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Biochemical identification of some faba bean genotype

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Abstract

Faba bean is an important crop in Egypt both for human and animal consumption. This experiment includes 8 promising lines and 4 varieties of faba bean genotypes (*Vicia faba* L.) was carried out at Giza Research Station, ARC during the winter seasons of 2018 and 2019 to identify and discriminate these genotypes. Biochemical analysis including (ISSR-PCR) technique, SDS-PAGE and Isozymes analysis were tested in leaves. By using Inter-simple sequence repeat (ISSR-PCR) technique, it was possible to determine the genetic diversity and relationships of the 12 faba bean genotypes included in this study. A total of 51 amplified bands were generated with five ISSR primers, of which 48 (92.3%) were polymorphic which represent a relatively high polymorphism level. Moreover, the patterns of leaf protein were studied using SDS-PAGE. It was found that each genotype was characterized by a protein with specific molecular weight. Isozymes analysis using Peroxidase and Poly phenyl oxidase results revealed that, differences in the density of bands can be used to successfully identify and characterize these faba bean genotypes.

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Introduction

Faba bean, broad bean or field bean (*Vicia faba* L.; 2n = 12) is a major food and feed grain legume owing to the high nutritional value of its seeds, which are rich in protein 27-34% (Link *et al.*, 1995 and Duc, 1997). It is considered as one of the major sources of cheap protein and energy in Africa, parts of Asia and Latin America, where most people cannot afford meat sources of protein (Duc, 1997 and Alghamdi, 2009). In Egypt, faba bean is among the main nutritional source of plant proteins (El-Danasoury *et al.*, 2008 and Bakry *et al.*, 2011). Nevertheless, the total production of this crop is still limited and falls to cover the increasing local consumption, so there is a prerequisite to enlarge the production by expansion throughout reclaimed areas which signify the scope of cultivated lands (Khalafallah *et al.*, 2008 and Bakry *et al.*, 2011). Nowadays, with increasing the number of faba bean varieties, it is difficult to differentiate these varieties based on morphological characters alone. These characters are either influenced by environmental factors and stage of plant development or reveal limited variation (Terzopoulosa and Bebeli, 2008). Recently, DNA-marker approaches have become gradually more utilized for taxonomic and phylogenetic analyses. They are not affected by environmental factors or by plant developmental stages.

Inter-simple sequence repeat (ISSR-PCR) is a route that overcomes most of these technical limitations (Reddy *et al.*, 2002 and Chen *et al.*, 2008) is a fast and simple system with a cost-efficient as well as it does not require any prior knowledge about the sequences to be amplified, being tremendously useful in genetic diversity, phylogeny, genomics and evolutionary studies (Aguilera *et al.*, 2011; Hu *et al.*, 2003 and Chen *et al.*, 2008). Identification and discrimination of 10 faba bean genotypes by using inter-simple sequence repeat (ISSR-PCR) technique showed genetic diversity and relationships among that genotypes. These results are important in protecting of plant breeders rights and at releasing these genotypes as new varieties (El-Emam *et al.* (2014). Mahmoud *et al.* (2003) identified some Egyptian faba bean genotypes by using molecular and biochemical methods. Enzymatic pattern of esterase (EST) and peroxides (PRX) differentiate between the four genotypes of *Vicia faba* L. The results showed that only one enzymatic band with same size and intensity, of EST was detected in three of the parental genotypes whereas two bands were recognized in the forth one and all somaclones regenerated from it. Behairy, Rehab (2007) identified ten broad bean varieties using biotechnological techniques. Results of electrophoresis analysis for SDS- protein fraction indicated some varieties showed some specific bands which could be used to characterize them among others. On the isozymes level, bands of three isozyme systems (peroxidase, glutamate oxaloacetate transaminase and esterase) were determined for the identification and characterization faba bean varieties based on polyacrylamide gel electrophoresis. Abdel-Razzak *et al.* (2012) Studied genetic diversity in faba bean using protein analysis. SDS-PAGE analysis of various faba bean leaf proteins reflected some variations among studied *vicia faba* populations. Hendawey and Younes (2013) evaluated some faba bean cultivars using biochemical techniques. Analysis of gel (SDS–PAGE) revealed that Sakha2 and Sakha3 were resolved in to 30 bands, while Giza843, Giza3, Nubaria1, and Missr1were resolved in to 31 bands. Also, Sakha4 was resolved in to 32 bands. Mohamed, Heba and Abdel-Hamid (2014) used some molecular and biochemical markers of some Vicia faba L. genotypes in response to storage insect pests infestation. Protein electrophoresis showed awide variation between genotypes and determined some biochemical markers (sodium dodecyl sulfate poly acrylamide gel electrophoresis, SDS-PAGE).

In addition, molecular genetic markers for stored insects resistance were obtained using inter simple sequence repeat polymerase chain reaction (ISSR-PCR) analyses. Pradhan *et al.* (2014) studied genetic diversity in 10 faba bean (*Vicia faba* L.) genotypes using seed protein and isozymes electrophoresis. Zymogram of superoxide dismutase showed no variation but catalase shows polymorphism. Tahir (2014) estimated genetic diversity among ten faba bean genotypes grown in Iraq using RAPD-PCR and biochemical (SDS-PAGE) markers. The results of SDS-PAGE revealed a total number of 10 protein

bands with different mol wt. Data of SDS-PAGE exhibited only 5 protein polymorphic bands with 50% polymorphism. He concluded that, the method can supply information for the discrimination of faba bean genotypes. Laurels *et al.* (2015) determined the genetic variability and analyze the relationship between 39 faba bean accessions using inter simple sequence repeats (ISSR) markers.

The use of ISSR markers was efficient to indicating the existence of variability and can be utilized in genetic improvement programs aimed at solving the needs of the producers. Asfaw *et al.* (2018) designed to reveal the genetic diversity existing among 32 Ethiopian faba bean varieties grown at three locations using 23 phenotypic traits and 11 inter simple sequence repeat (ISSR) primers. The results showed highly significant variations among the varieties for many of the traits. The principal coordinated analysis also categorized the varieties into three different groups similar to that of cluster analysis. 10 *Vicia faba* L. var. minor lines and five *Vicia faba* L. var. equine lines characterized using 14 ISSR (Inter Simple Sequence Repeat) primers.

The results showed that molecular variation between botanical types was higher (55%), however variation within types was also considerable, suggesting the convenience of keeping all tested materials for breeding purposes in this crop (Ortega, 2018). Ghada, Shebl *et al.* (2018) analyzed plants in fruiting stage of four faba bean genotypes (Giza 716, Sakha 1, Sakha 3, Sakha 4) using ISSR-PCR and HPLC to find out ISSR markers related to phenylalanine accumulation.

The study recommended Sakha 3 as a good for human food to maintain high dopamine biosynthesis in human. The purpose of the present study was to describe the genetic diversity of twelve faba bean genotypes by using Inter-Simple Sequence Repeats (ISSRs), SDS-PAGE and isozymes analysis.

Materials and methods

Laboratory experiment was carried out during the winter seasons of 2018 and 2019 at Giza Agricultural Research Station, ARC. The main objective of this study was to identify twelve faba bean genotypes by using some biochemical traits.

Plant material

Studied faba bean genotypes Pedigree of promising lines and names of varieties and their appreviations are shown in Table (1).

Table 1. Code and pedigree of promising lines and names of varieties.

Nubaria1× Determinate	G1	2- Giza 40×Ohishima- Zaira	G2
3-Santamora	G3	4- (Giza716 ×Atona)×Atona	G4
5-Giza716×Sakha1	G5	6- Sakha1× Ohishima- Zaira	G6
7- Sakha 2× Atona	G7	8- Sakha1× Sakha 2	G8
9- Roomy	Roomy	10- Peter 15	Pet15
11-Misr 1(Giza3 ×123A/45/76)	Misr 1	12- Giza 843 (561/2076/85×461/845/83).	Giza 843

DNA Extraction

DNA was extracted from the tissue of young, healthy leaf which was selected from each genotype, using DNeasy plant Mini Kit (QIAGEN). DNA quality was tested using 1.5% agarose gel electrophoresis and its concentration was determined spectrophotometrically.

ISSR-PCR

Five ISSR primers were selected for testing the genetic diversity among these genotypes. Names and sequences of these primers are shown in table (2). The PCR reaction was carried out in 25µl volume of a mixture containing 25ng of genomic DNA, 2.5mm dNTPs, 2.5mm MgCl2, 2 unit Taq polymerase, 10× Taq buffer and 0.6 µM primer.

The DNA amplifications were performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 94°C for 4 min followed by 45 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C. the reaction was finally stored at 72°C for 10min.

Gel electrophoresis

Gel electrophoresis was applied according to Adhikari *et al.* (2015). Agarose (1.5%) was used for resolving the PCR products. Bands were detected on UV-transilluminator and photographed by Gel documentation 2000, Bio- Rad. Similarity and dendrogram tree was performed using the SPSS program version 10.

Table 2. List of the primer names and their nucleotide sequences used in the study for ISSR procedure.

No	Name	Sequence
1	44B	5` CTC TCT CTC TCT CTC TGC 3`
2	HB-9	5`CAC CAC CAC GC 3`
3	HB-15	5´ GTG GTG GTG GC 3`
4	HB-10	5` GAG AGA GAG AGA CC 3`
5	HB-12	5`CAC CAC CAC GC 3`

Total protein and SDS-PAGE

Total protein was extracted from 2g fresh weight of plant leaves. Each sample were grinded with 10mL of Extraction buffer (0.5M Tris-HCl (pH 6.8), 10% sucrose, 2% SDS, and 5% 2-mercaptoethanol). The slurry was centrifuged at 5000 rpm for 20 min. Three milliliter of ammonium sulphate solution were added 1mL of the supernatant to precipitate the proteins then kept overnight in a refrigerator.

It was then centrifuged at 5000 rpm for 20 min. the pellet was washed two or three times in 70% acetone. SDS-PAGE was performed by the method described by Laemmli, (1970). Protein was analyzed on 1.5mm thick and 15cm long gels run in a dual vertical slab unit (Hoefer Scientific Instruments, San Francisco, CA, USA, MODEL SE 600 Series Hoefer, Pharmacia Biotech). From each sample, 50 µl of extract was loaded a polyacrylamide gel.

The separation gel (12%) and staking (4%) were prepared from an acrylamide monomer solution. Electrophoresis was carried out at constant current of 35mA through the stacking gel, and at 90 mA through the separation gel at 4°C After electrophoresis the gel was stained by Coomassie Brilliant Blue R-250 and the molecular weight (MW) of protein corresponding to each band was calculated by protein marker with kilo Daltons (kDs) molecular weights.

Data analysis

The ISSR bands were scored using the binary scoring system that recorded the presence and absence of bands as 1 and 0, respectively for each relative position. Genetic similarity between a pair of faba bean cvs. was calculated using Nei and Li's index of similarity (Nei and Li 1979). Cluster analysis was conducted based on genetic similarity.

Isozymes electrophoresis

Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted to identify isozyme variations among studied cultivars using three isozyme systems according to Stegemann *et al.* (1985).

Fresh and young leave samples for each cultivar and location were used separately for isozymes extraction. The utilized isozymes are Peroxidase (Px), Polyphenyl Oxidase (PPO) and Alkohol dehydrogenase (Adh).

Extraction of isozymes

Isozymes extraction from the different Peach cultivars homogenizing 0.5g fresh leaves samples in 1mL extraction buffer (10% glycrol) using a mortar and pestle. The extract was then transferred into clean eppendorf tubes and centrifuged at 10000 rpm for 5 minutes. The supernatant was transferred to new clean eppendorf tubes and kept at –20°C until use for electrophoretic analysis.

Peroxidase (Px)

Benzidine di Hcl 0.125gm, glacial acetic acid 2mL, D.W up to 50mL gel was placed into this solution and 5 drops of hydrogen peroxide was added. The gel was incubated at room temperature until bands appear Brown, (1978).

Poly Phenyl Oxidase (PPO)

M phosphate buffer pH 6.5, 100mg Sulphonillic Acid, 200mg Cathecol in 2ml Acetone, Gel was placed into this solution and Incubate at 30°C for 30 min until bands appeared.

Gel Analysis

Gels were photographed scanned, analyzed using Gel Doc VILBER LOURMAT system.

Results and discussion

ISSR analysis

In the present study, five selected primers of ISSR were used to differentiate between the 12 faba bean genotypes (Fig. 1). Inter- Simple Sequence Repeat (ISSR) technique yield more polymorphisms than other molecular techniques. The five primers amplified different numbers of bands and revealed

various levels of polymorphism. A total of 51 ISSR loci were observed and 48 (92.3%) of them were poly morphic. These primers yielded 10, 19, 6, 7 and 9 bands, respectively (Table3). The percentage of polymorphism was 90%, 100%, 83.3%, 85.7% and 100% respectively. The primer HB9 yielded the largest number of bands (19 band) while, primer HB10 had fewer number of bands. Among all the ISSR loci observed, 8 were unique. The highest number of unique band can observed in primer HB9 which produced 3 markers. While, the lowest number can observed in primer HB10 which produced 0 marker. The ISSR primer method is reported to produce more complex marker (Parsons *et al.,* 1997 and Chowdhury *et al.,* 2002), which is advantageous when differentiating closely related cultivars.

Table 3. Molecular data estimated from banding patterns of ISSR technique.

Primer		Amplicons								
Name	Sequence (5´→3`)	Molecular size range	Monomorphic	Polymorphic			Total	Polymorphism%	Polymorphic index content (PIC)	Resolving power Rp
				Without unique	Unique +	Unique -				
44B	$(CT)_8GC$	269:1923	1	7	2	-	10	90.0	0.290	4.17
HB-09	$(GT)_6GC$	173:2166	-	16	3	-	19	100.0	0.352	10.00
HB-10	$(GA)_6CC$	274:1088	1	5	-	-	6	83.3	0.283	2.34
HB-12	$(CAC)_3GC$	326:1016	1	4	2	-	7	85.7	0.264	2.50
HB-15	$(GTG)_3GC$	275:1082	-	8	1	-	9	100.0	0.367	3.66
Overall		173:2166	3	40	8	-	51	92.3	0.317	4.53

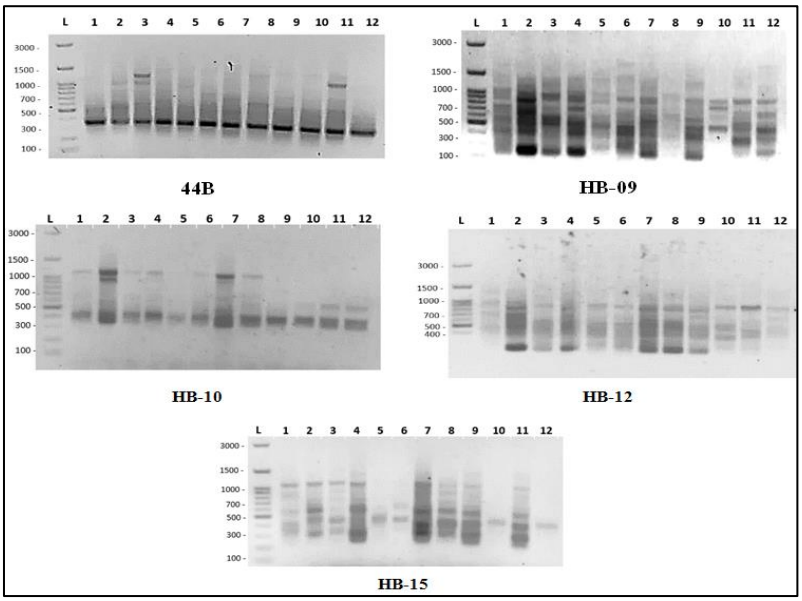

Fig. 1. ISSR fingerprinting of twelve faba bean genotypes.

Table 4. Molecular distance (MD) matrix for twelve strains of *Vicia faba* based on ISSRs data.

G	1	2	3	4	5	6	7	8	9	10	11
2	0.511										
3	0.514	0.391									
4	0.381	0.333	0.349								
5	0.647	0.395	0.429	0.400							
6	0.538	0.417	0.500	0.378	0.351						
7	0.429	0.333	0.488	0.333	0.500	0.422					
8	0.389	0.467	0.351	0.333	0.471	0.538	0.476				
9	0.538	0.292	0.450	0.333	0.405	0.429	0.333	0.385			
10	0.677	0.600	0.500	0.514	0.448	0.471	0.676	0.419	0.412		
11	0.550	0.510	0.463	0.478	0.474	0.535	0.522	0.300	0.349	0.429	
12	0.543	0.500	0.556	0.512	0.455	0.474	0.463	0.543	0.368	0.467	0.436

Genetic diversity

The ISSR data were used to estimate the genetic dissimilarity values among twelve faba bean genotypes (Table4). The lowest dissimilarity value between the twelve faba bean genotypes 0.292 was recorded between G2 and Roomy. However the highest dissimilarity value (0.677) was observed between G1 and Giza 843. The dendrogram based on ISSR data is shown in Fig. (2). The 12 studied genotypes were grouped in to four clusters namely A, B, C and D according to the truncated line at a coefficient of dissimilarity=0.544. G1 formed cluster A meanwhile, G2, G4, G5, G6, G7 and G9 formed cluster B and four genotypes namely G3, G8, G10 and G11 formed cluster C finally G12formed cluster D.

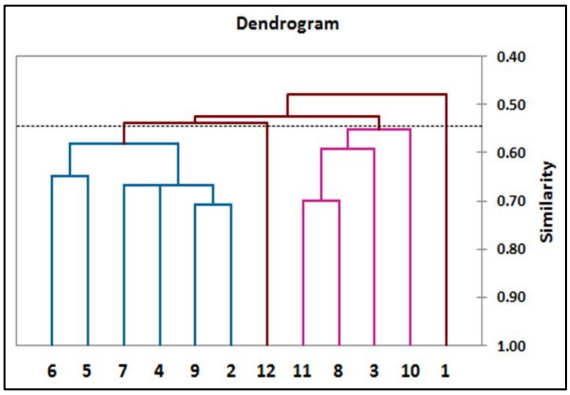

Fig. 2. Dendrogram derived by UPGMA method using Dice-similarity coefficient for binary data of ISSR technique for Twelve genotypes of *Vicia faba*.

The obtained present results for ISSR fingerprints of faba bean genotypes are along the same line with those of El-Emam *et al.* (2014) and Laurels *et al.* (2015) where they found that (ISSR-PCR) technique can be used as a tool for determining the extent of genetic diversity among faba bean for allocating genotypes in to different groups. Moreover Asfaw *et al.* (2018) designed to reveal the genetic diversity existing among 32 Ethiopian faba bean varieties grown at three locations using 23 phenotypic traits and 11 inter simple sequence repeat (ISSR) primers.

The results showed highly significant variations among the varieties for many of the traits.In the same respect, Ortega *et al.* (2018) reported that Inter Simple Sequence Repeat can be used as a tool for determining the extent of genetic diversity among 10 (*Vicia faba* L.) var. minor lines and five (*Vicia faba* L.) var. equine lines.

SDS-PAGE protein analysis

Protein banding patterns of the studied faba bean genotypes (including the eight chosen promising lines and four varieties) are revealed by SDS-PAGE for the total leave protein are illustrated in Fig. 3.

Electrophoretic analysis of leaves proteins exposed a total of 19 protein bands with variable intensity and molecular weights across the twelve faba bean. The protein bands ranging from 12.884 to 226.393KDa, in all genotypes under investigation.

Out of the 19 detected protein bands, 11 were monomorphic, while 8 were polymorphic with 42.105% polymorphism one of them was unique (51.242KDa) in G3 genotype. Similar observations have also been reported by Abdel-Razzak *et al.* (2012) and Tahir (2014). One positive unique band at molecular weight 51.242 in faba bean G3. This unique band is characteristic and considers a constant marker which gives this genotype a biochemical identity to distinguish from each other. The high polymorphism (42.105%) obtained by SDS-PAGE analysis of the twelve faba bean genotypes indicating reasonable variability could be used for faba bean genotypes differentiating and molecular markers detection.

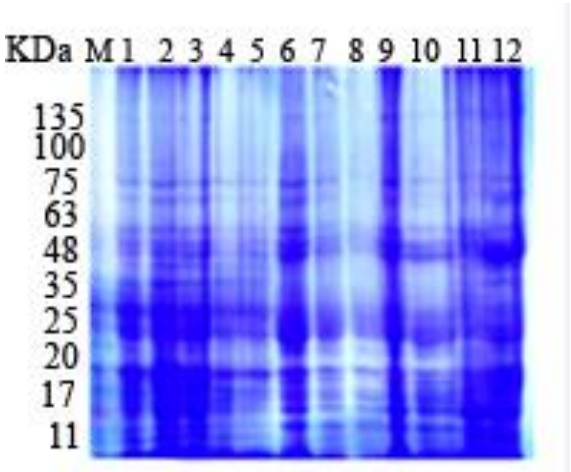

Fig. 3.: Faba bean genotypes showing protein bands as a result of SDS-PAGE. While, M= protein ladder, 1=G1, 2= G2, 3= G3, 4= G4, 5= G5, 6= G6, 7= G7, 8= G8, 9 = Roomy, 10= G843, 11= Peter15, 12= Misr1.

Isozymes analysis

One of the main targets of this investigation is to use two isozymes systems peroxidase (Prx), poly phenyl oxidase (PPO) for the identification and characterization of 12 faba bean varieties under study based on polyacrylamide gel electrophoresis profiles.

Peroxidase (PX)

The peroxidase (PX) electrophoretic patterns for different faba bean genotypes under investigation are presented in Table (27) and Fig (9). The density of (PX) bands differed according to faba bean genotypes, analysis of peroxidase isozyme patterns revealed three enzymatic bands. PX1pands observed in all genotypes with relative mobility (0.1) as follows: G1and Roomy high density band. While G2, G4, G5, G6, G8, Giza 843 and Misr1were moderate density bands. G3, G7 and Peter 15 were low density bands. PX2 group bands observed in all genotypes with relative mobility (0.3) as follows: high density band in Roomy genotype while, density bands were moderate in G4, G5, G6 and Giza 843. However, Px3 bands with relative mobility (0.5) observed in all genotypes as follows: moderate density bands in G1, G4, G5, Giza 843 and Peter15. While, G2, G3, G6, G7, G8 and Misr1were low density bands. High density band observed in Roomy genotype. Therefore, the differences in the density of bands can be used to successfully identify and characterize these faba bean genotypes.

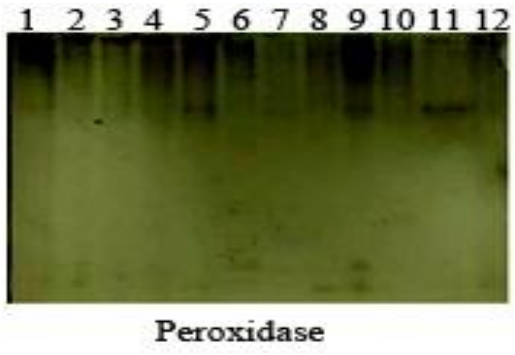

Fig. 4. Zymogram of Peroxidase (PX) isozyme for the ten faba bean varieties under study.

Polyphenyl Oxidase (PPO)

The density of (PPO) bands differed according to faba bean genotypes, analysis of polyphenyl oxidase isozyme patterns revealed four enzymatic bands. PPO1pands observed in all genotypes with relative mobility (0.1) as follows: G1and G4 high density band. While G2, G3, G5, Roomy and Peter15, Giza 843 and Misr1were moderate density bands. G3, G7 and Peter 15 were low density bands. PPO2 group bands observed in all genotypes with relative mobility (0.5) as follows: moderate density band in G1, G2, G3, G4, G5 and Peter 15. While, density bands were low in G6, G7, G8, Roomy, Giza 843 and Misr1. However, PPO3 bands with relative mobility (0.6) observed in all genotypes as follows: high density bands in G1, G3, G4, Roomy, Peter15 and Misr1. PPO4 bands with relative mobility (0.8) moderate density band observed in only G6. These findings led to characterize this genotype because of containing only this unique band. Hence, these values can adequately be used to characterize and identification between these varieties using of polyphenyl oxidase electrophoretic patterns.

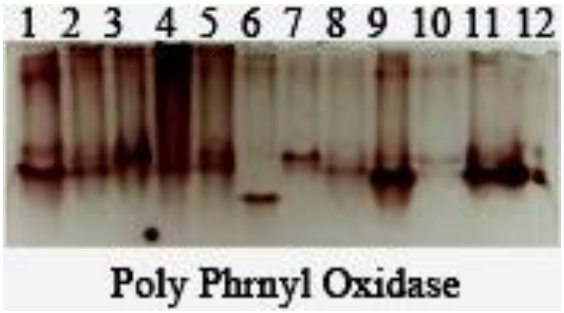

Fig. 5. Zymogram of poly phenyl oxidase (PPO) isozyme for the ten faba bean varieties under study.

Conclusion

ISSR markers, protein electrophoresis and isozymes analysis are useful tools for detection the genetic diversity among *Vicia faba* L. genotypes and help in studying genetic relationships among genotypes. Since, ISSR technique reflected enough polymorphism to distinguish among different genotypes. Also, it was possible to differentiate the closely related faba bean based on protein analysis.

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