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Mutagenic Effects of Sodium Azide on *Capsicum anuum* L.

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ABSTRACT

Dry seeds from pure lines of three varieties of pepper (Capsicum anuum *L.) were treated for 2 hours at 30ÉC with solutions of 0.12, 0.25, 0.50 and 0.75 mM sodium azide in phosphate buffer at pH 3. Data in the M_1 show a decrease in germination percentage, height at different stages of growth, number of leaves and seed set. Germination percentage and seed set were reduced significantly. Chlorophyll-deficient seedlings were observed in the M_2. The most frequent chlorophyll mutations were the viridis type followed by chlorina, xantha and albina. Very few xantha and albina mutants were observed.*

INTRODUCTION

The mutagenicity of sodium azide has been demonstrated in several plants. In lower plants, studies were done on yeast (Nillson-Tilgren and Kahn in Kleinhofs et al., 1978; Veleminski et al., 1977) and bacteria (Clark, 1950; Rosichan et al., 1983). Emphasis, however, has been on higher plants like barley (Niknejad, 1976; Nilan et al., 1976), peas (Kleinhofs et al., 1978), mungbean (Shaikh et al., 1983) and rice (Sarma et al., 1978; Awan et al., 1980).

There are, however, varying opinions as to its mutagenic properties. It has been reported that the key to the high mutagenic potency of sodium azide is the presence of a high hydrogen ion concentration in solution (Sideris et al., 1969), and that its mutagenic efficiency is possibly due to the low physiologic damage azide induced in treated plants.

An earlier report attributes the mutagenicity of the chemical to an indirect action, through its inhibition of catalase and peroxidase. This inhibitory action of azide on these enzymes would bring about the accumulation of hydrogen peroxide, which supposedly is the real mutagen (Berger et

al., 1953; and Clark et al., 1953). That peroxide was not the agent involved in azide mutagenesis, however, was reported by Kleinhofs and Smith (1976).

Studies by Owais et al. (1978) demonstrated the conversion of sodium azide to a mutagenic metabolite by an enzyme, O-acetylserine sulfhydrase A. The role of this enzyme in the conversion of azide to a mutagenic metabolite has been thoroughly analyzed and proven in barley (Rosichan, 1983). To date, however, this mutagenic metabolite has not been conclusively identified.

The pH of the solution influences the mutagenic efficiency of sodium azide. It has been shown that azide is most effective in inducing mutations at pH 3 (Nilan et al., 1973 and Kleihofs et al., 1974). The uncharged hydrazoic acid molecule which is the predominant form in which azide exists at low pH levels penetrates the cell membrane more rapidly and readily than the N_3 ion. It has been hypothesized that the cell membrane maybe more permeable to azide at low pH levels. Low pH of sodium azide is associated with increased seedling injury (Sideris et al., 1969 and Rines, 1985) with no significant chromosome aberrations. However, it has been reported that azide solutions are also highly mutagenic at neutral solutions and even at alkaline pH, especially at high mutagen concentrations (Rines, 1985).

Chlorophyll-deficient mutations were induced by sodium azide in barley (Nilan et al., 1976), *Vicia faba* (Brunner in Nilan et al., 1976), and rice (Awan et al., 1980), and among the chlorophyll mutants induced, the viridis type of mutation was the most frequent. Rines (1985) reported that high frequencies of chlorophyll mutations were induced in diploid oats, but very few were recovered in hexaploids oats. The low incidence of chlorophyll-deficient mutants in the hexaploid oats can be explained by assuming that in hexaploid oats, there are duplicative loci with the same functions which are able to compensate for and phenotypically mask the functional loss of a mutated locus. Rines (1985) stated that among the chlorophyll-deficient mutants observed in the hexaploid oats, no albina mutants were found, whereas albina mutants comprised more than half the number of chlorophyll mutations found in diploid oats.

The increasing work on the induction of mutations using chemicals reflects the growing interest in mutation breeding. It is also indicative of the mutagenic efficiency of sodium azide. A likewise efficient chemical mutagen, ethyl methanesulfonate (EMS), has been used widely in mutation breeding (Amano and Smith, 1965, Arañez, 1981; Rines, 1985; Longid, 1987) and has been reported to improve the qualities of algae like higher protein content

(Necas, 1974). However, there is no work done on the mutagenicity of sodium azide on pepper. Its use in mutation breeding may help improve the existing varieties of the capsicums.

This study aims to determine (1) the effects of sodium azide on the M_1 germination percentage, plant height, and seed set and (2) the type of mutation induced by sodium azide on the 3 varieties of pepper in the M_2 seedlings.

MATERIALS AND METHODS

Dry seeds from pure lines of three varieties of pepper (*Capsicum anuum* L.) were used in this experiment: the Long Slim (LS), Chinese (C) and California Wonder (CW). LS is known locally as "siling-mahahaba" and CW as Baguio or bell pepper. These are annual herbs, erect with angular branches and grow almost 40 cm tall.

One thousand two hundred seeds of each variety were divided into six separate sets. Each set containing 200 seeds was placed in nylon fishnet bags, washed in flowing water and soaked in distilled water for four hours. After soaking in water the seeds were dabbed dry with tissue paper and were treated for two hours at 30°C in appropriate solutions of 0.12, 0.25, 0.50 and 0.75 mM sodium azide with phosphate buffer at pH 3. Two sets from each variety were used for water and phosphate controls.

After treatment, the seeds were washed for 2 hours in running water and then germinated in sterilized petri dishes lined with moist tissue paper and grown in the laboratory under continuous flourescent illumination.

Thirty days after treatment, the seeds were scored for M_1 germination percentage and seedling height. Seeds whose radicle emerged were considered germinated. For the data on seedling height 30 days after treatment, the length was measured from the tip of the primary root to the base of the first leaf pair.

To determine plant height and number of leaves 60 days after treatment, 60 of the seedlings per dose per variety were planted in 3 seedling boxes containing soil sterilized for one hour at 16 p.s.i. Each wooden seed box measured 4 x 95 x 105 cm and contained 180 seedlings. The seedlings were planted in a concentration-to-row plan at a distance of 4 cm interval in a row and the distance between rows was 5 cm. For the data on the number of leaves, only fully expanded leaves were counted.

The remaining seedlings were planted in plastic cups containing sterilized soil for further growth before transplanting in the field. Five seedlings

were placed in each cup and were grown in the open field. After one week these were then transplanted in the field in a concentration-to-row plan with a distance of 20 cm interval between plants and 30 cm between rows. The M_1 plants were grown and scored for plant height at maturity which was 120 days after treatment, and seed set. Seed set of the M_1 fruits was determined by counting the seeds of the first 3 ripe fruits from each plant per treatment. Thirty plants per variety per treatment were used for this purpose. Fruits were considered ripe when almost one half of the fruit turned red. The seeds were counted, air-dried and stored for a dormancy period of not less than two months.

To determine the effects of sodium azide on the M_2 seedlings, after the dormancy period, seeds were chosen at random from the first three ripe fruits from each plant of each treatment. These seeds were soaked in water for four hours and planted directly in seed boxes and grown in the open field. The seedlings were scored for chlorophyll-deficient mutations. The chlorophyll mutations were determined as soon as the first pair of leaves was visible. The frequency of these mutants were estimated in terms of the number of chlorophyll mutations per 1000 M_2 seedlings.

The entire experiment was done in two replicates. Data obtained was analyzed for statistical significance using the analysis of variance, Duncan's multiple range test using the Statistical Package for Social Sciences.

RESULTS AND DISCUSSIONS

Sodium azide significantly decreased germination percentage in pepper (Table 1), but seedling and plant height (Tables 2 and 3) and number of leaves per plant (Table 4) were not significantly decreased. A significant decrease in fertility as reflected in the seed set (Table 5) is seen. In the M_2, sodium azide produced chlorophyll-deficient mutations in the seedlings.

It is evident from the results of this study that sodium azide produced both genetic and physiological effects in the three varieties of pepper. The various indices used as parameters of the effects of the mutagen were germination percentage, seedling and plant height at various stages of growth, number of leaves per plant at sixty days, seed set and the frequency of chlorophyll-deficient mutants in the M_2 seedlings.

There is little information on the mechanism of azide mutagenesis. However, it is common knowledge that mutations whether they are point or gross chromosomal aberrations involve alterations in the DNA molecule. Alterations in the DNA structure and constituent nucleotides brought about

by mutagens will change the information coded in it. Evidence so far indicates that as a base-substitution mutagen (Nilan et al., 1973) sodium azide acts primarily on replicating DNA and is most effective at the S stage. It is highly mutagenic at pH 3 (Nilan et al., 1973) and the dominant form is hydrogen azide (HN_3). It has been postulated that the increased effectiveness of azide in the acid form is probably due to the increased penetration of the cell membrane by the neutral molecule (Sideris et al., 1969).

The inhibitory effects of sodium azide on the different biological parameters of the three varieties could probably be explained by the inhibition of mitosis, disruption of the enzymatic process or direct changes on the genes involved. The significant decrease in the seed set (Table 5) indicate a decrease in the fertility of the M_1 in the three varieties. The results obtained confirm earlier reports that azide produces high levels of sterility in other plants like rice (Mustafa, 1976; Awan et al., 1980), barley (Kleinhofs et al., 1978) mungbean (Shaikh, 1983) and oats (Rines, 1985). This could probably be due to the effect of the mutagen on the pollen mother cells (Sato and Gaul, 1967; Narsinghani, 1975) brought about by chromosomal aberrations. Other abnormal meiotic figures like tetrads without nuclei or with fused nuclei were observed by other investigators (Sato and Gaul, 1967; Ramulu, 1971). Desynapsis (Rammalingan, 1976) and delay in chiasmata formation (Ramulu, 1971) were also observed. All these factors contribute to the reduced sterility of sodium azide-treated pepper. Nilan et al. (1973) also attribute sterility to gene mutation expressed as gametic or zygotic lethals.

Mutagens differ in their mechanism and modes of action in biological systems. The extent of damage done therefore is related to the mechanism of action of a given mutagen. As a respiration inhibitor, sodium azide may inhibit an energy supply system resulting in the inhibition of mitosis which can be associated with depression of seedling and plant growth and other biological processes like germination, leaf growth and seed set.

The induction of chlorophyll mutations is a genetic effect which is directly related to the mutagenicity of the mutagen. In this study, the frequency of chlorophyll mutations obtained in the M_2 generally increased with increasing mutagen concentration (Table 7) except in CW where there was an abrupt decrease in M_2 mutant seedlings when the mutagen was increased to the highest concentration of 0.75 mM. The increase in the frequency of chlorophyll mutations as the mutation increased could be attributed to the increase in the induction of point mutations or the induction of gross chromosomal aberrations. Since sodium azide produces little

or no chromosomal aberrations as postulated by several workers (Konzak et al., 1965; 1972; 1976; Niknejad, 1976; Walther, 1976) it is likely that only point mutations have been induced. These mutations involve alterations in the DNA molecule which will affect the synthesis of enzymes and ultimately affect several metabolic processes including the synthesis of chlorophyll. Another explanation could be the inactivation of enzyme repair systems, thus delaying or inhibiting the progress of DNA repair.

The rapid decrease in the number of mutations in CW at 0.75 mM per 1000 M_2 seedlings could probably be due to the following: death of cells carrying the mutated cells, probably a high resistance of the cells (Bhan and Kaul, 1976), the presence of an efficient repair system and the resitution of broken ends possibly due to the sticky nature of the broken chromosome ends (Soriano, 1967). Bahn and Kaul (1976) further postulated that death of the cells carrying the mutated cells could have occurred at the gametic or zygotic levels, thus it is unlikely that only point mutations are involved in the reduction of the number of mutants observed.

It was observed that the highest number of mutants scored at 0.50 mM concentrations in CW and at 0.75 mM in C and LS. The mutation spectrum (Table 7) showed a predominance of the viridis followed by chlorina, xantha and albina. LS showed a predominance of chlorina, followed by viridis and albina. There were no xantha mutants found in LS and no albina in CW.

The low frequency of xantha and albina mutants obtained in this study could be attributed to several factors such as death of the cells due to the damage caused by the mutagen, selective elimination of seeds due to sterility and failure of the seeds carrying mutant genes to germinate. These were observed in barley (Kleinhofs et al. 1974; Niknejad, 1976; Nila et al., 1976; Walther, 1976) and oats (Rines, 1985). The presence of an efficient enzyme repair system as in rice (Soriano, 1967), the inherent property of DNA to repair itself and dominance effects in diploid plants are factors which could possibly explain the low occurrence of xantha and albina.

It is evident from the results obtained that there are varietal differences in pepper in their response to sodium azide. These could be attributed to difference in the uptake or metabolism of the mutagen, cellular repair mechanisms or differences in the metabolic activities of the plant varieties. Varietal differences may also be reflective of the different degrees of diploidization among varieties (Rines, 1985).

SUMMARY AND CONCLUSIONS

The mutagenicity of sodium azide in three varieties of pepper (*Capsicum anuum* L.) was investigated in this study. Dry seeds of pure in-bred lines from 3 varieties: California Wonder (CW), Chinese (C) and Long Slim (LS) were treated for 2 hours at 30°C in laboratory conditions with solutions of 0.12, 0.25, 0.50 and 0.75 mM sodium azide with phosphate buffer at pH 3. The seeds were germinated for 30 days in the laboratory under constant illumination before transplanting them in seed boxes for another 30 days before finally planting them in the field.

The parameters observed were M_1 germination percentage, seedling and plant height at different stages of growth and seed set, and M_2 chlorophyll mutations.

Sodium azide decreased M_1 germination percentage, seedling and plant height at different stages of growth and seed set. Percentage germination and seed set were reduced significantly.

In the M_2 seedlings chlorophyll-deficient mutants viridis, chlorina, xantha and albina were observed. The highest number of mutants were viridis, followed by chlorina, xantha then albina.

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Table 1. Germination percentage in seeds of three pepper varieties after NaN_3 treatments

Variety	NaN3 (mM^3)	Percentage germination	Percent of control
C	water (control)	99.3	100.0
	buffer	99.7	100.3
	0.12	92.0	92.6
	0.25	90.7	91.3
	0.50	84.0	84.6
	0.75	66.8	67.2*
CW	water (control)	98.0	100.0
	buffer	97.3	99.3
	0.12	94.0	95.9
	0.25	90.7	92.5
	0.50	83.7	85.4
	0.75	67.3	63.7*
LS	water (control)	98.0	100.0
	buffer	97.0	99.0
	0.12	94.0	96.0
	0.25	93.3	95.2
	0.50	92.7	94.6
	0.75	90.0	91.9

*Significant at 5% (Chi-square test)

Table 2a. Seedling height (cm) in three varieties of pepper after NaN3 treatments

Variety	NaN_3 (mM)	Seedling Height (cm)		Percent of control
		Range	Mean/SE	
C	water (control)	2.9 – 4.1	3.6 ± 0.3	100.0
	buffer	3.2 – 4.0	3.6 ± 0.2	101.1
	0.12	2.9 – 4.0	3.4 ± 0.2	96.4
	0.25	2.9 – 3.8	3.3 ± 0.2	91.9
	0.50	2.8 – 3.6	3.2 ± 0.2	90.0
	0.75	2.2 – 3.4	2.7 ± 0.3	75.4
CW	water (control)	3.4 – 4.2	3.9 ± 0.2	100.0
	buffer	3.0 – 4.2	3.9 ± 0.2	98.9
	0.12	2.8 – 4.1	3.7 ± 0.3	93.9
	0.25	2.8 – 4.1	3.5 ± 0.3	89.1
	0.50	2.6 – 3.8	3.3 ± 0.3	84.0
	0.75	2.0 – 3.1	2.9 ± 0.4	73.3
LS	water (control)	1.2 – 2.9	2.2 ± 0.4	100.0
	buffer	1.6 – 3.2	2.3 ± 0.3	103.6
	0.12	1.8 – 3 1	2.1 ± 0.3	96.0
	0.25	1.4 – 3.1	2.0 ± 0.3	90.1
	0.50	1.5 – 2.3	1.9 ± 0.2	86.5
	0.75	1.4 – 2.1	1.8 ± 0.2	82.0

Table 2b. Plant height (cm) after 60 days in M_1 of three varieties of pepper after NaN_3 treatments

Variety	NaN_3 (mM)	Plant Height at 60 days		Percent of control
		Range	Mean/SE	
C	water (control)	5.0 – 24.7	14.4 ± 6.5	100.0
	buffer	4.0 – 26.3	16.1 ± 5.8	111.6
	0.12	3.5 – 26.6	13.4 ± 5.7	92.6
	0.25	3.5 – 24.8	12.8 ± 6.5	88.6
	0.50	3.4 – 24.8	12.5 ± 6.4	86.4
	0.75	3.3 – 25.0	12.4 ± 6.0	85.9
CW	water (control)	5.6 – 26.6	15.7 ± 5.8	100.0
	buffer	4.0 – 26.3	14.1 ± 5.9	89.9
	0.12	3.0 – 25.4	13.8 ± 5.4	88.2
	0.25	3.0 – 24.1	13.2 ± 5.1	84.6
	0.50	1.5 – 24.2	12.3 ± 6.3	78.9
	0.75	1.8 – 21.0	11.6 ± 4.9	74.2
LS	water (control)	4.0 – 26.4	14.3 ± 5.6	100.0
	buffer	5.0 – 26.6	15.3 ± 5.1	107.0
	0.12	3.4 – 24.0	14.7 ± 5.4	103.2
	0.25	3.0 – 24.7	13.4 ± 5.3	94.0
	0.50	3.0 – 24.0	13.3 ± 5.3	93.5
	0.75	4.0 – 22.8	11.8 ± 5.6	82.9

Table 3. M_1 plant height at maturity (120 days after treatment) in three pepper varieties after treatment with NaN_3

Variety	mM NaN_3	Plant Height at Maturity 120 days		Percent of control
		Range	Mean/SE	
C	water (control)	8.5 – 42.4	23.1 ± 8.5	100.0
	buffer	7.5 – 40.0	23.0 ± 8.4	99.7
	0.12	8.4 – 40.6	22.3 ± 8.1	96.7
	0.25	6.5 – 40.3	22.2 ± 8.3	96.2
	0.50	7.5 – 39.3	21.6 ± 6.9	93.7
	0.75	5.0 – 37.5	19.7 ± 8.8	85.4
CW	water (control)	7.5 – 42.2	25.0 ± 8.3	100.0
	buffer	10.3 – 39.2	25.7 ± 7.5	102.5
	0.12	6.7 – 35.3	22.1 ± 6.7	88.4
	0.25	8.4 – 31.5	20.6 ± 5.8	82.5
	0.50	6.2 – 35.0	20.5 ± 7.8	82.0
	0.75	7.5 – 32.2	18.3 ± 6.1	73.2
LS	water (control)	10.5– 55.5	33.9 ± 9.6	100.0
	buffer	9.5 – 50.2	32.6 ± 10.3	96.2
	0.12	11.2 – 46.2	26.6 ± 8.1	78.4
	0.25	6.0 –43.2	24.4 ± 9.5	72.1
	0.50	11.2 – 47.7	27.0 ± 9.4	79.7
	0.75	11.9 – 48.5	29.7 ± 8.6	87.6

Table 4. Number of leaves in three varieties of pepper 60 days after NaN_3 treatments

Variety	mM NaN_3	Plant Height at Maturity 120 days		Percent of control
		Range	Mean/SE	
C	water (control)	5 – 25	11.1 ± 4.7	100.0
	buffer	1 – 24	10.1 ± 6.0	90.9
	0.12	4 – 20	10.2 ± 4.0	91.1
	0.25	3 – 21	9.7 ± 4.1	86.6
	0.50	4 – 25	10.3 ± 4.4	92.1
	0.75	1 – 24	9.2 ± 5.6	82.5
CW	water (control)	2 – 19	9.2 ± 3.9	100.0
	buffer	3 – 24	9.3 ± 4.7	101.1
	0.12	1 – 22	7.8 ± 5.5	85.1
	0.25	2 – 23	8.7 ± 4.2	94.1
	0.50	3 – 14	8.2 ± 2.6	88.7
	0.75	1 – 23	8.0 ± 4.3	87.3
LS	water (control)	5 – 26	13.1 ± 5.5	100.0
	buffer	5 – 24	12.8 ± 5.0	97.4
	0.12	1 – 19	8.9 ± 4.6	68.0
	0.25	2 – 20	10.0 ± 4.3	76.6
	0.50	1 – 24	9.7 ± 5.5	74.1
	0.75	3 – 24	11.6 ± 5.7	88.7

Table 5. Seed-set in varieties of pepper after NaN_3 treatments

Variety	mM NaN_3	Seed - set		Percent of control
		Range	Mean/SE	
C	water (control)	28 - 230	111.4 ± 53.3	100.0
	buffer	8 - 162	79.2 ± 37.6	68.3
	0.12	14. - 174	81.0 ± 31.1	67.3
	0.25	9 - 253	78.7 ± 52.9	65.9
	0.50	4 - 267	77.7 ± 60.3	65.1
	0.75	6 - 138	62.5 ± 34.0	52.4*
CW	water (control)	25 - 315	108.2 ± 50.4	100.0
	buffer	12 - 240	103.5 ± 57.4	95.6
	0.12	1 - 204	86.7 ± 47.6	80.1
	0.25	14 - 222	79.2 ± 40.6	73.1
	0.50	7 - 210	73.2 ± 41.5	67.7
	0.75	3 - 190	55.0 ± 40.9	50.8*
LS	water (control)	8 - 108	66.2 ± 25.3	100.0
	buffer	18 - 155	88.4 ± 31.4	133.7
	0.12	21 - 158	85.3 ± 29.1	129.0
	0.25	8 - 122	53.5 ± 24.1	80.9
	0.50	4 - 95	38.3 ± 20.9	57.9
	0.75	7 - 92	43.3 ± 23.5	65.5*

*Significant at 5% (Duncan's Multiple Range Test)

Table 6. Frequency of M_2 chlorophyll-deficient mutations in three varieties of pepper after NaN_3 treatments

	mM NaN_3	No. of mutated M1 plants	No. of mutant M_2 seedlings	Mutation frequency per 100 M_1 plants	Mutation frequency per 1000 M_2 seedlings
C	0.12	2	2	3.3	3.2
	0.25	3	16	5.4	28.8
	0.50	3	25	6.0	53.9
	0.75	5	27	10.4	58.4
CW	0.12	3	0	5.0	0
	0.25	3	3	6.1	4.8
	0.50	4	23	8.9	41.0
	0.75	7	5	16.7	9.8
LS	0.12	2	4	3.2	7.1
	0.25	4	4	8.2	9.8
	0.50	5	10	10.4	24.4
	0.75	5	19	11.91	45.2

Table 7. Mutation spectrum induced by NaN_3 in the three varieties of pepper

		Types of chlorophyll mutations (Percent)*				
mM NaN_3	Total No. of Mutants	V	Ch	X	A	O
0.12	6	33.33	6.67	0.00	0.00	0.00
0.25	23	47.83	30.43	4.35	4.35	8.70
0.50	58	0.50	41.38	3.45	3.45	3.45
0.75	51	45.10	51.08	3.92	0.00	0.00

*V – viridis
Ch – chlorina
X – xantha
A – albina
O – others