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EFFECT OF SODIUM AZIDE ON SEEDLING GROWTH AND PEROXIDASE ISOZYME PROFILES IN *VIGNA RADIATA L.*

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ABSTRACT
In the present investigation an attempt was made to study the effect of different concentration of sodium azide (0.01 M to 0.06 M) at room temperature on peroxidase isozyme patterns in Mung bean *(Vigna radiata L.)* with an objective of finding it mode of action i.e. whether it has promoting or inhibitory effect on the enzyme synthesis and whether the action is specific or random. SA at all concentration reduced the rate of seed germination, root, and shoot and seedling growth as compared to control. The concentration of 0.03M was adjusted as LD50.PAGE analysis of peroxidase isozyme of 8-day old control seedlings showed only three bands of peroxidase isozymes. The concentration of 0.004M and 0.005M showed presence of seven distinct peroxidase bands. It is an established fact that when plants are exposed to stressful condition harmful substances like H_2O_2 increases and cause significant damage to plant cells. A major hydrogen detoxifying system in plant cell is the peroxidase isozymes which catalyzes the conversion of H_2O_2 into $H_2O$and nascent oxygen. The observed increase in number of peroxidase isozymes in the present investigation might be due to the genetic stress caused by deleterious effects of sodium azide as it is a potent mutant. The result suggests that SA induces synthesis of peroxidase isozyme and the action seems to be specific.

Keywords: *Peroxidase Isozyme, Potent Mutant, Promoting, Inhibitory*

INTRODUCTION
Chemical mutagenesis is being widely used for understanding of the process of mutation induction in different plants and microbial systems. Chemical mutagenesis mainly induces point mutations, causes chromosomal and DNA damage and induces high frequency of mutations. Although the mutations induced by the mutagens are not always useful, they are helpful in determining the effect and mechanism of action of the mutagen in question (Mujeeb-ur-Rehman *et al.*, 2000). Sodium azide was used as a mutagen in 1970s at Washington state university, Pulman, USA (Sideris, *et al.*, 1969, Nilan *et al.,* 1973, Kleinhofs *et al.,* 1974 and Sander and Muchlbour, 1977). The potentially high mutagenicity of azide was disclosed by Spence (1964). Sodium azide (NaN3) is the least dangerous and the most efficient mutagen and has been reported to be mutagenic in several crop species (Mostafa, 2011). The mutagenicity of sodium azide is arbitrated through the formation of an organic metabolite which enters the nucleus, interacts with DNA, and generates point mutations in the genome.
Legumes are next in importance to cereals as source of human food. Even more species of legumes are known of many are of importance as industrial medicinal or food plants. Mung bean (*Vigna radiata* (L.) Wilczek) is also known as green gram, or simply mung (Purseglove, 1977; Sinha, 1977; Duke, 1983). The crop is said to have originated from India and must have been derived from var. *sublobata* which occurs wild throughout India and Burma (Aykroyd and Doughty, 1964; Purseglove, 1977). From there it has spread to South and East Asia, East and Central Africa, the West Indies and the United States. Mung bean is a low altitude crop grown from sea level to approximately 2000 m, usually as a dry land crop. It thrives best on a good loam soil with well distributed rainfall of 70–90 cm year.
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). Peroxidase is an

enzyme, which is universally present in almost all plants. It possesses a high number of isozymes, which are usually called as peroxidase isozymes. Peroxidases perform a wide variety of catalytic functions in plants (Gasper *et al*., 1982). Important among then are destruction of toxic hydrogen peroxide (Cohen and Hochstein 1963), inactivation of IAA (Ray 1958), formation of hydrogen peroxide (Moder *et al*., 1980) and hydroxylation of prolines in cell walls (Ridge and Osborn, 1970). The peroxidsase is suitable as a model enzyme for studying the effect of any mutagen (Khanna and Maherchandani 1981). Selection of peroxidase isozymes for this study was mainly due to the fact that they serve as very good marker for any mutational studies (Malpathak and David 1991).

MATERIALS AND METHODS

1) Obtaining plant material

Seeds of mung bean were procured from the agro service centre, Bhende. Uniform seeds of same size and approximately same weight are selected from the lot. The seeds were surface sterilized with 0.1 % mercuric chloride solution for 1 minute at room temperature. They were thoroughly washed under running tap water for about 20 minutes and rinsed with doubled distilled water. The seeds were presoaked in distilled water for 4 hours and treated with different concentrations of sodium azide for different time intervals. These were washed thoroughly under running tap water for 30 min, in order to remove the excess of mutagen at the end of the treatment. From the preliminary trials made employing varying the concentration of sodium azide and treatment durations, six different concentrations of sodium azide (0.01, 0.02, 0.03, 0.04, 0.05 and 0.06 M) and duration of 4 hours was finally selected.

In further experiments, seeds presoaked in distilled water for 4 hours were subjected to treatment with concentrations (0.01, 0.02, 0.03, 0.04, 0.05 and 0.06 M) of sodium azide for 4 hours. Later they were thoroughly washed under running tap water for 20 minutes followed to rinse in double distilled water. Then the seeds were allowed to germinate in cocopeat in trays. Seeds soaked in distilled water for 4 hr. and wash thoroughly under running tap water were also allowed to germinate. They served as control. Later seeds were analyzed for present seed germination.

2) Calculation of percent seed germination

Percent of seed germination was calculated after fourth and eighth days of seed germination. Seeds which have risen to both plumule and radicle were considered as germinated. The percent seed germination was calculated as follows…

$$\textbf{Percent seed germination} = \frac{\textbf{No. of seeds germinated}}{\textbf{total no. of seeds kept for germination}} \times 100$$

3) Determination of root length, shoot length and seedling length

Final measurements of root length, shoot length and seedling length were taken after 8 days of growth of test species. Roots and shoots length of all plants in each replicate were measured in centimeters (cm) using ruler. For measurement of root length the part below cotyledon with hypocotyl region were considered and for shoot length the part above cotyledon with epicotyl were considered. The data recorded on shoot length and root lengths were further considered for determination of seedling length.

4) Analysis of peroxidase isoenzymes: -

A) Extraction of peroxidase isoenzymes from the plant material.

One-gram seedling was homogenized in two ml of separating gel buffer (pH=8.8). Homogenate were centrifuged for 30 minutes at 10,000 rpm at 4ºC. The 400 µl supernatant was stored in 1.5 ml centrifuge tubes, add half the amount of 50% glycerol in the supernatant and then add pinch of bromophenol blue (as a marker). The supernatant was stored at 20ºC until subjected to the electrophoresis.

B) Electrophoresis:

Electrophoresis is a technique for separating macro molecules by subjecting them to an electric field. The moment of charged particles is based on two factors.

1) The net electric charge,
2) The molecular weight.

Polyacrylamide gel be prepared with a high degree of reproducibility and the degree of porosity may selected enhance the separation of molecules at similar charges but different shapes and sizes. This feature makes the method particularly suitable for resolving mixture of protein. Other features of polyacrylamide gel which extend their usefulness for macromolecule separation include their minimal adsorption capacity, their lack of electro- osmosis and their suitability for in situ or quantitative analysis. Proteins were analyzed / resolved on 8% Polyacrylamide native gel using horizontal slab gel electrophoresis.

Activity staining of peroxidase isozymes:

The peroxidase isozymes present in the polyacrylamide gel slabs were detected using activity staining method of Shrawen (1966). The staining was done using benzidine solution in presence of hydrogen peroxide. The preparation of incubation mixture for the activity staining of peroxidase is as follows

The gel is photographed immediately to make a permanent record of the bands. They are also traced on a tracing paper to facilitate the calculation of the relative mobility (Rm values). The relative mobility of a given isozyme band is calculated as follows.

$$Rm = \frac{\text{Distance travelled by the isozyme}}{\text{Distance travelled by the marker dye}}$$

The molecular weight of the peroxidase isozymes could not be ascertained, due to non-availability of the molecular markers. All the observed peroxidase isozymes are arbitrarily named as isoforms 1, 2...so on, based on their Rm values, in ascending order.

RESULTS AND DISCUSSION

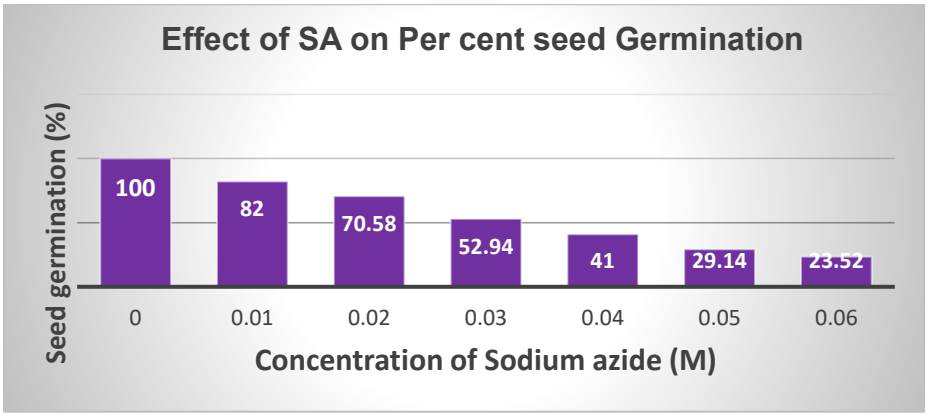
Results

Table 1: Per cent seed germination growth parameters of *Vigna radiata,* subjected to treatment with different concentrations of sodium azide. (After 8 days of germination)

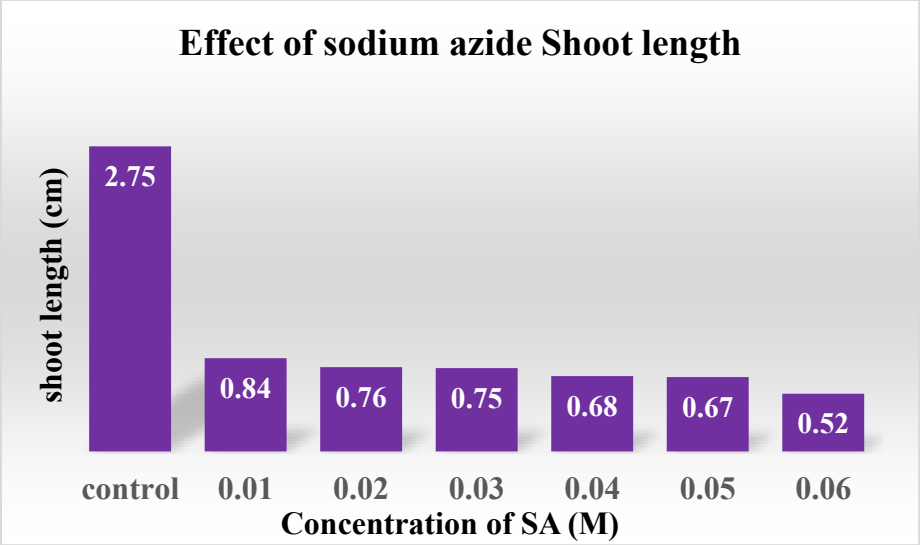
Concentration of SA (M)	Per cent seed Germination	Shoot length (in cm)	Root length (in cm)	Seedling length (in cm)	Percent inhibition
00	100	2.75± 0.87	4.81±0.9	7.56±1.78	-
0.01	82	0.84± 0.07	3.85±0.43	4.69±0.35	37.97
0.02	70.58	0.76± 0.11	3.25±0.13	4.01±0.01	46.96
0.03	51.94	0.75± 0.12	2.90±0.05	3.65±0.17	48.28
0.04	41	0.68± 0.16	2.37±0.31	3.05±0.47	59.66
0.05	29.14	0.67± 0.17	2.11±0.44	2.79±0.60	63.10
0.06	23.52	0.52± 0.23	1.67±0.66	2.19±0.90	71.04

± denotes the standard error

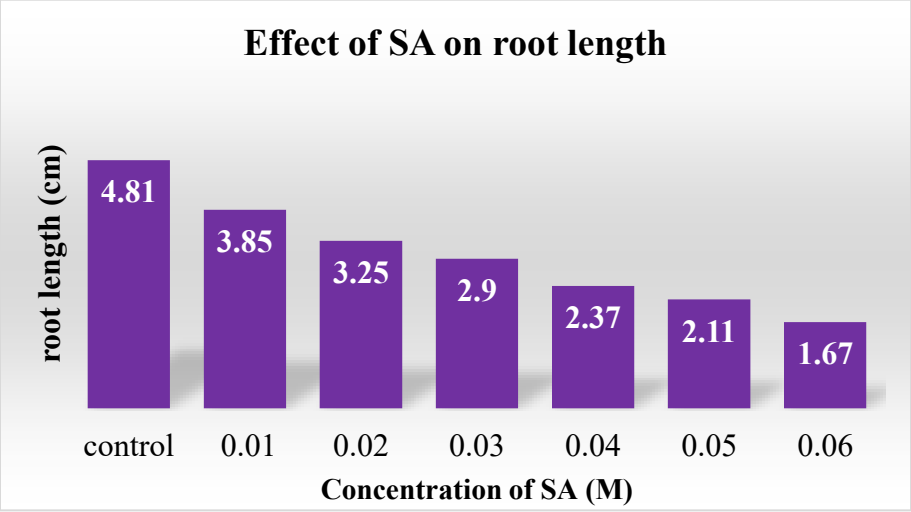
GRAPHS



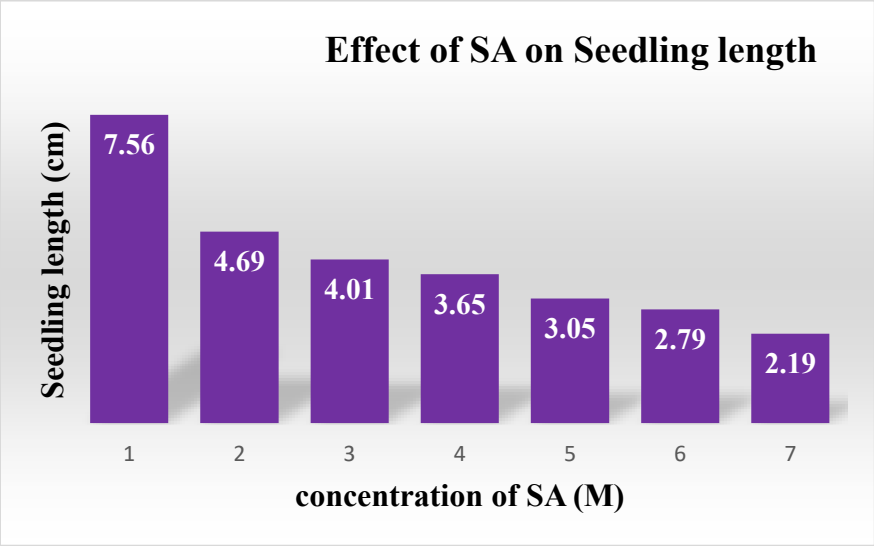
Graph 1: showing effect of sodium azide on percent seed germination in *Vigna radiata*



Graph 2: showing effect of sodium azide on shoot length in *Vigna radiata*



Graph 3: showing effect of sodium azide on root length in *Vigna radiata*



Graph 4: showing effect of sodium azide on seedling length in *Vigna radiata*

DISCUSSION

Percent germination of mung bean seeds treated with different concentrations of sodium azide (for four hours at room temperature) are shown in table-1 and graph 1. From the table it is evident that maximum percent of seed germination is observed in distilled water grown (control) seeds followed by 0.01 M sodium azide treated ones. There is a gradual reduction in percent seed germination with an increase in the concentration of sodium azide (Image 1 and graph 1). 51.94 % seed germination was observed in 0.03 M concentration of sodium azide (table 1 and graph 1). Hence this concentration was adjusted as LD50. The reason for the observed inhibitory effect of sodium azide on seed germination may be due to the action of sodium azide as respiratory inhibitor or as acting as an enzyme poison (Ramachandran and Goud, 1983). Khan (1990) and Nilan *et al.,* (1978) also reported seed germination inhibition in mung bean and barley.

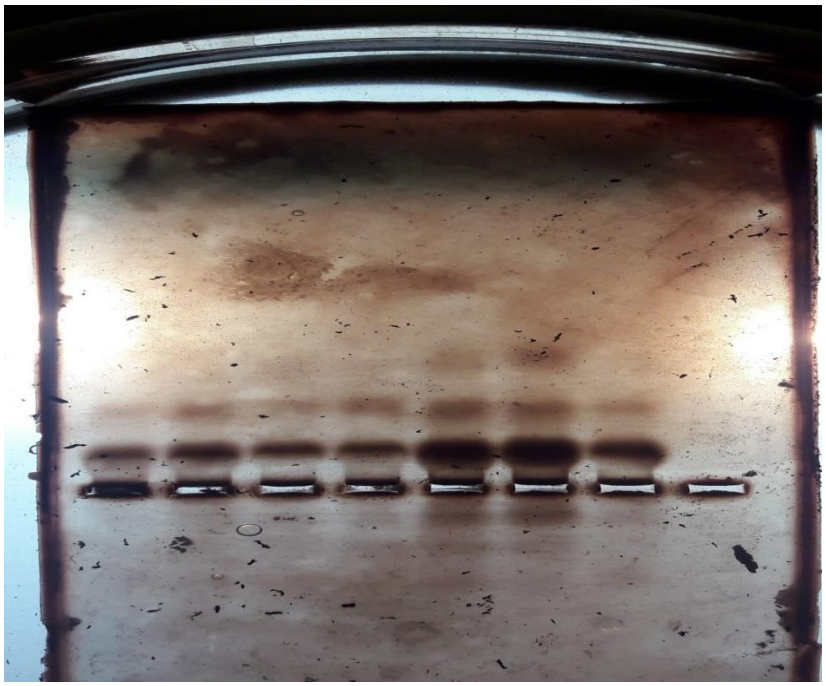
Maximum inhibition (71.04%) is observed in 0.06 M concentration of sodium azide (Table-1). Sodium azide continued to exert its inhibitory effect even on the growth of the seedling. The growth of radicle, plumule and total seedling length was measured from 8-day old seedlings and the data was depicted in table-1. Control (distilled water) grown seeds exhibited a shoot length of 2.75, root length of 4.81 and total seedling length of 7.56 cm. Seeds treated with various concentrations of sodium azide showed a gradual decrease in shoot, root and seedling lengths (Table-1, Image .1). The effect was dose dependent. There is a decrease in the growth rate, along with an increase in the concentration of sodium azide. So, it is clear from the observations that sodium azide exerted a clear inhibitory effect on growth of the seedlings. Maximum retarding effect of azide is seen in the seedlings grown from seeds treated with 0.06 M concentration of sodium azide (shoot length-0.52, root length-1.67 and seedling length – 2.19 cm).

The peroxidase isozyme composition of distilled water (control) grown seedlings showed the presence of three peroxidase isoforms. They are isozymes with Rm values, 0.06, 0.12 and 0.27. All three are anodal peroxidases. These enzymes are identified as isoforms 5, 7 and 8.

The sodium azide treated seedlings showed an increase in the number of peroxidase isozymes. The 0.01M, 0.02M and 0.03M sodium azide treated seedlings showed the presence of four peroxidase isozymes having Rm values -0.04, 0.06, 0.12, and 0.27. These are identified as isoforms1, 5, 7 and 8. Out of these the first one (isoform 1) is cathodal and the last three (5,7and 8) are anodal isozymes. The 0.04 M and 0.05M sodium azide treated seedlings showed the presence of nine peroxidase isozymes having Rm values, -0.04, -0.15, -0.22, -0.35,0.06,0.10,0.12,0.27 and 0.47. These are identified as isoforms 1, 2,3,4,5,6,7,8 and 9 respectively. Out of these the first four (isoform1, 2, 3 and 4) is cathodal and the last five (5, 6, 7, 8 and 9) are anodal isozymes.

Table 2: Nomenclature of the peroxidase isoforms based on their relative mobility values.

Rm Value	Isoform Number
-0.04	Isoform 1
-0.15	Isoform 2
-0.22	Isoform 3
-0.35	Isoform 4
0.06	Isoform 5
0.10	Isoform 6
0.12	Isoform 7
0.27	Isoform 8
0.41	Isoform 9



(Image 2)- showing peroxidase isozymes profiles in *Vigna radiata* seedlings treated with SA

Table 3: Peroxidase isozyme composition of 8-day old Control and Sodium Azide treated *Vigna radiata* seedlings.

Concentration of SA(M)	Rm Values	
	Towards Cathode	**Towards Anode**
00(Control)	-	Isoform 5- 0.06
		Isoform 7- 0.12
		Isoform 8- 0.27
0.01M	Isoform 1(- 0.04)	Isoform 5- 0.06
		Isoform 7- 0.12
		Isoform 8- 0.27
0.02M	Isoform 1(- 0.04)	Isoform 5- 0.06
		Isoform 7- 0.12
		Isoform 8- 0.27
0.03M	Isoform 1(- 0.04)	Isoform 5- 0.06
		Isoform 7- 0.12
		Isoform 8- 0.27
0.04M	Isoform 1(- 0.04)	Isoform 5- 0.06
	Isoform 2(- 0.15)	Isoform 6- 0.10
	Isoform 3(- 0.22)	Isoform 7- 0.12
	Isoform 4(- 0.35)	Isoform 8- 0.27
		Isoform 9- 0.41
0.05M	Isoform 1(- 0.04)	Isoform 5- 0.06
	Isoform 2(- 0.15)	Isoform 6- 0.10
	Isoform 3(- 0.22)	Isoform 7- 0.12
	Isoform 4(- 0.35)	Isoform 8- 0.27
		Isoform 9- 0.41
0.06M	Isoform 1(- 0.04)	Isoform 5- 0.06
	Isoform 2(- 0.15)	Isoform 6- 0.10

Peroxidase Isoforms 1, 2,3,4,6 and 9 which were totally absent in the control seedlings were seen in the sodium azide treated seedlings. This clearly indicates that sodium azide induces synthesis of certain peroxidase isozymes. This is inconsistence with the idea of Stickberger (1973) who opinioned that the sodium azide can inhibit the action of several enzymes in plant systems. Also, this is in contrast to the earlier finding of Apparao (2005), who reported that sodium azide disrupts the synthesis of certain peroxidase enzymes, and decreases their number as compared to control.
But our findings are in accordance with Wabale *et al.,* (2018) where he found that the sodium azide treated seedlings showed an increase in the number of peroxidase isozymes.

CONCLUSION

Different concentration of sodium azide showed decrease in the percent seed germination in *Vigna radiata*. Reduced germination with higher concentrations of sodium azide was considered to be due to initial metabolic disturbances in germinating seed due to action of sodium azide as respiratory inhibitor. (Ramachandran and Goud, 1983).

Sodium azide also exerted a clear inhibitory effect on the growth of root, shoot and overall seedling. The reduction in seedling growth may be due to the grass injury caused at cellular level either due to gene controlled biochemical processes or acute chromosomal aberrations or both (Mujeed-ur-Rehman *et al.,* 2000).

The result suggested that SA induces synthesis peroxidase isozymes and action seems to be specific. Appearance of additional bands of peroxidase isozyme in azide treated seedlings indicates that sodium azide acts as an inducer and induces certain genes to produce polypeptides or functional enzymes, like peroxidases, as evident from the present investigation.

According to Apel and Hirt (2004), when plants are exposed to stressful conditions the production of harmful substances like H_2O_2 (hydrogen peroxidase increases. A major hydrogen peroxidase detoxifying system in plant cells is peroxidase isozymes which catalyzes the conversion of H_2O_2 into $H_2O$and nascent oxygen ($O^{\cdot}$) (Vergnova *et al.,* 2002).

The observed increase in the number of peroxidase isozymes in the present investigation is might be due to the genetic stress caused by the deleterious effect of sodium azide as it is a potent mutant.

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