Effects of Mutagenic Sodium Azide (NaN_3) on In-Vitro Development of *Nigella sativa*

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Abstract
The study examined mutagenic effects of Sodium azide (NaN_3) on micropropagation of *Nigella sativa* with or without growth hormones in MS medium. Seeds treated with NaN_3 (0.1%) for 6 hrs grown on MS medium containing various concentrations of Benzylaminopurine (BA) and Napthelene Acetic Acid (NAA) showed the best shoot number. It was observed that average number of shoots increased and the average shoot length decreased with increase in the BA concentration.
Keywords: Sodium azide, mutation, micropropagation

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 and Van Harten, 1988) and study of genetics and plant developmental phenomena (VanDenBulk *et al.,* 1992; Bertagne *et al.,* 1996). Mutations generally occur naturally (spontaneous mutation) but can also be induced by mutagens i.e. the physical or chemical biological agents that change the genetic makeup (Streisinger and Owen, 1985). Physical and chemical mutagens are more popular due to their cost effectiveness.

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 that change the function of proteins but do not abolish their functions as deletions or frame shift mutations mostly do (Veen, 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). 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. Mutagens have been applied to suspension, callus and embryo cultures in many species including barley, soybean, carrot, maize, banana and morning glory (Blixt, 1965a; Blixt, 1965b; Blixt, 1967a; Blixt, 1967b; Broertjes and Lefferring, 1972; Kleinhofs *et al.,* 1978a; Kleinhofs *et al.,* 1978b; Bhagwat and Duncan, 1998; Bhate 2001). 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. The aim of this study was to determine the optimum concentration and efficiency of in vitro Sodium azide (NaN_3) treated seeds of *Nigella sativa* and select mutated plants.

Materials and Methods

Filter sterilized solution of NaN_3 (1.5 M) was prepared in de-ionized water and diluted with sterile 0.1 M phosphate buffer (pH 3.6) to give 0.1 %, 0.2 %, 0.4% and 0.5 % working solution to treat the sterilized seeds. The seeds were given treatment for 6 and 24 hrs. The untreated seeds submerged in autoclaved distilled water for the same period of time

served as control. Prior to treatment with mutant all the seeds were sterilized by mercuric chloride (0.1%) for 10 minutes and then washed with autoclaved double distilled water. All the seeds were inoculated on MS basal medium (Murashige and Skoog, 1962) having 3% sucrose (carbon source) and 0.8% agar (solidifying agent) for germination. The pH of the medium was adjusted to 5.6-5.8 with 0.1 N NaOH or 1 N HCl before gelling with agar. The seeds were incubated in light intensity of 8/16 h (day/night) photoperiod.

The shoot tips of both treated and untreated seedlings were excised and inoculated on MS media supplemented with various concentrations of 6-benzyladenine (BA), kinetin (Kn) 2,4-Dichlorophenoxy acetic acid (2,4-D) alone or in combination with IBA or NAA. Each experiment was done three times in10–12 replicates. Data was recorded after 4 weeks of culture. The shoots obtained were inoculated on MS medium supplemented with different concentrations of IBA, IAA, NAA and 2,4-D to determine best rooting media. All the cultures were kept under cool-white fluorescent at 25±2°C and 60–70% relative humidity. The cultures were examined daily for contamination and morphogenetic responses and the data was recorded at the end of 4–12 weeks of culture period with respect to callus induction, its biomass, organogenesis (shoot and root induction) etc. The data collected on different parameters were subjected to statistical analysis to determine the degree of authenticity of results in terms of mean and standard error.

Results and Discussion

Seeds submerged in NaN_3 started germination after about 1 week of culture period. Seed germination rate decreased with increasing the mutagen concentration. The highest percentage of seed germination rate was observed at 0.1% NaN_3 solution for 6 hours. This response was almost equal to that of control used (Figure 1 & 2; Table 1). The complete germination of the seeds took eleven days with green leaves, epicotyl, hypocotyl, and root.

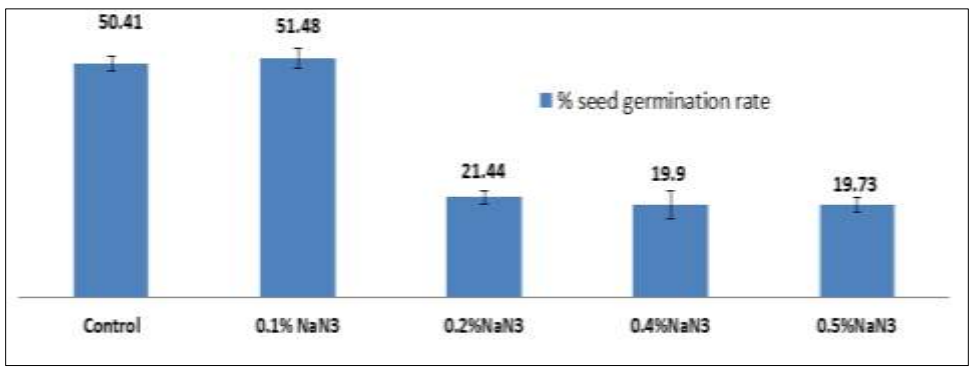

Figure 1: Percentage seed germination treated with sodium azide for a period of 6 hrs

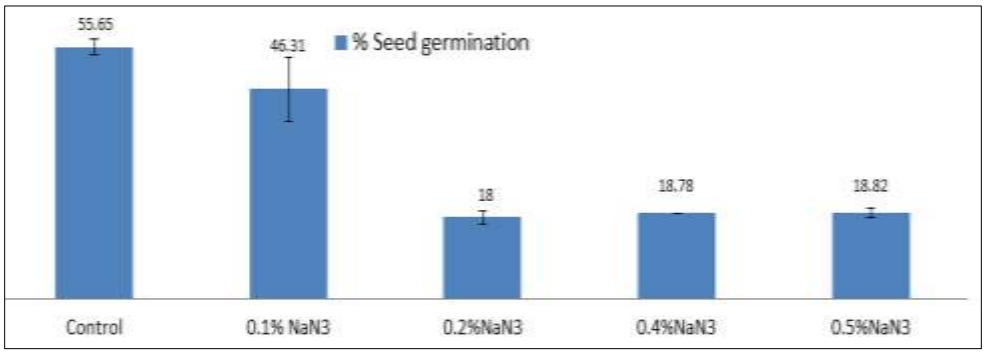

Figure 2: Percentage seed germination treated with sodium azide for a period of 24 hrs

Table 1: Effect of different concentrations of NaN_3 treatment for a period of 6 and 24 hours on seed germination of *Nigella sativa*

6 hours period		24 hours period	
Conc. of NaN_3	**Percent seed germination rate Mean ± S.E.**	**Conc. of NaN_3**	**Percent seed germination rate Mean ± S.E.**
Control	50.41±1.60	Control	55.65±1.67
0.1%	51.48±1.95	0.1%	46.31±7.03
0.2%	21.44±1.5	0.2%	18±1.41
0.4%	19.9±3.02	0.4%	18.78±0.02
0.5%	19.73±1.48	0.5%	18.82±0.83

Shoot tips obtained from both treated and untreated seeds were sub cultured in MS medium supplemented with a range of BA concentrations and the concentrations of 4.4µM, 5.55 µM and 8.8 µM resulted in shoot proliferation and callus growth (Figure 3). It was observed that average number of shoots increased and the average shoot length decreased with increase in the BA concentration (Table 2 and Figure 4).

Table 2: Shoot proliferation of Nigella sativa using MS medium supplemented with phytohormones

Phytohormone	**Callusing**	**No. of shoots Mean ± S.E***	**Length of shoots Mean ± S.E***
BAP(µM)			
4.9	+	1.8±0.4	3.62±0.57
5.5	++	2.0±0.5	2.25±0.41
8.8	+	2.67±1.0	1.67±0.27
Kn (µM)			
4.9	-	1.1±0.1	4.9±0.67
5.8	+	1.4±0.3	4.2±0.9
9.2	-	1.0±0.0	7.3±2.1
BA(6.66µM)+IAA(2µM)	++	2.4±0.6	2.17±0.28
BA(6.66µM)+NAA(2µM)	+	3.3±0.7	1.2±0.12
2,4-D(4 µM)+Kn(2µM)	+++	-	-
1/2MS+BA(4µM) + IBA(1µM)	++	1.67±0.19	1.6±0.1

The maximum average shoot number (2.67) was achieved on MS medium supplemented with BA 8.8 µM. A good callus and lateral growth was observed on MS +BA 5.55 µM while the shoot tips cultured in different concentrations of kinetin alone does not lead to any multiplication but lead to the elongation of shoot tips with a very scanty callus formation in some concentrations (Figure 3). The combined interaction of BA (6.66 µM) and IAA (2 µM) favored non regenerative callus formation with multiple shoot differentiation while BA (6.66 µM) and NAA (2µM) lead to the poor growth of callus (no regenerative) but a high multiple shoot differentiation. Highest average shoot number of 3.3 was achieved in this concentration. MS (half strength) augmented with BAP (4 µM) and IBA (1 µM) favored multiple shoot formation and good growth of callus. The shoot tips grew in size, shoot multiplication, callus growth was observed but there was no root induction.

Figure 3: Effect of different concentrations of cytokinins alone and in combination with auxins on multiplication of *Nigella sativa* [BA: a. 4.9 µM, b. 5.5µM, c. 8.8 µM; Kn: d. 4.9 µM, e. 5.8 µM, f. 9.2 µM, g. BA (6.6 µM) + IAA (2 µM), h. BA(6.6 µM) + NAA (2 µM), i. 2,4-D (4µM) + Kn (2 µM), j. ½ MS+BA(4 µM) + IBA(1 µM)

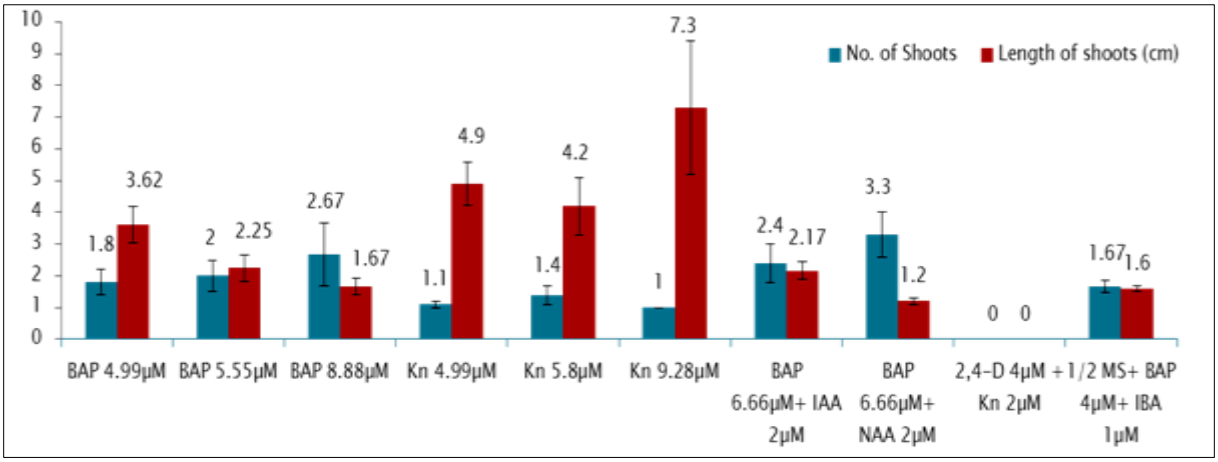

Figure 4: Effect of different concentrations of BA and Kinetin alone and in combination with IAA, IBA and NAA in shoot multiplication of *Nigella sativa.*

As far as effect of mutagenic treatment is concerned, in the present study it was observed that the shoot multiplication rate increased as the percent concentration of NaN_3 decreased and the maximum shoot multiplication rate was observed in the seeds treated with 0.1% NaN_3 for 6 hours. So our results revealed that we got a positive mutation which has improved shoot multiplication rate. Therefore using mutagenic NaN_3 (0.1% for 6 hrs.) produced mutants with possible improved characteristics.

The main purpose of in vitro propagation of plantlets is to develop complete plants from cells, tissues and organs or to develop new plants with new characteristics favorable to given environmental and climatic conditions. A lot of research is devoted in developing various mediums to suite the specific cultures. Numerous studies indicate that different cultures are successful in specific mediums i.e., the mediums vary in their composition from culture to culture. It is well known that organogenesis in vitro depends on a complex system of endogenous and exogenous interacting factors (Alicchio *et al.*, 1982). The regeneration ability may also vary from plant to plant depending upon the species and genotype, physiological conditions and type of explant.

The morphogenic response of the explant is mainly based on the type and concentration of hormone used. The combination effect of cytokinins and auxins to promote shoot induction and elongation has been reported from early studies. They are known to interact with different endogenous processes, including apical dominance, cell cycle, lateral root initiation, regulation of senescence, and vasculature development (Coenen and Lomax 1997; Swarup *et al.*, 2002).

In the present study the micropropagation of *Nigella sativa* was studied after treating the seeds with sodium azide. The study was conducted to observe the effect of mutagen on the micropropagtion rate as well as number of shoots. In vitro generated shoot tips were used as explants. Different phytohormones (auxins and cytokinins) were used individually and in combinations. In the present study, the combination effect of BAA and NAA was found most effective in inducing multiple shoot proliferation. Our results are in conformity of the results made by various researchers who had studied the combination effect of BAP and NAA (Khan *et al.* 1997; Hossain *et al.* 2003; Durkovic 2008; Frabetti *et al.* 2009; Girijashankar, 2011). Experimental observations revealed that combination of 2,4-D and Kn did not lead to the induction of shoots but a very good callus growth. Al-Ani (2008) also reported similar results. It was also observed that using BA alone also lead to the shoot proliferation and increasing the concentration of BAP in MS medium was found to increase the number of shoots while decreasing the average length of individual shoots. The maximum average shoot number of 2.67 was found in MS medium supplemented with 8.88 µM BA. In all the above cases, poor callus growth was observed. Using Kinetin (Kn) alone lead to the development of very low number of shoots but a very good shoot elongation and MS medium augmented with 9.28 µM kinetin showed maximum elongation of about 7.3 cm. A wider survey of existing literature reveals that BA is most reliable and useful cytokinin for multiplication of shoots (Barna and Wakhlu, 1994). BA is the most effective cytokinin for the shoot tip, meristem and bud culture. However Meyer and Staden (1991) and Natali *et al.* (1990) reported Kn better as compared to BA for shoot proliferation in Aloevera which is in contrast to our results.

The main objective of mutating plant species is to produce mutants with improved characteristics. Chemical mutagens especially sodium azide have been used extensively to produce mutants with improved characteristics (Kiruki *et al.*, 2006; Kumar, 1988; Ali *et al.*, 2007). In view of the above facts, seeds of *N. sativa* were given different mutagenic treatments of sodium azide and a dose dependent reduction in germination rate was observed i.e., increasing the NaN3 concentration (form 0.1-0.5%) decrease the % germination rate of seeds. Our results are in conformity with that of Bashir *et al.* (2013). Many workers have reported adverse effect of chemical mutagens on various plants (Koner *et al.*, 2007; Sangle *et al.*, 2011). The decrease in germination rate of seeds may be due to the damage of cell constituents at molecular level or altered enzyme activity (Khan and Goyal, 2009; Chowdhary and Tah, 2011). Therefore using mutagenic NaN_3 (0.1% for 6 hrs) produced mutant with possible improved

characteristics. Our results were supported by a number of studies which shows that NaN3 has been used in many plant species for improving their physiological characteristics (Suzuki *et al.*, 2008; Okubara *et al.*, 1993).

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