaction conditions [11] and there are differences in amplification level among PCR machines from various manufacturers. Therefore, STS markers are more reproducible and specific than the original RAPD marker. Conversion of RAPD markers into breeder friendly STS markers should enhance MAS efficiency for bacterial wilt resistance in carnation. Therefore, we also attempted to convert a RAPD marker tightly linked to a major gene controlling bacterial wilt resistance into an STS marker [12]. There are some techniques such as differential display PCR (DD-PCR), this technique was devised to amplify messenger RNAs and display their 3' termini on polyacrylamide gels. It shows the plant responded to the stress conditions by altering the gene expression of regulatory metabolic processes, especially the photosynthesis [13].

A new marker technique called sequence-related amplified polymorphism (SRAP), which combines simplicity, reliability, moderate throughput ratio and facile sequencing of selected bands. Further, it targets coding sequences in the genome and results in a moderate number of co-dominant markers. SRAP and AFLP markers used to construct a genetic map of Brassica oleracea in order to demonstrate the application of the former in genetic mapping. This includes tagging of the GLS-ALK gene, which regulates the desaturation of aliphatic glucosinolates in crucifers. SRAP was also used to fingerprint genomic DNA and cDNA from other crops to show its potential broad applications.

This study was conducted to investigate the possibility of determination some molecular markers which is responsible for resistance or susceptibility in some potato cultivars.

MATERIALS AND METHODS

Six potato (*Solanum tuberosum*. L) cultivars; Diamont, Lady rosetta, Valor, Santana, Nicola and Lady balfor which commonly cultivated in each of Syria and Egypt. These cultivars were planted in clay pots (30 cm diam.) each containing sterilized sand-clay (1:1 V/V). Two tubers were planted per pot and fifteen pots were used as replicates for each cultivar. The growing plants were maintained under greenhouse conditions during spring season.

Bacterial Isolates and Inoculum Preparation: Virulent isolate of *Ralstonia solanacearum* (AC20) was prepared from 48 hours old cultures on Tetrazolium medium (*Bacterial Selective Media TZC*) and adjusted to 10^8 colony forming units (cfu)/ml using spectrophotometer (0.3 as optical density at 600 nm). The suspension was immediately used for inoculation of potato plants. Two inoculation methods; stem injection and soil drench to evaluate resistance and/or susceptible of potato cultivars [14, 15].

Disease Assessment: Disease incidence was recorded at 10 days intervals after 30 days of pathogen inoculation and determined according to a disease ratting scale where: 0 = no symptoms; 1 = up to 25% of the foliage wilted; 2 = 26-50% of the foliage wilted; 3 = 51-75% of the foliage wilted; and 4 = 76-100% of the foliage wilted. Disease severity was calculated per potato cultivar and percentage of disease index (DI) was calculated according to Hayward [14] as follow: DI = (Ó R.T/N×4) ×100, where: T = total number of plants with each category; R = disease ratting scale (R=0, 1, 2, 3 and 4); N = total number of tested plants.

SDS-Protein Electrophoresis: Healthy and *Ralstonia solanacearum* infected of potato cultivar roots and shoots were collected at 5 days post inoculation, then kept under -8°C till use. Sodium dodycyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on protein fractions according to the method of Laemmli [16] and modified by Studier [17].

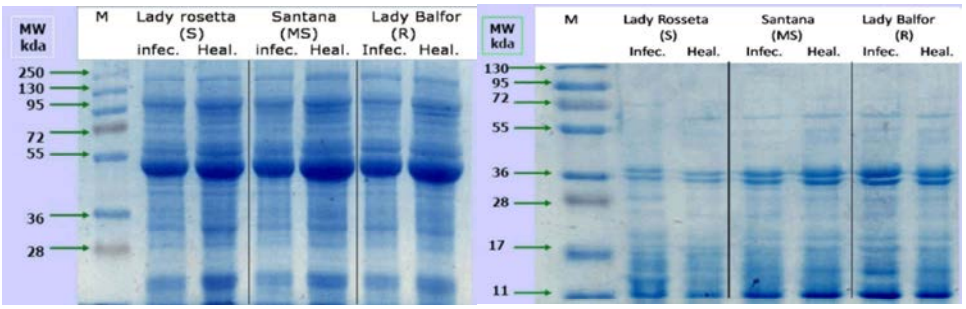
RAPD-PCR Analysis: Total genomic DNA was extracted from young leaves for the six selected potato cultivar plants. Small amount of young and fresh leaves (0.1 g) from each potato cultivars was collected and grinded to fine powder with small amount of liquid nitrogen according to Dellaporta *et al.* [18]. RAPD-PCR was carried out according to Williams *et al.* [19]. PCR was conducted for the six selected potato (*Solanum tuberosum* L.) cultivars DNA to detect markers for resistance or susceptibility to bacterial wilt disease caused by *Ralstonia solanacearum*. Seven RAPD primers were used for RAPD analysis from Operon Technology (USA) as following: Primer A-16: 5-AGC CAG CGAA-3, primer A-14: 5-TCT GTG CTG G-3, primer B-08: 5-GTC CAC ACG G-3, primer B-12: 5-CCT TGA CGC A-3, primer C-05: 5-GAT GAC CGC C-3, primer C-11: 5-AAA GCT GCG G-3 and primer C-20: 5-ACT TCG CCA C-3. PCR was performed in 30-μl volume tubes containing DNTPs (2.5 mM), MgCl2 (25 mM), buffer (10 x), primer (10 pmol), Taq DNA polymerase, template DNA (25 ng), H_2O (d.w). The amplification was carried out in a DNA thermocycler (MWG-BIOTECH Primuse) which programmed as following: (94 °C 5 minutes) 1 cycle, (94 °C 1 minutes, 37 °C 1 minute, 72 °C 1 minute) 40 cycles, (72 °C 10 minutes) 1 cycle.

SRAP- PCR Analysis: SRAP is a PCR-based marker system with two primers, a forward primer of 17 bases and a reverse primer of 18 bases. The forward primers consist of a core sequence of 13 bases. The first ten bases starting at the 5' end are "filler" sequences of no specific constitution, followed by the sequence CCGG and then by three selective nucleotides at the 3' end. Variation in these three selective nucleotides generates a set of primers sharing the same core sequence. The reverse primers consist of the same components as the forward primers with the following variations: the filler is followed by AATT instead of the CCGG sequence. Following the AATT sequence, three selective bases are added to the 3' end of the primer. The only rules for construction of the forward and reverse primers are that they do not form hairpins or other secondary structures and to have a GC content of 40-50%. Further, the filler sequences of the forward and reverse primers must be different from each other and can be 10 or 11 bases long. Based on the report of Li and Quirous [20], SRAP-PCR amplification was performed with 1× Taq Master Mix (Kangwei, Beijing) in a total volume of 25 ìl. The available SRAP1 Forward primer was 5-AACTGTTCGAGGCCTGG-3 and Reverse primer was 5-ACTTGGGCAGCAATTAGG-3. Amplification of DNA at the first five cycles was run at 94°C, 1 min, 35°C, 1 min and 72°C, 1 min, for denaturing, annealing and extension, respectively. Then the annealing temperature is raised to 50°C for another 35 cycles. For amplification we used the cocktail used in other routine PCR-marker applications as reported by Vos *et al*. [21]. PCR products were loaded in gel-wells in parallel with appropriate volume of standard ladder of DNA markers. The PCR products separation was performed in Pharmacia submarine unit (20 cm X 20 cm) and the voltage was adjusted to 80 V. The gels were immediately photographed after the run was ended using Bio-Rad Gel Doc 2000 apparatus.

RESULTS AND DISCUSSION

Six potato (*Solanum tuberosum*. L) cultivars (Diamont, Lady balfor, Lady rosetta, Nicola, Santana and Valor) were tested to evaluate their response to of bacterial wilt disease caused by bacteria *Ralstonia solanacearum* race 3 biovar 2 (R3b2), isolate (AC 20) under artificial inoculation (greenhouse) condition. Stem injection method and soil drench method were applied to evaluated response of potato cultivars for the disease. The results showed great variation with statistically significant between potato cultivars against bacterial wilt disease as show in Table 1. Lady balfor cultivar appeared resistance to bacterial wilt disease, but Santana, Valor and Diamont cultivars were moderately resistance and/or susceptible to the disease and Nicola and Lady rosetta cultivars were high susceptible to bacterial wilt disease, under artificial inoculation conditions.

Shoot-SDS-Protein Electrophoresis: The SDS-PAGE separation of proteins extracted from potato cultivars in each of healthy and infected shoots of healthy and inoculated tested potato cultivars are shown in Table (2) and Figure 1A. Infected plants of susceptible cultivar (Lady rosetta) gave only 14 bands with absence of four bands, comparing with healthy plant. The number of



(a) (b)

Fig. 1: Electrophoresis pattern of extracted protein from potato cultivars (A) shoot and (B) root, Healthy (H) and inoculated (In) with virulent *Ralstonia solanacearum,* at 5 days after inoculation.

Table 1: Response of potato cultivars against bacterial wilt disease using virulent isolate of Ralstonia solanacearum under artificial inoculation condetions.

	Disease severity		
Cultivar	I (%)	II	Response
Diamont	32.4^{e}	1.7^{e}	R
Lady balfor	19.5^{f}	1^{f}	R
Lady rosetta	73.3^{b}	3.7^{b}	S
Nicola	83.6^{a}	4.2^{a}	S
Santana	45^{c}	2.3^{c}	MS
Valor	36.2^{d}	1.8^{d}	MR

Values followed by the same letter(s) in each column are not significantly different at P= 0.05 according to Duncan's multiple range tests.
I = Disease index according to disease ratting scale from 1-5 (French, and Delindo) [22].
II= Mean disease rating.

Table 2: Separation of protein bands of potato cultivars shoot. Healthy (H) and inoculated (in) with virulent ralstonia solancearum, at 5 days after inoculation. By get electrophoresis.

		Potato cultivars					
		Lady rosetta		Santana		Lady baifor	
Band No.	Mw	In	H	In	H	In	H
1	160.90	1	1	1	1	1	1
2	117.77	1	1	1	1	1	1
3	107.24	1	1	1	1	1	1
4	95.89	1	1	1	1	1	1
5	88.01	1	1	1	1	1	1
6	74.13	1	1	1	1	1	1
7	66.03	1	1	1	1	1	1
8	49.40	1	1	1	1	1	1
9	59.73	1	1	0	1	1	1
10	44.18	1	1	1	1	1	1
11	39.50	0	1	0	1	1	1
12	35.60	1	1	1	1	1	1
13	32.67	0	1	0	1	0	1
14	29.44	1	1	1	1	1	1
15	26.44	0	1	0	1	0	1
16	24.35	0	1	0	1	0	1
17	16.96	1	1	1	1	1	1
18	15.73	1	1	1	1	1	1
Total band No		14	18	13	18	15	18

Table 3: Separation of protein bands of potato cultivars root. Healthy (H) and inoculated (in) with virulent ralstonia solancearum, at 5 days after inoculation. By get electrophoresis.

		Potato cultivars					
		Lady rosetta		Santana		Lady baifor	
Band No.	Mw	In	H	In	H	In	H
1	82.53	1	0	0	1	1	1
2	68.73	1	1	1	1	1	1
3	59.87	0	1	1	1	1	1
4	54.28	1	1	1	1	1	1
5	38.76	1	1	1	1	1	1
6	34.40.	1	1	1	1	1	1
7	32.28	1	0	1	1	1	1
8	29.65	1	0	1	1	1	1
9	24.65	1	1	1	1	1	1
10	19.04	1	0	1	1	1	1
11	17.03	0	1	0	1	1	1
12	15.98	1	1	1	0	1	0
13	13.81	0	0	1	1	1	1
14	10.65	1	1	0	1	1	1
15	11.69	0	1	1	1	1	1
Total band No		13	11	12	14	15	14

absence bands was, 11, 13, 15 and 16 with molecular weights 39.50, 32.67, 26.33 and 24.35 Kda. respectively. Infected plant in moderately susceptible cultivar (Santana) comparing with healthy plant gave only 13 bands with absence of band number 9, 11, 13, 15 and 16 with molecular weights 49.40, 39.50, 32.67, 26.33 and 24.35 Kda, respectively. Meanwhile, in case of resistance cultivar (Lady balfor) comparing with healthy plant gave 15 bands with absence of band number 13, 15 and 16 with molecular weights 32.67, 26.33 and 24.35 Kda, respectively.

It could be concluded that, number of detected protein bands in infected shoots of all cultivars was less than those in healthy shoots and the number of absent band due to infection in resistance cultivar was less than in susceptible or moderately susceptible cultivars. On the other hand, the density of protein bands in infected plant was less than the density of healthy plant in all

tested cultivars especially bands numbers 2, 3, 7, 8, 17 and 18 with molecular weights 117.77, 107.24, 68.03, 59.73, 16.96 and 15.73 in all tested cultivars. The obtained results were differently with those Ibrahim *et al*. [23] who found that protein profile of six potato cultivars revealed that no significant difference between the control (non-infected) and infected potato cultivars. But, Louis et al. [24] by using SDS-PAGE observed that heat shook proteins differential switching off, indicates that the mechanistic translational and transcriptional machinery differs within Solanum. tuberosum L. cultivars.

Roots-SDS-Protein Electrophoresis: The SDS-PAGE separation of extracted proteins from potato cultivars root, Healthy and inoculated with virulent *Ralstonia solanacearum*, at 5 days after inoculation were performed and shown in Table (3) and Figure 1 B. Infected plants of susceptible cultivar (Lady rosetta) comparing with healthy plant gave only 11 bands with absence of band number 1, 8, 10 and 14 with molecular weights 82.53, 29.65, 19.04 and 10.65 Kda. respectively. Infected plant in moderately susceptible cultivar (Santana) comparing with healthy plant gave only 12 bands with absence of band number 1, 11 and 14 with molecular weights 82.53, 17.03 and 10.65 Kda. respectively. Meanwhile, in case of resistance cultivar (Lady balfor) comparing with healthy plant gave 14 bands with absence of band number 12, with molecular weights 15.987 Kda. It could be concluded that, number of protein bands detected in infected roots of all cultivars was less than those in healthy roots and the number of absent band due to infection in resistance cultivar is less than the number of absent band in susceptible or moderately susceptible cultivars. On the other hand, the density of protein bands in infected roots was less than the density of healthy roots in all tested cultivars especially bands numbers 5, 6 and 15 with molecular weights 38.76, 34.40 and 11.69 in all tested cultivars. These results explain the proteins differential switching off that indicates to the mechanistic translational and transcriptional machinery.

Electrophoretic patterns of soluble proteins and have been used as a powerful tool for the study of genetic variability of solanum species. The profiles of tuber soluble proteins were applied in the studies of relationship and plant genetics of cultivated and wild potato species and hybrids [25- 27]. In Europe, collections of European potato cultivars were discriminated by their profiles of protein and esterase patterns [28, 29].

RAPD-DNA Molecular Markers: Data presented in Figure 2 indicate that the selected potato cultivars are different in their genome with all used primers. The six potato cultivars were divided into three distinguished groups depending on their resistance to bacterial wilt disease. Group A was resistance (R) cultivars, Lady balfor and Diamont. Group B was moderately susceptible or resistance (MS and MR) cultivars, Valor and Santana. Group C was susceptible cultivars, Lady rosetta and Nicola. Individual plants from each cultivar were tested against seven preselected primers as shown in Figure 3. Seven RAPD primers (A14, A16, B8, B12, C5, C11and c20) showed polymorphism with the studied genotypes, while only three primers developed molecular markers.

Primers A14 exhibited one positive molecular marker which was found only in the susceptible cultivars with molecular sizes of 943 bp. The primer C11 exhibited two positive molecular markers which were found only in the resistance cultivars with molecular sizes 1728 bp and 1133 bp. On other hand the primer C20 exhibited four molecular markers, positive molecular markers which were found only in the moderately cultivars with molecular sizes 859 bp. One negative molecular marker which was absent only in the resistance cultivars with molecular sizes 732 bp. The same marker was linked with only moderately and susceptible cultivars positively with molecular sizes 732 bp. Another marker was found in this primer and linked positively with only susceptible cultivars with molecular sizes 505 bp. In conclusion, these results revealed that RAPD-PCR could be considered as a satisfaction molecular technique to obtain molecular markers for bacterial wilt resistance trait in potato. This technique could be used as a tool for marker-assisted selection (MAS) in potato breeding programs by molecular breeding. The application of random amplified polymorphic DNA (RAPD) analysis by Williams *et al.* [8] has proven useful in the development of DNA markers that are linked to disease and insect resistance in several crops. Bulked segregate analysis is an efficient method of rapidly identifying molecular markers linked to a specific gene and has been used to find resistance genes in several crop species as reported by Michelmore *et al*. [9]. Therefore, we decided to use bulked segregate analysis to identify RAPD markers linked to the gene controlling bacterial wilt resistance. The major disadvantage of the RAPD technique is its low reproducibility as revealed by Debener [10]. Further, the RAPD technique is highly sensitive to reaction conditions [11].

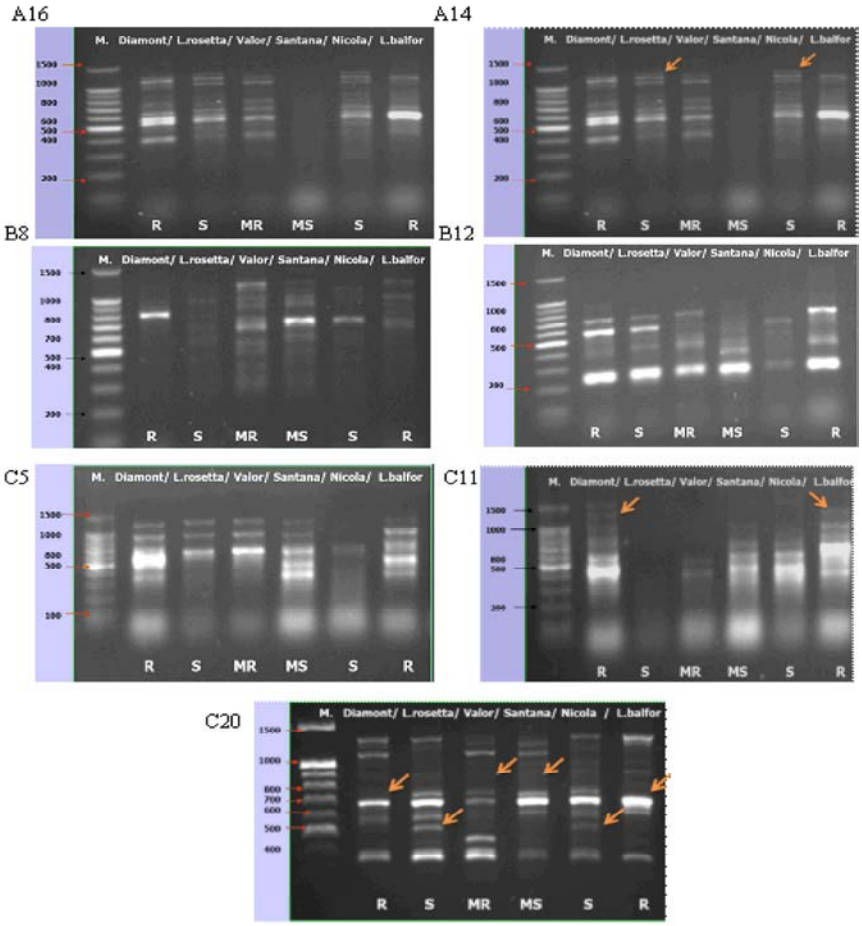


Fig. 2: RAPD-PCR profiles of A 14, A16 B8, B12, C5, C11 and C20 primer for selected potato cultivars DNA.

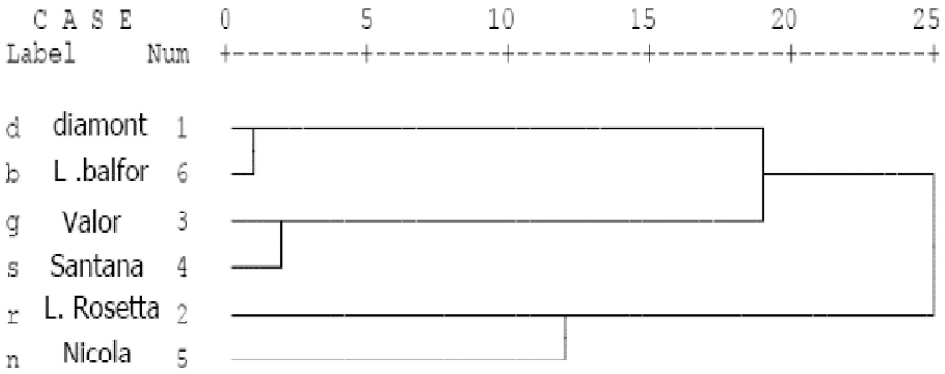


Fig. 3: Phylogenic tree of selected potato cultivars DNA with genetic distance.

The DNA based molecular markers have already become important tools for developing improved cultivars and for studying phylogenetics relationships [30- 32]. In addition, Kahraman [33] found that RAPD-PCR is currently used as genetic markers quite useful in breeding programs for assessment of genetic variability between genotypes. The DNA markers are useful complements to the morphological and physiological characterization of cultivars because they are plentiful, are not influenced by plant tissue or environmental effects and allow cultivar identification very early in plant development [34, 35].

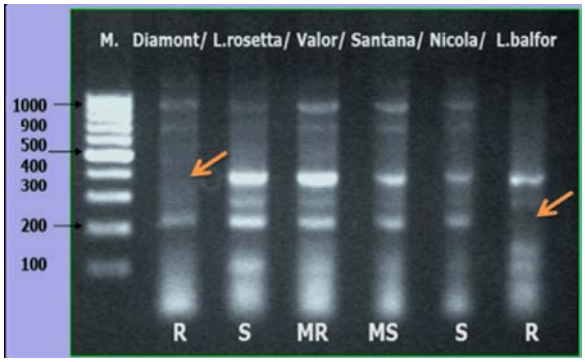


Fig. 4: SRAP-PCR profiles of SRAP 1 primer for selected potato cultivars DNA.

Dendrogram tree based on RAPD- PCR products as shown in Figure 3 grouped potato cultivars into three distinguished groups. Group A was resistance (R) cultivars, Lady balfor and Diamont. Group B was moderately susceptible or resistance (MS and MR) cultivars, Valor and Santana. Group C was susceptible cultivars, Lady rosetta and Nicola. This result suggested that RAPD can be useful technique for genetic variation and identification to identify resistant or sensitive of potato cultivars as reported earlier in ginger [36] and in tomato [37]. Ijaz et al. [38] perform dendrogram tree based on RAPD on 40 linseed varieties genotypes were grouped into five main clusters.

SRAP Molecular Markers: The SRAP marker system is a simple and efficient marker system that can be adapted for a variety of purposes in different crops. It has several advantages over other systems: simplicity, reasonable throughput rate, is closes numerous co-dominant markers, allows easy isolation of bands for sequencing and, most importantly, it targets ORFs. In our study as shown in Figure 4, the six potato cultivars showed some polymorphism. Primer SRAP 1 shows positive markers as two fragments for susceptible or resistance (MS) cultivars Valor and Santana (Mw 400 bp and 200 bp). But it gives two negative markers for both of resistance and susceptible cultivars such as only fragment with Mw 200pb for Diamont cultivar and absent fragment with Mw 400bp and only fragment with Mw 400bp for Lady balfor cultivar and absent fragment with Mw 200bp. There are variations in resistance and susceptibility with B.W disease is due to the variation in genes action in potato cultivars. SRAP technique can be used by Budak [39] for fingerprinting and the development of a comprehensive picture of the genome, which leads to the development of improved turfgrass cultivars. On the other hand, Quiros *et al*. [40] reported that, since exonic sequences are generally conserved among different individuals, their low level of polymorphism precludes using them as sources of markers. They found some SRAP markers were co-dominant, which was demonstrated by sequencing and have successes tagged the glucosinolate desaturation gene *BoGLS-ALK* with these markers.

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