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Molecular and physiological responses of *Pisum sativum* and *Vicia faba* to sodium azide

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Key words: Antioxidants, isozymes, ISSR finger printing, lipid peroxidation, *Pisum sativum, Vicia faba.*

Abstract

Seeds of *Pisum sativum* and *Vicia faba* were pre-soaked in either 1.0, 2.0 or 4.0 mM of sodium azide (NaN_3) for 30 min, 1 h or 2 hs then, sown in clay soil in plastic pots under conditions of 25/18°C day/night and natural light (16/8 h day/night) and the seedlings were harvested after 21 days. The results revealed that total phenols, H_2O_2, malondialdehyde and the activities of catalase, peroxidase and DPPH scavenging activity in both plant were gradually increased with the increase in NaN_3 concentration and soaking time. The isozymes of α- and β-esterases and α-amylase had number of loci ranged from two (α-amylase) to four (α-esterase) in pea, while in bean, it was three for the three investigated enzymes. The ISSR analysis revealed that both plants showed highly significant increase in the appearance of bands by the highest treatment of NaN_3, especially with the longest duration. Pea produced 14 polymorphic, 9 monomorphic and 4 unique bands, while bean produced 14 polymorphic, 8 monomorphic and 3 unique bands.

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Introduction

Faba bean (*Vicia faba* L.) is a major food and feed legume, a crucial source of protein and can be used as a winter or spring cover crop, green manure, silage, forage, hay and vegetable (Crepona *et al*., 2010). Pea (*Pisum sativum* L.) is a field crop grown for its edible seeds. They are nutritious legumes, containing 15 to 35% protein, and high levels of the essential amino acids lysine and tryptophan (Elzebroek and Wind, 2008).

Sodium azide (NaN_3) is a common bactericide, pesticide and industrial nitrogen gas generator known to be highly mutagenic in several plants such as barley and some other crop species (Ilbas *et al*., 2005). Kleinhofs and Sander (1975) reported that NaN_3 induced a wide range of morphological and physiological mutants in barely. The azide ions are strong inhibitors of cytochrome oxidase, which in turn inhibits the oxidative phosphorylation process. In addition, it is a potent inhibitor of the proton pump (Kleinhofs *et al.,* 1978), alters the mitochondrial membrane potential and changes the metabolic activity (Zhang, 2000). The toxicity of NaN_3 and most of its physiological effects can be traced to its reversible inhibitory effect on enzymes containing a coordinated divalent ion, such as those of cellular respiration (Kleinhofs *et al.,* 1978). Maxim *et al.* (2009) found that NaN_3 resulted in an increase in the activities of the antioxidant enzymes catalase and peroxidase.

Mutagenicity of NaN_3 is arbitrated through the formation of an organic metabolite of azide which enters the nucleus, interacts with DNA and generates point mutations in the genome (Owais and Kleinhofs, 1988). Sodium azide causes meiotic aberrations and their genetic consequences result in the appearance and disappearance of some protein bands depending on its concentration and duration (Saad-Allah *et al*., 2014). Soliman (2003) and Shehab *et al.* (2004) attributed such changes of mutational events to the loss of some genetic material due to induction of laggards, breaks and micronuclei or changes in gene sequences. Inter Simple Sequence Repeat–Polymerase Chain Reaction (ISSR-PCR) method permits the detection of some mutational events as polymorphisms in inter-microsatellite loci, using a primer designed from di-nucleotide or tri-nucleotide simple repeats, and possesses some advantages of stability and reproducibility (Gupta *et al*., 1994).

Phenolic compounds are ubiquitous in plant food, and have been associated with the sensory and nutritional quality of fresh and processed plant foods (Ho *et al*., 1992). Foods rich in phenolic compounds have been shown to impart anti-mutagenic, anti-inflammatory and antioxidant properties (Friedman, 1997). Most of the antioxidant substances in plants are phenolic compounds which serve as oxidation terminators by scavenging radicals to form resonance stabilized radicals (Rice-Evans *et al*., 1997). Jeng *et al.,* (2010) found that the total phenolics level is enhanced in bean subjected to NaN_3 treatments. Flavonoids act as mild pro-oxidants and stimulate the endogenous antioxidant defenses which reduce the impact of oxidative stress (Al-Qurainy and Khan, 2009). However, Jain and Agrawal (1990) stated that the total flavonoids content is increased in two *Trigonella* species to their maximum value under the effect of 0.0001M and decreased at 0.001M of NaN_3.

Isozymes are functionally similar enzymes with different molecular forms and they are very useful as genetic marker to distinguish mutants (Allendorf and Luikart, 2007, Talukdar (2010). Esterases are lipolytic enzymes which are widely used for the hydrolysis of ester bonds and transesterification (Brady *et al*., 1990). Amylases are calcium metalloenzymes, completely unable to function in the absence of calcium (Yamaguchi *et al*., 2004). It is stated that α-amylase breaks down long-chain carbohydrates by acting at random locations along the starch chain, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin (Pandey *et al*., 1999).

All stress types bring about important alterations in the reactive oxygen metabolism; among these alterations is the disappearance of catalase activity

and over production of H_2O_2 (Bailly *et al.,* 1996). Some of these stresses enhanced the active oxygen species which can oxidize biological molecules such as DNA, proteins and lipids (Murthy *et al.,* 2003). Free radicals have the potential to change the membrane structure and function; as a result membrane integrity is gradually disrupted causing increased permeability of the plasma membrane and loss of compartmentation of cytoplasmic organelles (Kumar and Knowles, 1993, Bailly *et al.,* 1996).

The present study aimed to assess the impact of the treatment with different concentrations of the mutagen NaN_3 for different time intervals on some molecular and physiological responses of the two legume plants, *Pisum sativum* and *Vicia faba*.

Materials and methods

Germination

Seeds of the two legume crops pea (*Pisum sativum* L.) and bean (*Vicia faba* L.) were kindly provided by the Agriculture Research Centre (ARC), Giza, Egypt, and grown for one season (December 2012 to April 2013). The seeds were surface sterilized using 0.01% (w/v) mercuric chloride ($HgCl_2$) for 8 min., with continuous stirring, rinsed thoroughly several times in distilled water, then three groups of them were soaked in three concentrations of NaN_3 (1.0, 2.0 or 4.0 mM) for different time intervals (30 min., 1 h. or 2 h.) beside the control which was maintained by pre-soaking the seeds in distilled water. The soaked seeds were then washed under running tap water and sown in plastic pots (35 cm diameter x 18 cm depth), each containing 6 kg clay soil. The experiment was carried out in randomized complete block design (RCBD) with split-plot arrangement in the green house under conditions of 25/18°C day/night temperature and natural light (16/8 h day/night). Phosphate fertilizer was applied at 20 lb/acre, as a side band with the seed. The seedlings were harvested after 21 d., a group of them was kept fresh for the biochemical analysis of flavonoids, H_2O_2, catalase, perodidase, malon dialdehyde (MDA), isozymes and ISSR finger printing. Another group of seedlings was dried in an oven at 50 °C for the analysis of total phenolics and DPPH activity.

Total Flavonoids and Total Phenolics

Total flavonoid content was estimated using aluminium chloride colorimetric technique according to Chang *et al.* (2002) and was calculated as mg/g f.wt. Total phenolic content was estimated quantitatively using the method described by Jindal and Singh (1975) and was expressed as mg/g d.wt.

Antioxidant activity

The total antioxidant activity (percentage of scavenged DPPH) was evaluated by the method described by Brand-Williams *et al.* (1995) and Bondet *et al.* (1997). H_2O_2 content was determined using the method given by Velikova *et al.* (2000) and its amount was expressed as nmol g^{-1} f.wt. Two antioxidant enzymes, catalase [EC1.11.1.6] and peroxidase [EC1.11.1.7] were extracted according to Beauchamp and Fridovich (1971) and their activities were assayed according to Kato and Shimizu (1987) and were expressed in units of µM of the substrate converted min^{-1}. g^{1} f.wt. The concentration of lipid peroxides was determined by the method of Mihara and Uchiyama (1978) and the MDA content was expressed as µmol g^{-1} f.wt.

Electrophoresis of Isozymes

Isozyme-PAGEs were prepared by extracting 0.2 g fresh leaves of 21-day-old seedlings according to El-Fadly *et al.* (1990). α-Amylase was determined in the extract by the method of Pasteur *et al.* (1988) and Esterases (EST) were estimated by the method of Soltis *et al.* (1983) using α-naphthyl acetate for α-esterase and β-naphthyl acetate for β-esterase. The isozyme electrophoretic banding patterns were scored for analysis and coded as 0 or 1 depending on their absence or presence, respectively, in the two studied plants.

ISSR- Analysis

The ISSR finger-printing procedures were based on the method described by Dogan *et al.* (2007). Five ISSR primers (Operon Nippon EGT CO. LTD) were

screened for the production of polymorphic products from all samples of the two plant species. The sequences of the primers and their properties are mentioned in Table (1). The ISSR fingerprinting was visualized using a Gel Works 1D advanced gel documentation system (UVP, UK) and photographed under UV light. The size of each band was estimated using 100 bp DNA ladder (Fermentas) as a standard marker, using Lab Image software program version 2.7 produced by Kapelan GmbH Co, Germany.

Table 1. Primers ID, sequences, length in bp and annealing temperature **(**Tm**)** of the selected ISSR primers with the number of polymorphic bands and percentage of polymorphism in *Pisum sativum* and *Vicia faba*.

Ser	ID	Sequence (5'→3')	Length (base pair)	Tm (°C)	Number of polymorphic bands		Polymorphism percentage (%)	
					Pisum sativum	*Vicia faba*	*Pisum sativum*	*Vicia faba*
01	17899 A	CACACACACACAAC	14	42	2	2	50	40
02	HB-10	GAGAGAGAGAGACG	14	44	2	2	33.3	40
03	HB-11	GTGTGTGTGTGTCG	14	44	3	4	50	66.6
04	HB-12	CACCACCACGC	12	38	2	2	50	66.6
05	HB-13	GAG GAG GAG GC	11	38	5	4	71.4	66.6

Results and discussion

Total Flavonoids

The total flavonoid content of methanolic extract of *Pisum sativum* and *Vicia faba* under the effect of different sodium azide concentrations and different soaking time intervals were shown in Fig.1. It increased gradually with the increase in both concentrations and soaking time in NaN_3. The highest flavonoid content was achieved with the highest NaN_3 concentration (4 mM) at soaking time 2 hours. Dixon and Paiva (1995) and Grace and Logan (2000) reported that flavonoids are frequently induced by abiotic stress and promote roles in plant protection. Moreover, the modifications of flavonoid structure i.e., glycosylation, prenylation and methylation could affect their antioxidant properties, thus they may help inhibit lipid peroxidation in stressed-plants (Caturla *et al*., 2003, Potapovich and Kostyuk, 2003). It is known that the natural substances such as flavonoids and tannins or their derivatives possess antimutagenic properties and these metabolites could be involved in mutagen deactivation (Edenharder *et al*., 1993, Yen and Chen, 1996, Hornl and Vargas, 2003). The possible mechanism of the demonstrated antimutagenic behaviour of flavonoids could be due to inactivation of the reactive intermediates formed from mutagens (Gowri and Chinnaswamy, 2011).

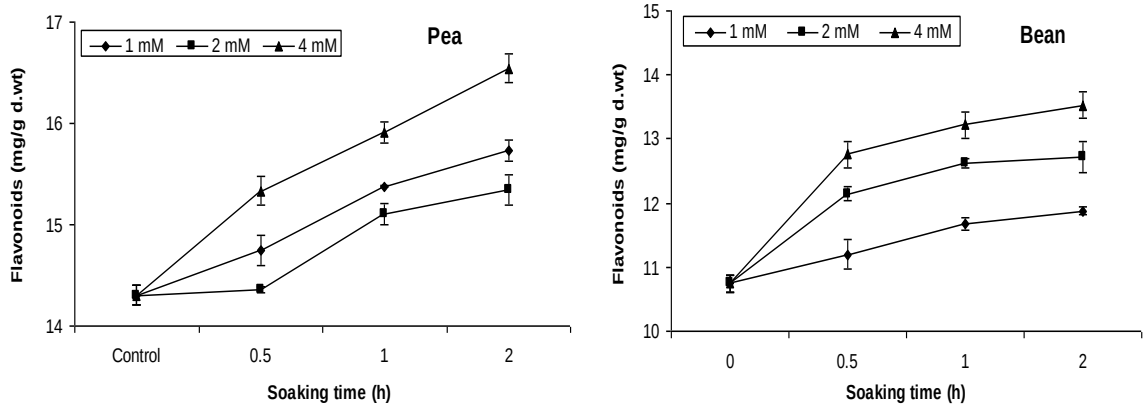

Fig. 1. Effect of presoaking of pea (*Pisum sativum*) and bean (*Vicia faba*) seeds in different concentrations (1, 2 and 4 mM) of NaN_3 for different time intervals (0.5 h, 1h and 2 hs) on the total flavonoid contents of 21-days old seedlings.

Total Phenolics

The total phenolic content in the two plant species gradually increased with the increase in NaN_3 concentration and soaking time (Fig. 2). Generally, the total phenolic compound contents of *Vicia faba* was greatly higher (about four-fold) than that of *Pisum sativum* either in the control or in sodium azide treatments. Soaking of *Vicia faba* seeds in the highest concentration of NaN_3 (4 mM) for 2 hs resulted in 24.4% increase in the total phenolic contents, while in *Pisum sativum* it was increased by 62.6% compared with their controls. These results manifested the correlation between the concentration of the mutagen and the accumulation of the phenolic compounds. Several reports stated that phenolic compounds have many biological activities; they can act as antioxidants (Kagan and Tyurinov, 1998), scavengers of active oxygen species and electrophiles (Zhou and Zheng, 1991) and chelators of metals (Brune *et al*., 1989). Birosov *et al*. (2005) found that phenolic acids inhibited the mutagenic activity of sodium azide in *Salmonella typhimurium* by 82%. Several mechanisms have been proposed for the action of phenolic compounds as antimutagens, however, the two main mechanisms include the inhibition of enzyme systems such as the cytochrome-P450-dependent bioactivation of the various mutagens and the scavenging of metabolically generated mutagenic electrophiles (Del Pozo-Insfran *et al*., 2004). In addition, Hour *et al*. (1999) proposed a third mechanism which includes the blocking of the mutagen transfer into the cytosol by phenolic binding or insertion into the transporters of the outer membrane of the cell. As concluded by de Mejia *et al*. (1999), phenolic compounds can interact directly and non-enzymatically with the mutagen or form a complex between themselves and the mutagen thereby reducing the mutagen bioavailability. Phapale and Misra-Thakur (2010) reported that *Feronia limonia* has high antimutagenic effect against sodium azide due to its high content of phenolic compounds.

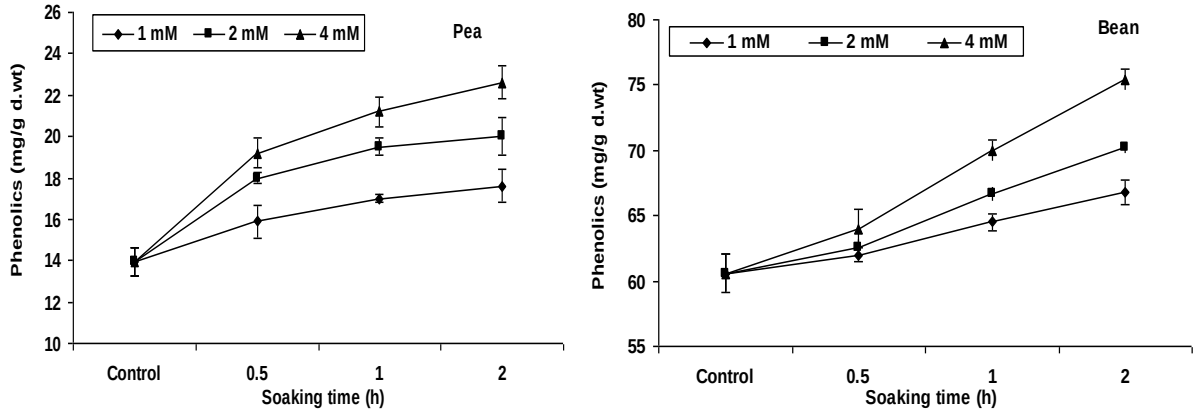

Fig. 2. Effect of presoaking of pea and bean seeds in different concentrations (1, 2 and 4 mM) of NaN_3 for different time intervals (0.5 h, 1 h and 2 hs) on the total phenolic content of 21-days old seedlings.

Antioxidant activity

The DPPH radical scavenging activities of *Pisum sativum* and *Vicia faba* extracts under the different concentrations of NaN_3 and different soaking time intervals wer showed in Fig. 3. The DPPH scavenging activity of *Vicia faba* was higher than that of *Pisum sativum* under control treatments (81.6% and 62.0%, respectively). However, the DPPH scavenging activity of *Pisum sativum* was more affected by NaN_3 treatments than *Vicia fava*. In both plant species, this activity was increased proportionally with the increases of NaN_3 concentration and the soaking period. The highest increase was recorded with 4 mM NaN_3 after 2 hs of soaking, where it caused 32.4% and 4.3% increase in the DPPH scavenging activity in *Pisum sativum* and *Vicia faba*, respectively. The results of this study indicated that sodium azide treatments might have induced some antioxidant compounds as phenolics that are capable of donating hydrogen to a free radical in order to remove the odd

electron, which is responsible for the radical's reactivity (Singh *et al*., 2007, Olayinka and Anthony, 2010, Duhan *et al*., 2011). Phenolics and flavonoids are the major constituents noted in most plants and it has been reported by many researchers that they posses antioxidant and free radical scavenging activity (Yerra *et al*., 2005). The observed antioxidant activity in this study may be due to the neutralization of free radical character of DPPH, either by transferring of an electron or hydrogen atom (Naik *et al*., 2003). The ability of the extract to scavenge the DPPH radical has also been related to the inhibition of lipid peroxidation (Rekka and Kourounakis, 1991).

Sodium azide treatments significantly increased H_2O_2 content in the leaves of both *Pisum sativum* and *Vicia faba* (Fig. 4). However H_2O_2 content of *Vicia faba* was relatively higher than that of *Pisum sativum* under the control and sodium azide treatments. The increase in H_2O_2 content was proportional to the increase in sodium azide concentration and soaking period. The highest increase in H_2O_2 content was recorded for both seeds with soaking in 4 mM NaN_3 for 2 hs; however, it was 2 folds in *Pisum sativum* and 2.5 folds in *Vicia faba*, compared with their controls. These results were in agreement with those of Szatrowski and Nathan (1991) who reported that the presence of sodium azide in the reaction mixture prevents the competitive action of catalase on H_2O_2 leading to the accumulation of H_2O_2, while they disagreed with those obtained by Francisco *et al.* (2008), who reported that sodiume azide treatments decreased the level of H_2O_2 in grapevine buds.

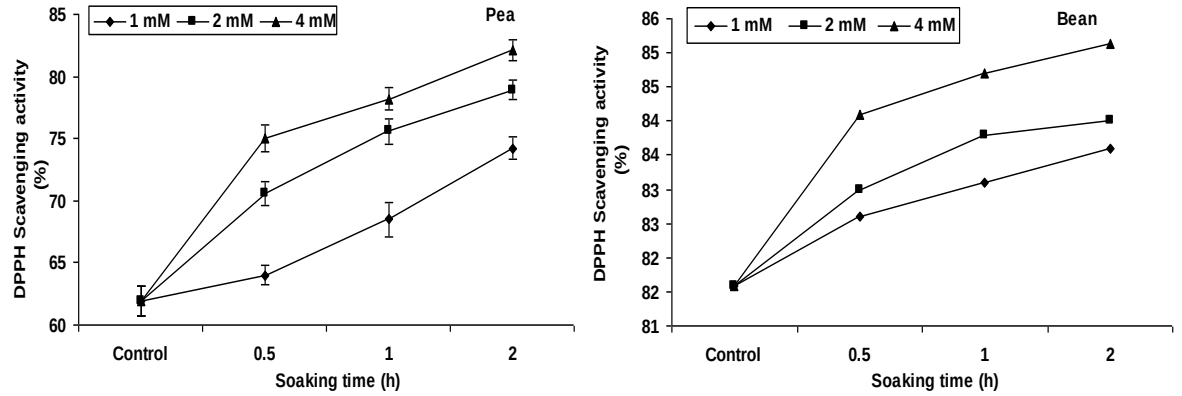

Fig. 3. Effect of presoaking of pea and bean seeds in different concentrations (1, 2 and 4 mM) of NaN_3 for different time intervals (0.5 h, 1h and 2 hs) on DPPH radical scavenging activity of 21-days old seedlings.

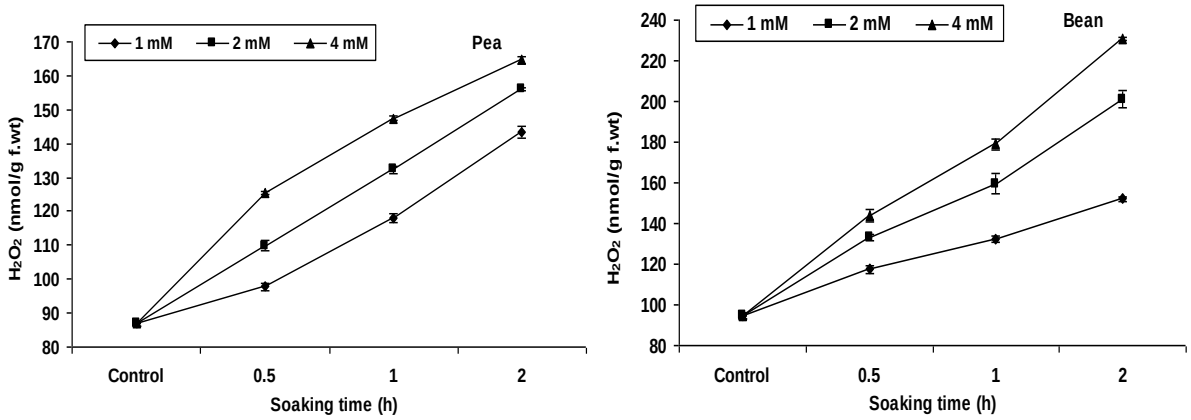

Fig. 4. Effect of presoaking of pea and bean seeds in different concentrations (1, 2 and 4 mM) of NaN_3 for different time intervals (0.5 h, 1 h and 2 hs) on H_2O_2 content of 21-day old seedlings.

In addition to the well-known oxidative damages caused by increase in ROS levels in plant tissues especially H_2O_2, they play an important role as a signalling molecule produced and controlled by

metabolism, being beneficial at low concentrations and harmful when produced in excess (Foyer and Noctor, 2005, Gechev and Hille, 2005, Quan *et al*., 2008). It was proposed that H_2O_2 contributes to the increase in antioxidant enzyme activity (Azevedo Neto *et al*., 2005), the decrease in lipid peroxidation and chloroplast ultrastructure protection in *Cucumis sativus* (Gao *et al*., 2010).

Results in Fig. 5 showed that NaN_3 treatments significantly increased catalase activity in both *Pisum sativum* and *Vicia faba*, compared with the control. This increase was proportional with NaN_3 concentration and treatment duration. This was consistent with the results reported by Maxim *et al*., (2009) which showed that catalase activity is varied depending on NaN_3 treatment duration. Similarly, Mierlici1 *et al*. (2011) found that the high concentrations of the mutagen ethyl-methane-sulfonate caused high catalase activity in *Hordeum vulgare*. In contrast, Santos *et al*. (2008) concluded that catalases directly catalyze the decomposition of H_2O_2 to ground-state O_2 and can be non-specifically inhibited by NaN_3.

Peroxidase showed similar trend to that of catalase in both of *Pisum sativum* and *Vicia faba* (Fig. 6). It was clear from the results that the peroxidase activity of *Pisum sativum* was higher than that of *Vicia faba* under control and NaN_3 treatments. The soaking in the highest concentration of NaN_3(4 mM) for 2 h resulted in six-folds and ten-folds increases in peroxidase activity in *Pisum sativum* and *Vicia faba*, respectively, compared with their controls. The increase in peroxidase activity under azide treatments might arise from the increased exertion of H_2O_2 under the mutagenic effect of sodium azide. Zaka *et al*. (2002) reported that sodium azide induces the formation of reactive oxygen species, which indicates that this enzyme was involved in cell protection against oxidative stress However, the present results disagree with those obtained by Mydlarz and Harvell (2006), who pointed out that 1 mM and 10 mM NaN_3 inhibited peroxidase activity by 52% and 85% in *Gorgonia ventalina*, respectively. Similarly, sodium azide treatments competitively inhibited peroxidase activity in *Luffa aegyptiaca* (Yadav *et al*., 2011) and in *Beta vulgaris* (Chaurasia *et al*., 2013).

The effect of pre-treatment with sodium azide on malondialdehyde (MDA) content in *Pisum sativum* and *Vicia faba* was shown in Fig. 7.The results indicated that MDA content was increased significantly with the increase in NaN_3 concentration and soaking time. Where, soaking of *Pisum sativum* and *Vicia faba* seeds in 4 mM NaN_3 for 2 hs. resulted in 43.6% and 54.0% increase in its content, respectively, compared with their controls. MDA is a common product of the peroxidation of unsaturated fatty acids by singlet oxygen and a sensitive diagnostic index of the oxidative injury (Janero, 1990, Bradley and Min, 1992).The recorded increase in MDA may be due to the increased generation of the oxidative molecules as a result of NaN_3 treatment. Price and Hendry (1991) stated that oxidative molecules initiate damage in the chloroplast including chlorophyll destruction and lipid peroxidation.

Isozymes

In this study, the enzymes of α- and β- esterase and α-amylase had potential as a biochemical marker to detect different responses for the effect of the mutagen NaN_3 on gene expression (Fig. 8 and Table 2). It is known that enzymes which are coded by different alleles of a distinct locus or those coded by separate loci frequently show different electrophoretic mobilities and these differences were due to variations in the amino acids content of the enzyme molecules, which in turn dependent on the sequences of nucleotides in DNA (Micales *et al.,* 1992).

Table 2. Scoring of isozymes produced by different three enzymes after presoaking of *Pisum sativum* and *Vicia faba* seeds in different concentrations (1, 2 and 4 mM) of sodium azide for different time intervals (0.5, 1.0 and 2.0 h) in 21- day old seedlings.

Band Number	Species	*Pisum sativum*									*Vicia faba*									
	Treatment	NaN_3									NaN_3									
	concentration	1mM			2mM			4mM			1mM			2mM			4mM		
		Soaking duration (h)									Soaking duration (h)									
	Control	0.5h	1h	2h	0.5h	1h	2h	0.5h	1h	2h	Control	0.5h	1h	2h	0.5h	1h	2h	0.5h	1h	2h
								α-Esterase												
α-EST1	1	1	1	1	1	1	1	2	2	2	1	1	1	1	1	1	1	1	1	1
α-EST2	1	1	1	1	1	2	2	0	0	2	1	1	2	2	2	2	0	1	1	1
α-EST3	1	1	1	1	1	1	1	2	2	2	1	1	1	1	1	1	1	0	1	1
α-EST4	1	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0
								β-Esterase												
β-EST1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
β-EST2	2	2	2	2	2	2	2	0	0	0	2	1	1	1	1	1	1	1	1	1
β-EST3	2	0	0	2	0	1	2	2	2	2	2	0	0	0	1	1	1	1	1	1
								α-Amylase												
α-AMY1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
α-AMY2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
α-AMY3	2	2	2	1	2	1	1	1	1	2	1	1	0	0	0	0	0	1	1	1

The data in Fig. 8 and Table 2 revealed that in the case of pea, the number of loci for the three tested isozymes varied between two and three in α- and β-esterase and α-amylase or four in α-esterase. In case of bean, the number of loci was either two or three for the three investigated isozymes. These variations in allele number per locus reflect that, the effect of the mutagen differed from plant to another, which means a differential expression (Ashwani *et al*., 1998). These loci may vary in the intensity of their allele which reflects the number of subunits accumulation in each locus (Deka *et al*., 1999).

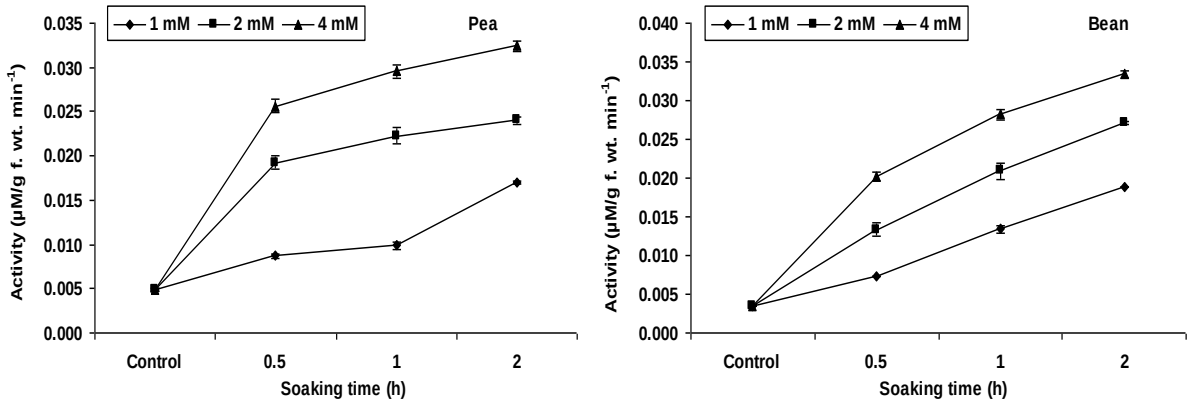

Fig. 5. Effect of presoaking of pea and bean seeds in different concentrations (1, 2 and 4 mM) of NaN_3 for different time intervals (0.5 h, 1 h and 2.0 hs) on catalase enzyme activity of 21-day old seedlings.

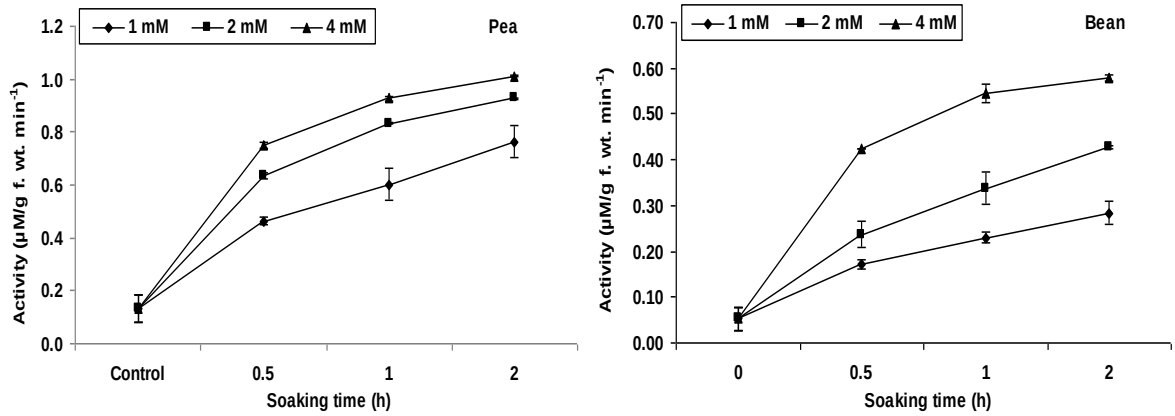

Fig. 6. Effect of presoaking of pea and bean seeds in different concentrations (1, 2 and 4 mM) of NaN_3 for different time intervals (0.5 h, 1 h and 2 hs) peroxidase enzyme activity of 21-days old seedlings.

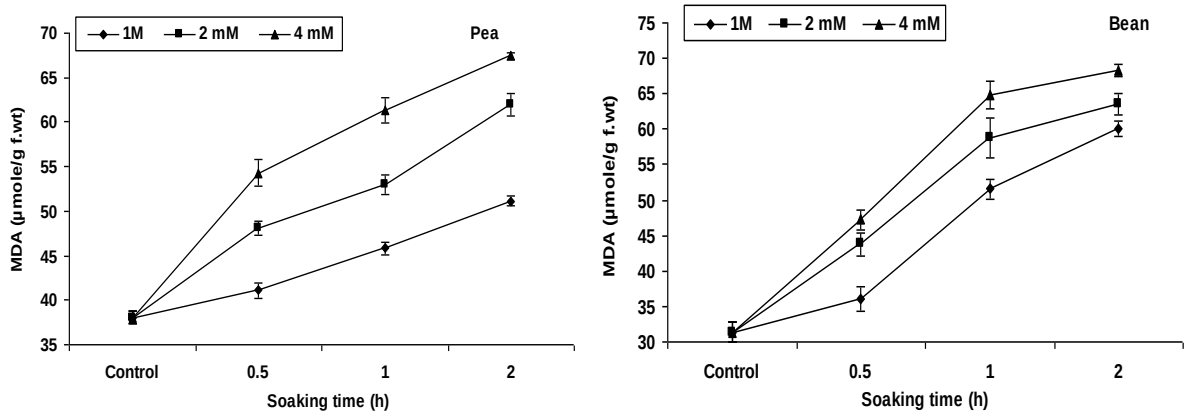

Fig. 7. Effect of presoaking of pea and bean seeds in different concentrations (1, 2 and 4 mM) of NaN_3 for different time intervals (0.5 h, 1 h and 2 hs) on Malondialdehyde (MDA) content of 21-days old seedlings.

Isozymes are widely used as molecular markers to distinguish mutants as reported by Talukdar (2010). For the isozyme α-esterase in pea, the three loci were expressed by only one allele similar to that of the control and it was revealed by two alleles with the highest treatment 4mM in the first and third locus, while it completely disappeared in the fourth locus. For bean, the first and third loci were expressed by only one allele similar to that of the control, while in the second locus, it was expressed by two alleles with 1mM for 1 h and 2h, and with 2 mM for 0.5 and 1 h.

For the isozyme of β-esterase, the results cleared that, in pea, the second locus was expressed by two alleles in all treatments similar to that of the control, and these alleles were disappeared with the highest concentration of NaN_3 (4mM). However, it was expressed by only one allele with 1 and 4 mM of NaN_3 treatments, compared with the control, while they were disappeared with 2 mM NaN_3 treatments. The third locus of the isozyme β-esterase in pea was expressed by two alleles with the highest concentration of NaN_3 treatment for the longest duration similar to that of the control; it was expressed by only one allele with 2mM for 1h, while it was disappeared with each of 1mM for 0.5 and 1h and with 2mM for 0.5 h. In case of bean, the locus was expressed by only one allele with 1 mM and 4 mM of NaN_3 treatments that differed with that of the control while they were disappeared completely in the lowest treatments (1mM).

For α-amylase in pea, the first locus with 1mM $NaNo_3$ for 0.5 h and the second locus expressed by only one allele similar to that of the control while disappeared completely in the other treatments of the first locus.

For the third locus, it was expressed by tow alleles with the longest duration for each treatment of $NaNo_3$. In case of bean, the first locus, it was expressed by only one alleles with the highest treatments (4mM of $NaNo_3$) which was not found in the control, while the second and third locus were expressed by only one allele for each one with the highest treatments (4mM of $NaNo_3$) which was similar to that of the control. These results were almost in agreement with those of Bartosova *et al.* (2005) and Malaviya *et al.* (2006). The mutagenesis treatments seemed to activate expression of some genes which resulted in the appearence of some new bands (Talukdar, 2010). It was reported that mutations have been identified as one of the sources of isozyme variation in higher plants (El-Mokadem and Mostafa, 2014). On the other hand, the disappearance or reduction in the number of alleles may be due to the mutagenic effects of NaN_3 (Aly and Elsayed, 2006) that resulted in a change in the metabolism of cells by forming oxygen radicals which acted as mediators of cell damage (Frigo *et al.,* 2009). The greater sensitivity of the enzymes to the higher mutagenic levels might be attributed to various factors such as changes in the metabolic activity of the cells, inhibitory effects of mutagens and disturbance of balance between promoter and inhibitors of growth regulators (Padmanaban *et al.*, 2013).

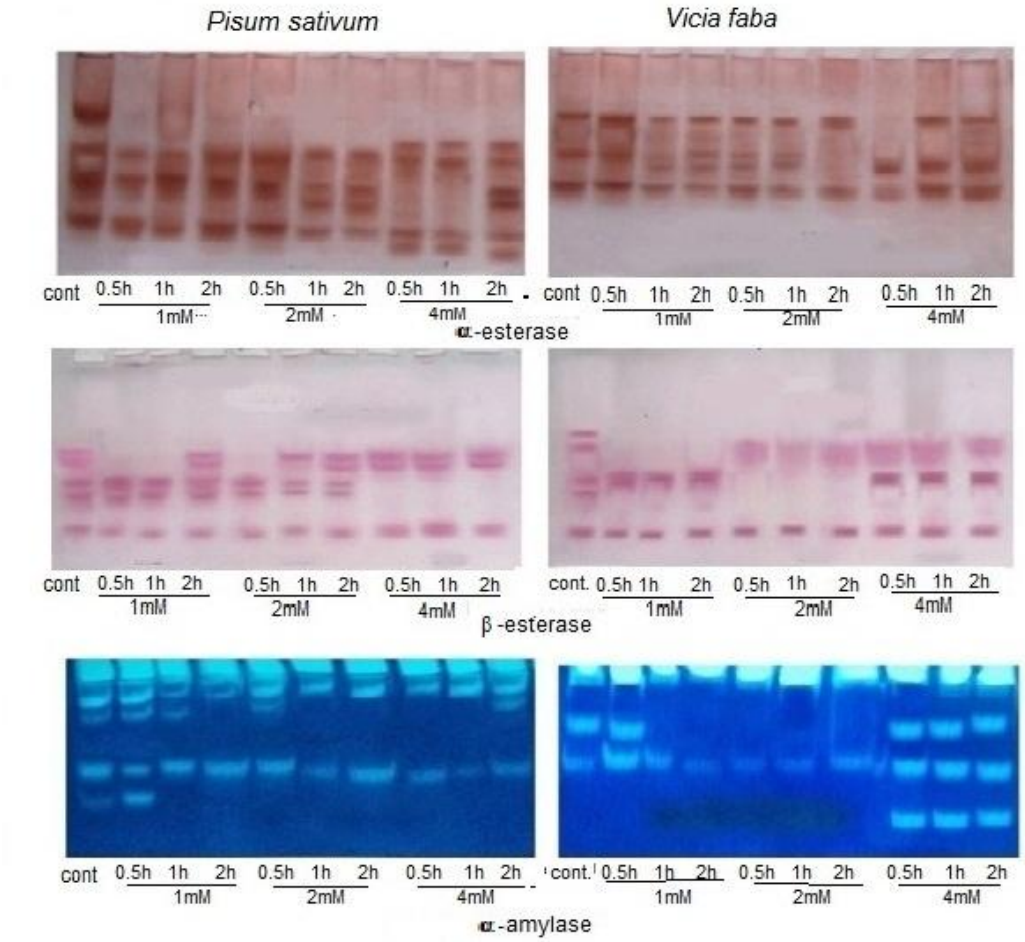

Fig. 8. Effect of presoaking of pea and bean seeds in different concentration (1, 2 and 4 mM) of NaN_3 for different time intervals (0.5, 1.0 and 2.0 h) on isozyme profile for three enzymes of 21-day old seedlings.

ISSR-Analysis

The data of the scoring of ISSR produced by different five primers after presoaking of pea and bean seeds in different concentrations shown in Table 3 and Figs. 9A and 9b revealed that pea produced 27 bands (14 polymorphic, 9 monomorphic and 4 unique), while bean produced 25 bands (14 polymorphic, 8 monomorphic and 3 unique).

Table 3. Scoring of ISSR fingerprinting produced by 5 primers and their band type as polymorphic (P) monomorphic (M) or unique (U) for presoaking of pea and bean seeds in different concentrations (1,2 and 4 mM) of sodium azide for different time intervals (0.5,1 and 2 h) of 21-days old seedlings.

Band No.	Mol. Size (bp)	Species	*Pisum sativum*									Band type	Mol. Size (bp)	*Vicia faba*									Band type
		Treatment	NaN_3 concentration (mM)											Treatment	NaN_3 concentration (mM)								
		concentration	1mM			2mM			4mM					Concentration	1mM			2mM			4mM		
			Soaking duration (h)											Soaking duration (h)									
		Control	0.5h	1h	2h	0.5h	1h	2h	0.5h	1h	2h			Control	0.5h	1h	2h	0.5h	1h	2h	0.5h	1h	2h	
Primer 17899 A sequence 5`CAC ACA CAC ACA AG 3																							
01	471	0	0	0	0	0	1	1	0	0	0	P	801	0	0	0	0	0	0	0	0	0	1	U
02	401	1	1	1	1	1	1	1	1	1	1	M	657	0	0	0	0	0	0	1	1	1	1	P
03	303	0	0	0	0	1	0	1	1	1	0	P	530	0	0	0	0	1	1	1	1	1	1	P
04	207	1	1	1	1	1	1	1	1	1	1	M	439	1	1	1	1	1	1	1	1	1	1	M
05													345	1	1	1	1	1	1	1	1	1	1	M
Primer HB-10 sequence 5` GAG AGA GAG AGA CC 3`																							
01	279	0	0	0	0	0	0	0	0	0	1	U	645	1	1	1	1	1	1	1	1	1	1	M
02	255	0	0	0	0	0	0	0	0	0	1	U	621	1	1	1	1	1	1	1	1	1	1	M
03	224	0	1	1	1	1	1	1	1	1	1	P	531	0	0	1	1	0	1	1	0	0	1	P
04	186	0	0	0	1	0	0	1	0	0	1	P	467	0	0	1	0	0	0	0	0	0	0	U
05	157	1	1	1	1	1	1	1	1	1	1	M	385	0	0	0	0	0	1	0	1	1	1	P
06	116	1	1	1	1	1	1	1	1	1	1	M												
Primer HB-11 sequence 5` GTG TGT GTG TGT CC 3'																							
01	561	0	0	0	0	0	0	1	0	1	1	P	559	0	0	0	0	0	0	0	0	1	0	U
02	536	0	0	0	1	0	0	0	0	0	0	U	494	0	0	0	0	0	1	0	0	0	1	P
03	463	0	0	1	0	1	1	0	1	1	1	P	476	0	0	0	0	1	0	0	1	0	0	P
04	379	0	1	0	0	1	1	1	1	0	1	P	405	0	1	1	1	1	0	1	0	0	0	P
05	315	1	1	1	1	1	1	1	1	1	1	M	349	0	0	0	0	0	0	0	0	1	1	P
06	163	1	1	1	1	1	1	1	1	1	1	M	229	1	1	1	1	1	1	1	1	1	1	M
Primer HB-12 sequence 5` CAC CAC CAC GC 3`																							
01	381	0	0	0	0	0	1	0	0	1	1	P	472	0	0	0	1	1	1	1	0	1	1	P
02	310	0	0	1	0	0	1	0	0	1	1	P	430	0	1	1	1	1	1	1	1	1	1	P
03	249	1	1	1	1	1	1	1	1	1	1	M	354	1	1	1	1	1	1	1	1	1	1	M
04	195	1	1	1	1	1	1	1	1	1	1	M												
Primer HB-13 Sequence 5` GAG GAG GAG GC 3`																							
01	797	0	0	0	0	0	0	0	0	0	1	U	838	0	0	0	0	0	0	0	0	1	1	P
02	708	0	0	0	0	0	0	0	1	1	1	P	705	0	0	0	0	0	1	1	1	0	1	P
03	604	0	0	0	0	1	1	1	1	1	1	P	569	0	0	0	0	0	1	1	1	1	1	P
04	510	0	0	0	0	0	0	0	1	1	1	P	466	0	0	0	0	1	1	1	1	1	1	P
05	392	0	1	1	1	1	1	1	0	0	0	P	334	1	1	1	1	1	1	1	1	1	1	M
06	324	0	0	0	0	0	0	0	0	1	1	P	228	1	1	1	1	1	1	1	1	1	1	M
07	210	1	1	1	1	1	1	1	1	1	1	M												

The data revealed that both plants showed highly significant increase in the appearance of bands by the highest treatment of NaN_3, especially with the longest duration treatments. In pea, the data showed four new positive unique bands appeared with the highest level of NaN_3 treatment, one with molecular size of 797 bp with the HB-13 primer, two with molecular sizes of 279 and 225 bp with the HB-10 primer. These three bands appeared with the treatment of 4 mM NaN_3 for 2 h, and the fourth band with molecular size of 797 bp with the HB-11 primer appeared with the treatment of 1mM NaN_3 for 1 h. However, in case of bean, the data reflected three positive unique bands with different treatments of NaN_3, where there was a band with molecular size of 801 bp appeared with the 17899 A primer with the treatment of 4 mM for 2h, band with molecular sizes of 559 bp appeared with the HB-11 primer with the treatment of 4 mM for 1 h, and a band with molecular size of 467 bp appeared with the HB-10 primer with the treatment of 1mM for 1h. These unique bands were exclusive to particular concentration of NaN_3, which made them distinct from the other treatments in the two species under study.

The obtained data reflected the appearance of new bands with the highest concentrations of NaN_3 for longest duration, such as the bands with molecular size of 708 bp, 604 bp and 510 bp with the HB-13 primer with the treatment of 4 mM NaN_3 for 2 h in case of pea. In case of bean, bands with molecular size of 657bp with 17899 A primer, 705 bp and 838 bp with the HB-13 primer with the treatment of 4 mM

for 2 h. The appearance of these new bands may be due to the formation of an organic metabolite by NaN_3 which enters the nucleus, interacts with DNA and generates point mutations in the genome (Berenschot *et al.*, 2008) and may damage or modify important components of plant cells and affect the biochemistry and physiology of plants (Sander *et al.*, 1978). These effects can include changes in the cellular structure and metabolism of the plants (Saad-Allah *et al.*, 2014).

ISSR marker accesses variation in the numerous microsatellite regions dispersed throughout the various genomes (particularly the nuclear genome) and circumvents the challenge of characterizing individual loci that other molecular approaches require (Semagn *et al.*, 2006, Sharma *et al.,* 2008). The data revealed that the two bands with molecular size of 430 bp with HB-12 primer for bean and of 224 bp with HB-10 primer for pea appeared in all treatments of NaN_3, which were not detected in the control. The appearance or disappearance of different bands due to change during DNA replication causes changes in the DNA bands, where the main changes in the ISSR profiles according to the mutagenicity effect (Sander *et al.*, 1978).

Finally, it can be concluded that sodium azide was a powerful mutagen for the induction mutations in *Pisum sativum* and *Vicia faba*, and ISSR and isozyme analysis could act as useful biochemical markers for mutant identification.

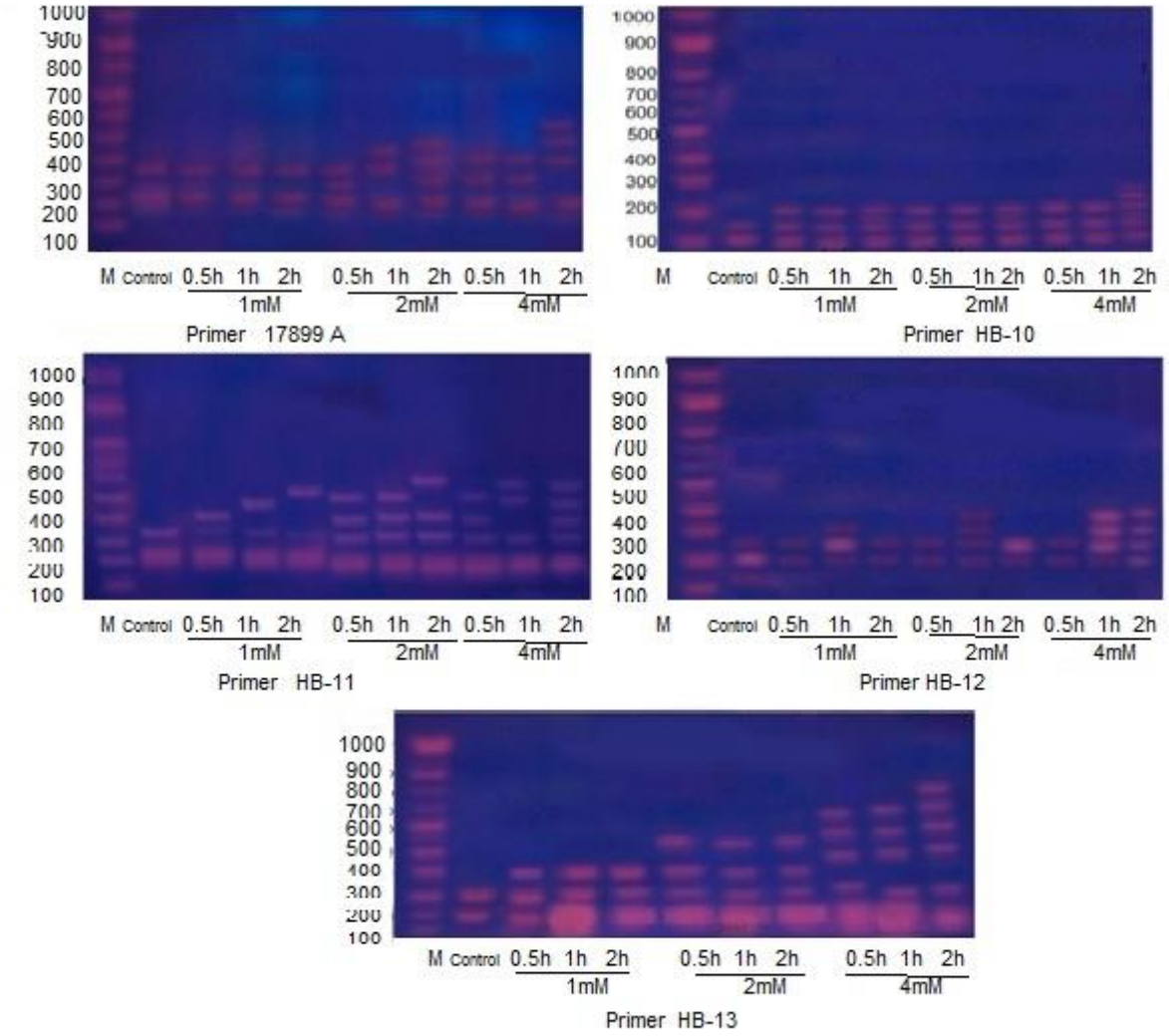

Fig. 9a. ISSR fingerprinting profile produced by five primers used for presoaking of *Pisum sativum* seeds in different concentration (1, 2 and 4 mM) of NaN_3 for different time intervals (0.5,1.0 and 2.0 h) of 21-day old seedlings.

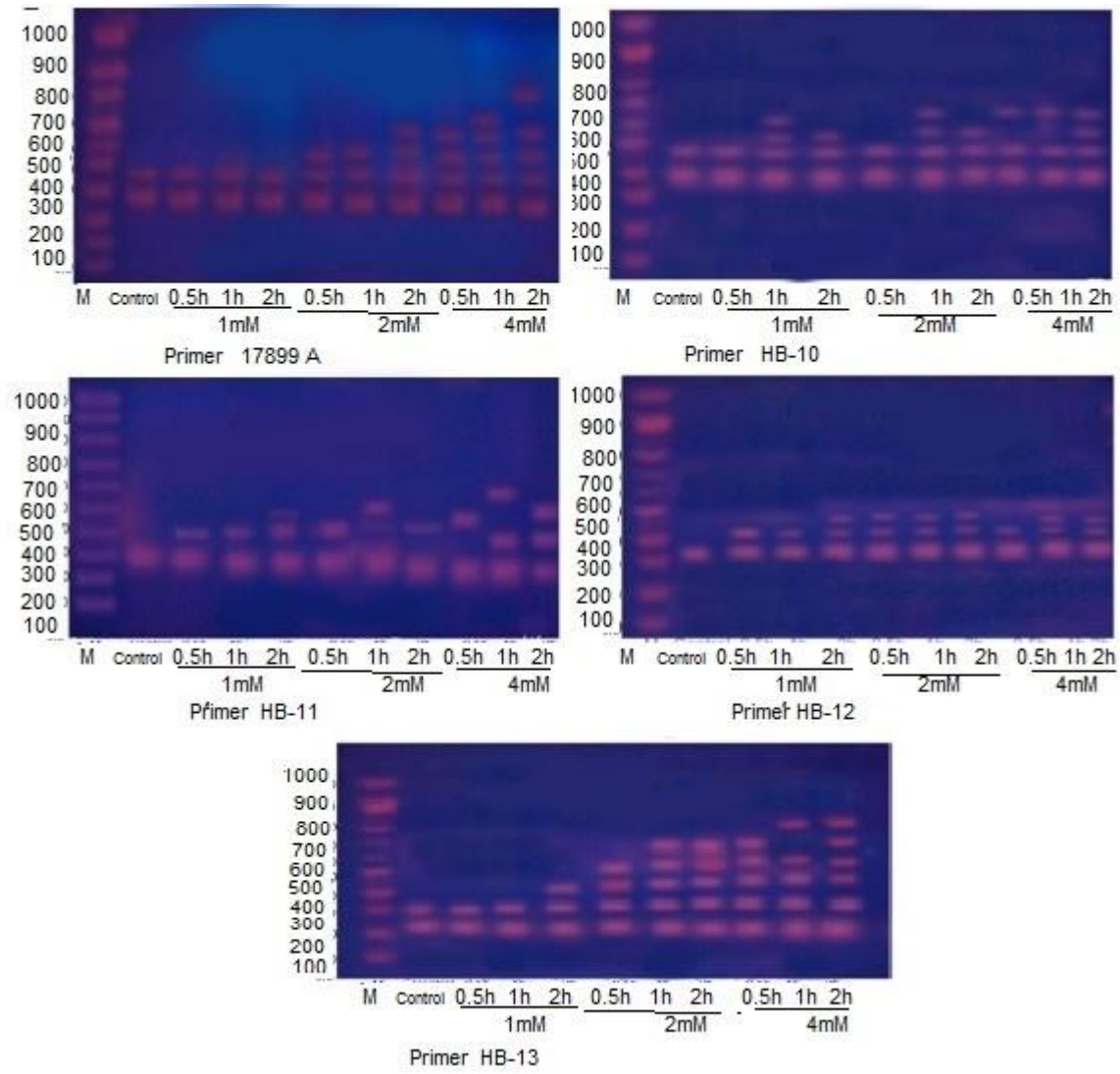

Fig. 9b. ISSR fingerprinting profile produced by five primers used for presoaking of *Vicia faba* seeds in different concentration (1, 2 and 4 mM) of NaN_3 for different time intervals (0.5,1.0 and 2.0 h) of 21-day old seedlings.

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