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Effects of Mutagenic Sodium Azide (NaN_3) on *In Vitro* Development of Four Pea (*Pisum sativum* L.) Cultivars

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ABSTRACT

The study examined mutagenic effects of 0.001-0.005 M NaN_3 with or without BAP or TDZ in MS medium on M1 generation of four pea cultivars Winner, Sprinter, Bolero and Karina. LD_{50} results showed that 0.001 M NaN_3 was most appropriate for the creation of mutagenesis and was not highly lethal. Seeds treated with 0.001 m NaN_3 grown on MS medium containing various concentrations of Benzylaminopurine (BAP) and Thidiazuron (TDZ) showed that BAP with NaN_3 was better compared to TDZ with NaN_3. Although, no effect of mutagen NaN_3 was observed on rooting, presence of BAP or TDZ in the culture medium significantly affected this attribute. Shoots previously cultured on BAP with NaN_3 rooted better on MS medium containing 2.5 μM NAA; compared to shoots previously cultured on MS medium containing TDZ with NaN_3.

Key Words: Sodium azide; Mutation; Growth arrest
Abbreviations: cv: cultivar; NaN_3: Sodium azide; BAP: 6 Benzylaminopurine; TDZ: Thidiazuron; NAA: α Naphthalene acetic acid; IBA: Indole 3 butyric acid.

INTRODUCTION

Genetic variability is fundamental to successful breeding programs in vegetatively and sexually propagated plants. This variation can occur naturally or can be induced through mutations, using physical, biological or chemical mutagens and has attracted the interest of plant breeders for many decades. Mutations have been used to produce many cultivars with improved economic value (Broerties & Van Harten, 1988; IARA & FAO symposium, 1995) and study of genetics and plant developmental phenomena (VanDen-Bulk *et al.,* 1990; Bertagne-Sagnard *et al.,* 1996).

Seeds have high regenerative potential and are advantageous for use in mutagenesis. *In vitro* techniques can be used for both seed and vegetatively propagated species. Tissue culture techniques, combined with a mutagenesis treatment, speed up the breeding program. Chemically induced mutations generally lead to base pair substitutions especially GC→AT resulting in amino acid changes, which change the function of proteins but do not abolish their functions as deletions or frame shift mutations mostly do (Van der Veeen, 1966). A common chemical used with seeds is the promutagen sodium azide, which must be metabolized by plant cells to the mutagenic agent presumably azidoalanine to be mutagenic (Owais *et al.,* 1983). This metabolite is chemically identified in barley and bacteria as an amino acid analogue L azidoalanine (N_3 - CH_2 - CH $(NH)_2$ - COOH). The production of this metabolite was found to be dependent on the enzyme *O*-acetlserine sulfhydrylase (E.C. 4.2.99.8). The enzyme catalyses the condensation of azide (N^{-3}) or sulfide (S^{2-}) with *O*-acetylserine to produce azidoalanine or L-cysteine respectively (Kredich, 1971; La Velli & Mangold, 1987). Data obtained from barley seeds germinated in the presence of azide confirm that this compound may act as point mutagen during DNA replication (Sander *et al.,* 1978). There is some evidence that not all species in tissue culture are able to metabolize sodium azide to the mutagenic agent (Dotson & Somers, 1987; Wang *et al.,* 1987), which is potent mutant in barley, pea and rice (IATA, 1977; Kleinhofs *et al.,* 1978ab; Awan *et al.,* 1980; Prina & Favret, 1987) but is hardly effective in Arabidopsis (Gichner *et al.,* 1994).

The *in vitro* conditions help exposure of many varieties to mutagens easily as they can be exposed to mutagens in a relatively small space for reliable screening against mutations in M_1 generation. Appropriate selection pressure can be applied to the culture to select mutants, which can save time, money and space compared to growing thousands of plants in the green-house or field. Mutagens have been applied to suspension, callus and embryo cultures in many species including barley, soybean, carrot, maize, *Kalanchoe,* banana and morning glory (Blixt, 1965ab, 1967ab; Broertjes & Lefferring L., 1972; Kleinhofs *et al.*, 1978ab, Bhagwat & Duncan, 1998; Bhate 2001). However, most of these studies have been performed in 1960s and 1970s. Successful use of mutagens requires optimum conditions to retain maximum germination capacity of seeds or adventitious shoot regeneration capacity of explants. Besides, the timing and dose of mutagen application are very critical and must be determined empirically.

To our knowledge there is no report pertaining to the mutagenic effects of NaN_3 on pea germination under *in vitro* conditions. The aims of this study were to determine the optimum concentration and efficiency of *in vitro* NaN_3 treated seeds of four pea cultivars (Winner, Sprinter, Bolero & Karina) under 3 conditions of work to create and select M_1 mutated plants.

MATERIALS AND METHODS

Filter sterilized solution of NaN_3 (1.5 M) was prepared in deionised water and diluted with sterile 0.1 m phosphate buffer (pH 3) to give 0.001, 0.002, 0.003, 0.004 and 0.005 M working solution to treat the seeds. Seeds of four commonly cultivated cultivars of pea in Turkey, Winner, Karina, Sprinter and Bolero were first placed in sterile deionised water for 30 min to imbibe seeds and then submerged in mutagen solution for 60 min. Seeds submerged in deionised water for the same period of time served as control.

The seeds were sterilized with 50% H_2SO_4 for 1 min followed by treatment with commercial bleach (Axion containing 5-6% NaOCl) for 10 min and three times rinsing with sterile distilled water. At first stage of optimization, – LD_{50} was determined by germinating seeds after treatment with above mentioned 5 concentrations of NaN_3 *in vitro* on MS medium (Murashige & Skoog, 1962). At second stage, the seeds were germinated on MS medium containing 5 - 10 µM TDZ and 25 - 50 µM BAP to optimize the best concentration of TDZ and BAP for seed germination. The third stage consisted of seeds cultured and germinated on MS medium containing 10 µM TDZ and 50 µM BAP (optimized during 2nd stage) and by treating with 0.001 and 0.003 M NaN_3 (optimized during the first stage) to find out the best cytokinins and NaN_3 combination for the induction of mutations.

Rooting. The pea shoots (0.5 - 1 cm) obtained during 2nd stage, were rooted on MS medium containing different concentrations of IBA and NAA to determine the best rooting media. Regenerated shoots from 3rd stage were rooted on MS medium containing 2.5 µM NAA optimized during the 2nd stage, contained in magenta vessels. The rooting observations were recorded four weeks after culture by counting number of shoots per magenta, rooting frequency and the length of the primary roots. Mutations were recorded based on morphological observations during first and third stage of experimentation by separating plantlets into white or green and by recording any other abnormality/ies on the basis of morphological appearance.

Statistical analysis. Each treatment was replicated 4 times with 5 explants in seed germination and 4 explants in rooting such that each experiment was repeated twice. Phenotypic changes were recorded periodically and statistical analysis was based on randomized complete block design. Data given in the percentage were subjected to transformation by arcsin ($\sqrt{X}$) method (Snedecor & Cochran, 1967) prior to analysis of variance (ANOVA) using SPSS (v. 11. SPSS Inc USA). Post hoc tests were performed using Duncan's multiple range test.

RESULTS AND DISCUSSIONS

First stage selection. The frequency of seed germination and root: shoot ratio regenerated from non-treated seeds was considerably high; whilst, mutations based low germination was observed in all treated seeds, which decreased with each increase in the concentration of NaN_3 (Table I). This showed that the severity of mutations in seeds with multicellular structures increased with each increase in the dose and the higher mutagen doses were more efficient because they provided higher number of mutants. However, there was good reason not to use the highest mutagen dose because that leads to un-wanted mutations at some of the loci resulting in lethality with poor or no germination. This made relatively low doses attractive to reduce the amount of work in screening of M_1 plants. Percentage of seed germination, shoot and root length from 0.001 M NaN_3 treated seeds ranged from 57 - 100%, 1.65 to 3.00 cm and 2.18 to 4.69 cm, respectively equivalent to almost half of those noted for control (un-treated conditions) for each cultivar. This deterioration increased further on 0.003 M NaN_3 treated seeds. Seeds treated with 0.004 and 0.005 M NaN_3 were accompanied with mutation based abnormalities including very poor or no germination and dwarfing of seedlings with high percentage of light yellow or white to light green colored mutated abnormal shoots and leaves. Therefore, these two doses were not taken into consideration in subsequent experiments. The erosion in the characters of Winner, Bolero and Sprinter was consistent from 0.001 to 0.003 M NaN_3 treated seeds; however, cv. Karina showed an inconsistent behavior. Nonetheless, we found an increased frequency of seed germination, root and shoot length of cv. Karina at 0.002 M NaN_3 treatment compared to other treatments.

A general perusal of the data shows that NaN_3 affected the four cultivars in the following order cv. Winner > Karina > Sprinter > Bolero. Moreover, the results also led to identify the optimum dose of NaN_3 to determine LD50 for use in *in vitro* studies.

Kleinhofs *et al.* (1978a, b) suggests that 0.003 M NaN_3 dose increases mutations in pea. They further founds that NaN_3 was non-effective when dissolved in alkali and effective when solved in acidic solutions. Prina and Favret (1983) used 0.001 and 0.005 M doses of NaN_3 on barley but could not detect any physiologic changes on the shoot development. Cheng and Gao (1988) treated barley seeds with sodium azide and found a significant decrease in the germination percentage. We detected insignificant differences in the shoot length at 0.001 and 0.003 M of NaN_3 treatment, implying that these doses have similar effects on shoot length and growth.

Second stage selection. Use of 10 µM TDZ and 50 µM BAP in the culture media showed best germination with multiple shoots (Data not shown). The cv. Sprinter showed poor germination compared to the others. The highest number of multiple shoots in case of TDZ was inferior to those obtained on BAP.

Table I. Effects of various doses of NaN_3 on germination shoot and root length of four pea cultivars Winner, Bolero, Sprinter and Karina

Doses of Sodium Azide	Cultivars	Winner	Karina	Sprinter	Bolero
Control	Germination (%)	100.00 a	100.00 a	90.00 ab	87.00 ab
	Shoot length (cm)	6.05 a	5.39 a	4.38 a	3.42 ab
	Root length (cm)	8.70 a	7.69 a	5.55 ab	5.78 ab
0.001 M NaN_3	Germination (%)	100.00 a	87.00 bc	90.00 a	57.00 b
	Shoot length (cm)	2.59 a	2.31 bc	3.00 bc	1.65 cd
	Root length (cm)	4.69 a	3.63 b	3.97 b	2.18 c
0.002 M NaN_3	Germination (%)	90.00 a	97.00 a	70.00 b	37.00 c
	Shoot length (cm)	3.03 a	3.75 a	2.95 ab	1.31 c
	Root length (cm)	3.38 ab	4.93 a	2.32 bc	1.32 c
0.003 M NaN_3	Germination (%)	80.00 b	87.00 a	50.00 c	43.00 c
	Shoot length (cm)	2.39 a	1.37 b	1.38 b	0.97 c
	Root length (cm)	3.15 a	2.56 ab	1.77 b	0.80 c
0.004 M NaN_3	Germination (%)	73.00 a	60.00 b	30.00 c	20.00 c
	Shoot length (cm)	1.82 a	1.41 b	0.77 c	1.56 b
	Root length (cm)	3.36 a	1.84 b	1.07 c	1.41 c
0.005 M NaN_3	Germination (%)	57.00 b	60.00 a	27.00 c	20.00 c
	Shoot length (cm)	1.39 b	1.86 a	0.83 c	0.34 c
	Root length (cm)	2.75 a	2.51 a	0.74 b	0.43 b

Each value is the mean of 4 replicates each with 5 explants.
[1]Values with in a row followed by different letters are significantly different at 0.05 level of significance using Duncan's Multiple Range Test.

These results are in line with the results of Malik and Saxena (1992a), who found multiple shoot in pea on MS medium containing plant growth regulators and single shoots on medium without growth regulators.

Gönülşen (1987) reported that shoot regenerated *in vitro* on medium containing cytokinins was difficult to root. It was therefore, considered necessary to take the regenerated shoots on MS medium containing different concentrations of IBA and NAA (data not shown). NAA (2.5 µM) was more effective for the rooting of regenerated shoots compared to 9.8 µM IBA. Similarly, rooting from BAP regenerated shoots was better compared to TDZ regenerated shoots (data not shown). The shoots obtained after 4 weeks on 10 µM TDZ cultured medium rooted at the rate of 25%. Malik and Saxena (1992b) reported that if the shoot is growing on medium containing TDZ for more than 2 - 3 weeks they are difficult to root. Rooting on IBA was inferior compared to NAA. Özcan (1992) found that 4.9 µM IBA was effective for rooting of pea. These results are further supported by Polanco *et al.* (1988) and Ahmad *et al.* (1997) on lentils. Malik and Saxena (1992b), Lumsden *et al.* (1994) and Griga *et al.* (1986) found positive relationship of NAA in rooting of pea. Similarly rooting was achieved by Barna and Wakhlu (1994) on pea with 1 µM IBA, and by Khawar and Özcan (2002) on lentils with MS medium containing 1.47 µM (0.25 mg dm- 3) IBA.

Development of shoots with normal leaves is of great significance in the tissue culture and mutagenesis. Development of abnormal leaves is not a desirable characteristic, because shoots with such leaves may be difficult to root in addition to carrying number of un-desirable characteristics. Shoots obtained from the seeds cultured on MS medium containing TDZ were smaller and numerous compared to those obtained from seeds cultured on MS medium containing BAP.

Third stage selection. Cv. Winner had high seed germination and shoot regeneration when cultured on 10 µM TDZ (Fig. 1a) or 50 µM BAP (Fig. 1 b, c) and treated with 0.001 or 0.003 m NaN_3 compared to germination and shoot regeneration from control (Table II). Germination was inhibited in the presence of 10 µM TDZ or highly inhibited under 50 µM BAP, when they were treated with 0.003 m NaN_3 for germination. This suggested that the interaction of plant growth regulators with mutagen at 0.001 M concentration was stimulating but inhibiting at 0.003 M concentration. We assume that NaN_3 shock was not so effective at 0.001 M NaN_3 alone and a combined treatment effect of NaN_3 with plant growth regulators on seeds had an added effect in improving germination and growth, especially in case of cv. Winner. The best shoot regeneration was achieved on control medium and increase in the concentration of mutagens was followed by corresponding decrease in the shoot regeneration capacity due to mutations. Large majority of shoots (95.5%) of the cv. Winner and Bolero were green and remaining 4.45% were mutated white. Whereas, cv. Sprinter experienced more deterioration and was inferior in terms of green shoot regeneration. It showed 73.33% green shoots and 26.67% white mutated shoots (data not shown). Considering development of shoots and leaves the cv. Sprinter (Fig. 1d) and Bolero had the highest mutations followed by cv. Winner and Karina.

The second aspect of induction of mutation is a cell division arrest, i.e. increased dose of NaN_3 resulted in reduced germination along with shoot or root length. No

Table II. Effects of TDZ and BAP on seed germination and shoot regeneration from 0.001 and 0.003 M Sodium azide treated seeds

Treatments	Percentage (%) of Germination								Number of shoots/regenerated seed							
	Winner		Karina		Sprinter		Bolero		Winner		Karina		Sprinter		Bolero	
	TDZ	BAP	TDZ	BAP	TDZ	BAP	TDZ	BAP	TDZ	BAP	TDZ	BAP	TDZ	BAP	TDZ	BAP
Control	21.93 b	30.788b	21.93a	38.86a	26.57 a	13.08a	16.92a	8.86a	3.8 0a	4.70 a	2.61 a	4.73 a	1.63 a	3.25 a	0.51 a	1.92 a
0.001 M	73.07a	63.44a	26.57a	8.86b	4.00 b	0.00	3.96 a	0.00a	8.90a	7.60 a	3.25 a	1.17 b	0.51 a	0.00 a	0.42 a	0.64 a
0.003M	8.86b	39.23b	30.00a	12.78b	0.00 b	0.00	4.85 a	8.88a	1.00 a	5.12 a	3.75 a	2.43 b	0.00 a	0.00 a	0.03 a	1.11 a

Treatments	Frequency (%) of shoot regeneration							
	Winner		Karina		Sprinter		Bolero	
	TDZ	BAP	TDZ	BAP	TDZ	BAP	TDZ	BAP
Control	46.9 b	60.00 c	43.05	76.92 a	13.03 b	8.81 b	51.90 a	30.01 a
0.001 M	51.9 a	81.13 a	38.81	21.91 c	21.91 a	43.01 a	38.81 b	26.15 ab
0.003M	50.7 a	72.23 b	42.23	51.11 b	0.00 c	8.84 b	8.83 c	30.07 a

Each value is the mean of 4 replicates each with 5 explants
[1]Values with in a column followed by different letters are significantly different at 0.05 level of significance using Duncan's Multiple Range Test

cycle arrest was observed at lower dose treatment of Sodium azide with cytokinins (Table III). It is likely that only higher doses resulted in the restriction of cell growth, which did not affect lower doses after interaction of the mutagens with plant growth regulators.

Third aspect is seeds in a plant growth regulator rich medium, overcome the cell cycle arrest and divide repeatedly to eventually form a normal seedling; thus, inducing mutagenesis. Pea seeds, not cultured on plant growth regulators, did not under go such phenomenon. Thus, the treatment with NaN_3 reprogrammed growth pattern of pea. Apparently, changes in morphologic expression appeared to have happened with Sodium azide treatment, which caused a developmental switch, precise function of which remains elusive.

The Sodium azide was very hazardous at both levels (10 µm TDZ or 50 µm BAP) of seed treatment for varieties Sprinter and Bolero. No seed germination was observed from Sprinter on 0.003 m NaN_3 treated seeds (with 10 µM TDZ or 50 µM BAP) and from Bolero on MS medium containing BAP from seeds treated with 0.001 M NaN_3. Seed germination and shoot setting was very poor in case of cv. Sprinter and Bolero on TDZ and BAP containing media (control) as well. Reduced germination was also reflected on the shoot induction capacity of the varieties and resulted into either no or very reduced germination on treated seeds. The results explicitly show that interaction of NaN_3 with TDZ and BAP was inhibitory and deteriorating. This could be due to the reason that cv. Sprinter and Bolero have thin seed coat that was damaged by commercial bleach used for seed sterilization. This helped the mutagens combined with growth regulators to penetrate deeply and destroy or affect embryo by hampered metabolic functions, resulting in partial, reduced or complete loss of activity. However, survival and germination was observed on both cultivars when NaN_3 treated seeds were grown on MS medium containing no cytokinins during the first stage of optimization (Table I).

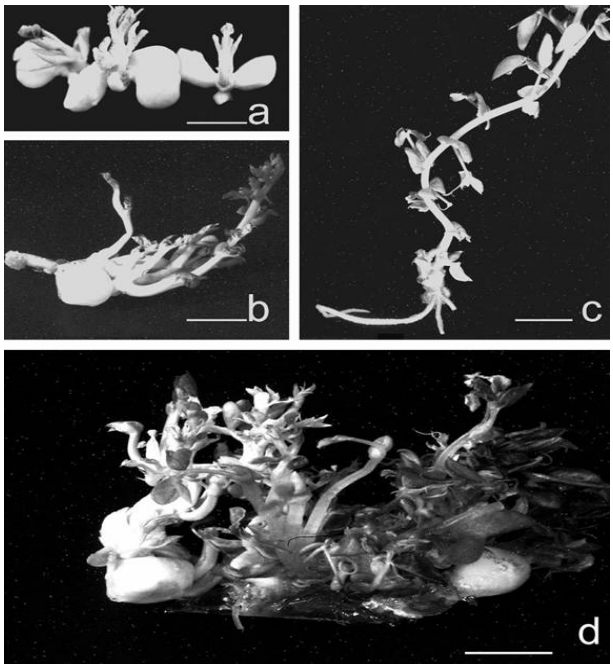
This suggested that Winner and Karina were better for undertaking experiments compared to Sprinter and Bolero. At the same time treatment with 0.001 M NaN_3 resulted in higher germination with similar to control compared with 0.003 M treatment, having negative effect on seed germination in combination with BAP or TDZ. Our findings do not agree with Gao *et al.* (1992), who treated mature embryos of rice with 0, 1, 2 and 4 mM NaN_3 and found that the shoot regeneration from higher dose (2 - 4 mM sodium azide treated seeds) was better compared to those treated with lower dose (1 mM NaN_3). Our results agree with Malik and Saxena (1992a), who emphasized that 50 µM BAP was the most effective plant growth regulator concentration for shoot regeneration in pea. Similarly Rubolo *et al.* (1984) and Khehra and Mathias (1992) showed that the shoot regeneration was predominantly affected by the genotype or the type of explant or both. We noted that the varieties of pea show different shoot regeneration capacity on the same medium when subjected to different experimental conditions.

Table III. Rooting of shoots of four pea varieties using 2.5M NAA after seed treatment with 0.001 and 0.003 M NaN_3 and culturing them on BAP and TDZ

Cultivars	Mean number of roots/shoot	Number of rooting shoots	Length of primary root (cm)
Winner	1.67 a	40.06 a	0.52
Karina	0.56 ab	23.21 ab	0.54
Sprinter	0.60 ab	21.14 ab	0.11
Bolero	0.32 b	16.54 b	0.11

Each value is the mean of 4 replicates each with 5 explants
[1]Values with in a column followed by different letters are significantly different at 0.05 level of significance using Duncan's Multiple Range Test.

Fig. 1. Effects of mutant Sodium azide on pea. (a) germination of cv. Winner on MS medium containing 10 µM TDZ after treatment with 0.001 M sodium azide (b) germination of cv. Winner on MS medium containing 50 µM BAP after treatment with 0.001 M sodium azide (c) Rooting of cv. Winner from 0.001 M sodium azide treated seeds cultured on 50 µM BAP on MS medium containing 2.5 µM NAA. Mutated shoots and leaves on the seedlings of cv. Sprinter (Fig.1d). Bar = 1 cm.



Rooting. No effect of NaN_3 was observed on rooting. However, the roots that developed from shoots previously cultured on BAP were better compared to those previously cultured on TDZ. The number of roots per shoot and the frequency of roots showed effects of cytokinin in the medium and cultivars on rooting ($p < 0.01$) independent of NaN_3 doses. We observed 1.67 roots per shoot and root regeneration rate of 40.06% in cv. Winner (Table III). Although no statistical difference was observed among varieties for primary root length yet the roots length in case of Sprinter and Bolero seemed very inferior. The longest

root (0.54 cm) was recorded on Karina followed by cv. Winner (Fig. 1c, 0.52 cm) and the smallest root (0.11 cm) on Bolero. Considering all of the above we find that shoot regeneration capacity of the seeds was better on the MS medium containing BAP compared to TDZ containing media. These results are in agreement with Malik and Saxena (1992b), who found negative effect on rooting if the shoots remained on the MS medium containing TDZ for more than 3 weeks.

In conclusion, much additional information must be obtained before the exact relation of mutation breeding to the conventional or modern methods of plant breeding is well established. The study is helpful in bringing the results of germination and treatments with mutagens and is helpful to determine the cares in preparing the explant and determine the type of hormone that results in better shoots regeneration. These studies will be of considerable help in the future mutation breeding research by producing large M_1 population mutants in a small place.

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