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Sodium Azide as a Mutagen

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1. Introduction

Sodium azide (NaN_3), a common bactericide, pesticide and industrial nitrogen gas generator is known to be highly mutagenic in several organisms including plants and animals. Sodium azide is a potent mutagen in micro-organisms and a very efficient mutagen in barley as well as in some other crop species, however it is marginally mutagenic in mammalian systems and not mutagenic in *Neurospora, Drosophila* and in *Arabidopsis thaliana* – a model plant species. Mutagenicity of sodium azide is mediated through the production of an organic metabolite of azide and highly dependent on acidic pH. The frequency of chromosome breakage caused by sodium azide is relatively very low. The issues concerning metabolism, activity, cytotoxic and mutagenic effects of sodium azide and comparisons with other mutagens are presented in this chapter.

2. Metabolism and Activity of Sodium Azide

2.1. Effects on Seed Germination and Plant Growth

Sodium azide (SA) is a well-known inhibitor of heavy-metal enzymes with influences on metabolism and respiration of living cells. It is metabolized *in vivo* to a powerful chemical mutagen in many plant species, including barley, rice, maize and soybean (e.g. Owais and Kleinhofs, 1988; Szarejko and Maluszynski, 1999). The mutagenic effect of SA greatly depends on the pH of the treatment solution and similarly to the MNU (N-methyl-N-nitrosourea), can be increased further by pre-germination of seeds prior to NaN_3 treatment. This mutagen generates M_1 sterility and high frequency of M_2 chlorophyll mutations in barley. The high frequency of chlorophyll and morphological mutations induced

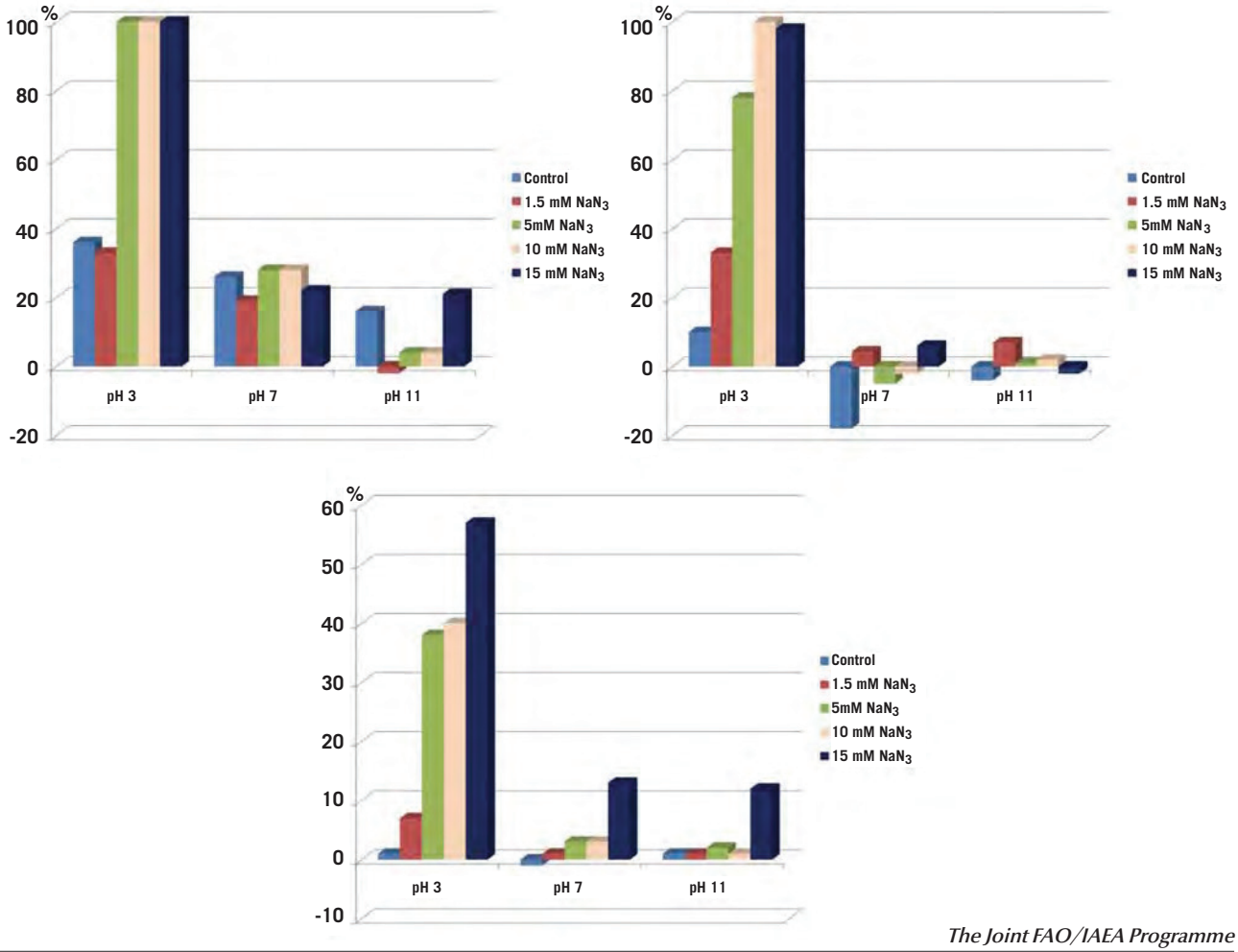

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Figure 13.1 (A) Influence of sodium azide treatment pH on barley seed germination. Seed were treated with four SA concentrations: 1.5 mM, 5 mM, 10 mM and 15 mM for 4 h. (B) Influence of sodium azide treatment pH on barley seedling emergence reduction. Seed were treated with four SA concentrations: 1.5 mM, 5 mM, 10 mM and 15 mM for 4 h. (C) Influence of sodium azide treatment pH on barley seedling height. Seed were treated with four SA concentrations: 1.5 mM, 5 mM, 10 mM and 15 mM for 4 h.

by sodium azide is comparable with the frequency produced by alkylating agents. Influence of sodium azide on barley seeds germination (**Fig 13.1A**), seedlings emergence reduction (**Fig 13.1B**) and seedlings height (**Fig 13.1C**) is greatly dependent on acidity of treatment solution, while in alkaline solution it is completely ineffective.

2.2. Metabolism of Sodium Azide in Plant Cells

Since the pI (isoelectric point) of sodium azide is at pH=4.8, the predominant compound at pH=3 is hydrogen azide (HN_3). The improved mutagenic effectiveness of SA in the acid form is due to facilitated penetration through the cell membrane by the uncharged HN_3 molecule. The mutagenic effect of sodium azide is mediated through the synthesis of an organic metabolite, which was identified in bacteria and barley as an amino acid analogue L-azidoalanine [N_3-CH_2-CH(-NH_2)-COOH]. A free amino acid group is essential for mutagenic activity, when compared to the carboxyl group. Synthetic D-azidoalanine displays very low mutagenic activity, indicating that a stereo-selective process is involved in azidoalanine mutagenicity. The production of this metabolite is dependent on the enzyme O-acetylserine sulfhydrylase [E.C.4.2.99.8] inhibited *in vitro* by cysteine. This enzyme catalyses the addition of azide (N^{3-}) or sulphide (S^{2-}) to O-acetylserine which leads to the synthesis of azidoalanine or L-cysteine, respectively. Alpha-methyl substitution blocks the mutagenicity of azidoalanine with alpha-methyl-azidoalanine being nearly devoid of mutagenic activity. Homologation of azidoalanine to yield 2-amino-4-azidobutanoic acid markedly increases the molar mutagenic potency. As in the case of azidoalanine, the mutagenic activity of this homologue is associated with the L-isomer. The lack of SA mutagenic effect on adult fruit flies and arabidopsis seeds is attributed to the absence of cellular components conducting enzymatic conversion of sodium azide to the metabolite azidoalanine (Sadiq and Owais, 2000).

2.3. Physiological Effects

Sodium azide is an inhibitor of the terminal segment of the electron transport chain. The physiological effect of SA is the inhibition of catalase, peroxidase and cytochrome oxidase, thus influencing respiratory processes. Proton-translocating ATPase complex (F_0F_1), which catalyzes the terminal step of photo- or oxidative phosphorylation in chloroplasts, mitochondria and bacterial membranes is particularly prone to inhibition by sodium azide. This leads to a reduction in ATP production and, as a consequence, DNA, RNA and protein synthesis. This inhibition is dose-dependent and is potent even at the lowest dose used for mutation induction in barley embryos. Recovery from this reduction was observed in the case of DNA replication, whereas there was only marginal recovery of protein synthesis. Due to the fact that SA affects DNA synthesis, it is most effective when it is applied at S-phase of the cell cycle. Sodium azide acts as an inhibitor of the proton pump and alters the mitochondrial membrane potential. Additionally, in E. *coli* the primary site of the inhibitory action of SA is SecA protein, an essential component of the protein export system, displaying ATPase activity. Mutations causing resistance to sodium azide in E. *coli* occur mainly in the *sec*A gene (Fortin *et al.* 1990).

3. Cytotoxic and Mutagenic Effect

3.1. Influence on the Cell Cycle and Metabolism

A dose-dependent decline in mitotic index was observed when SA at concentrations 0.1-0.5% were applied to the seed of *Trigonella foenum-graecum*. At the highest concentration the reduction in mitotic index was 50%. The inhibitory influence of SA dose (concentration and duration of treatment) on the mitotic index was also observed in barley seedling, what corroborated a cytotoxic effect of this mutagen (Ilbas *et al.* 2005), and confirmed the inhibitory effect of sodium azide on cell cycle. The ATP demand of dividing cell is much higher compared to a non-proliferating cell. The ATP deficiency caused by sodium azide may be one of the reasons for the decrease in mitotic index. SA was found to decrease the cellular level of calmodulin, which is a calcium binding protein participating in signal transduction and cell division. Sodium azide at a concentration 1 mM caused a remarkable reduction of cell divisions in barley anthers (Castillo *et al.* 2001).

The organization and movement of spindle fibres during the cell division is an ATP dependent process. Due to the reduced synthesis and availability of ATP in sodium azide treated cells, the spindle fibre organization is affected, which may in turn influence the organization of chromo-

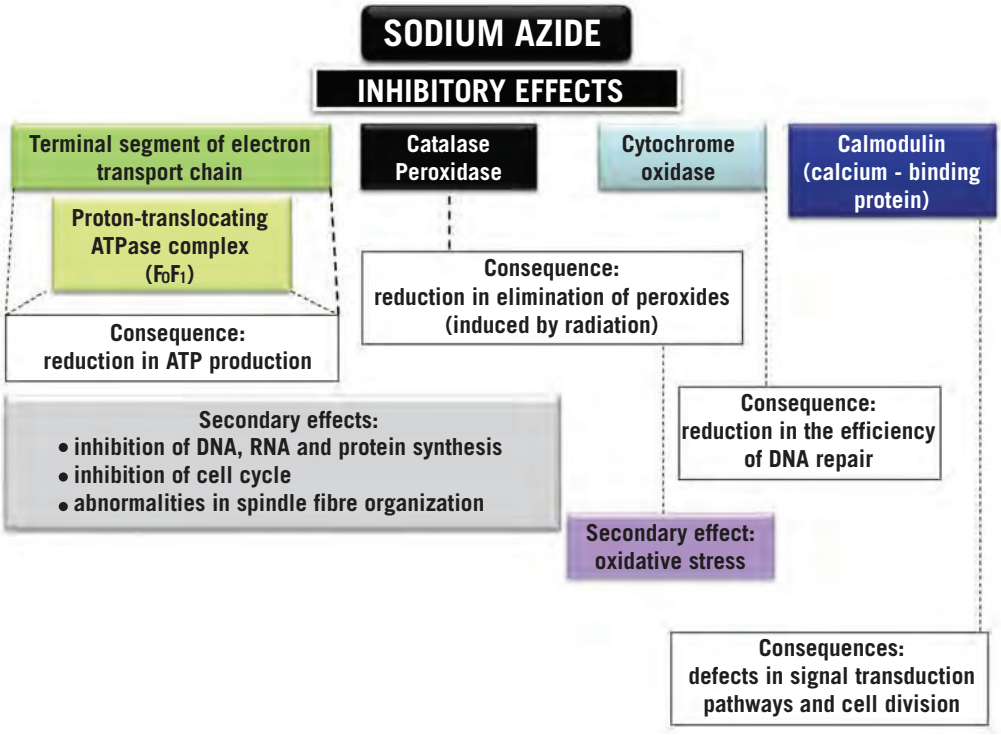

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Figure 13.2 Targets and consequences of sodium azide activity in plant metabolism.

somes at the metaphase plate and their migration during anaphase. Metabolic mechanisms that are the targets of sodium azide activity are shown in **Fig 13.2**.

Apart from being a suppressor of cell divisions, positive effects of sodium azide on pollen embryogenesis have been demonstrated in *Solanum nigrum* and *Hordeum vulgare* (Kopecky and Vagera, 2005; and Vagera *et al.* 2004, respectively).

3.2. Mutagenic Effect

It is well known that sodium azide induces chromosomal aberrations only at a very low rate compared to other mutagenic treatments. Is has been shown that L-azidoalanine does not interact with DNA directly under *in vitro* conditions. Mutagenic activity of this compound was attenuated by a deficiency in the excision of UV-like DNA damage in both plants and bacteria, therefore it seems that a lesion recognizable by the excision-repair mechanisms must be formed to evoke the effect. Mutagenesis proceeds from this by 'direct mispairing' (Owais and Kleinhofs, 1988).

Sodium azide was used for the first time as a mutagen by Nilan *et al.* (1973) in barley, when an increase in the frequency of chlorophyll mutations was observed in a dose-dependent manner, at the concentration range 1-4 mM at pH 3, with highest frequency, 17.3% recorded for 4 mM. A high frequency of chlorophyll mutations was also observed in barley variety Aramir after combined treatment with 1 mM NaN_3 (3 hours, pH=3) followed by 0.7 mM MNU with a 6-hour inter-incubation (germination) period between treatments. Using this combination 6.4% of chlorophyll mutations were obtained in the subsequent M_2 population (**Fig 13.3A**), with a fertility and height reduction of M_1 plants lower than after the double treatment with MNU (**Fig 13.3B**).

Similarly, a very high level of point mutations was observed for other barley genotypes after combined treatment with NaN_3 and MNU. On average 5.6% chlorophyll seedlings were found in the M_2 derived from this treatment for six barley varieties tested and about 30-50% of M_1 plants carried a chlorophyll mutation. The reduction in M_1 plants fertility did not exceed 55%, which makes this combination a very efficient treatment for inducing point mutations in barley. Combined treatment of NaN_3 and MNU yielded a wide spectrum of gene mutations in many barley genotypes, leading to dwarf and semi-dwarf characters or changes in root system development and structure (Szarejko and Maluszynski, 1999). The protocol recommended as a highly efficient

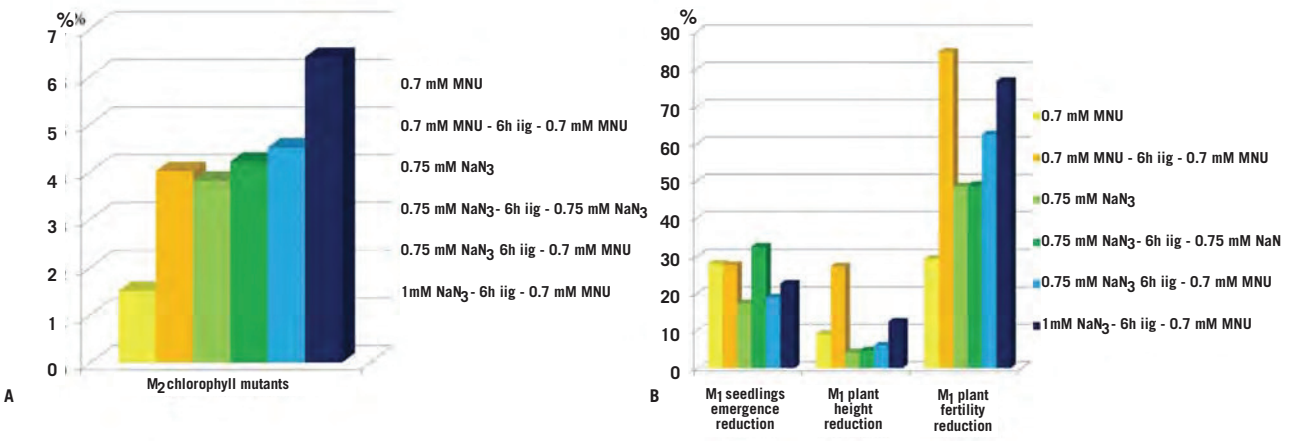

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Figure 13.3 **(A)** Mutagenic effect of single, double and combined treatments with sodium azide and MNU on chlorophyll mutation frequency in the M_2. The mutagens were applied on barley seed var. Aramir. **(B)** Mutagenic effect of single, double and combined treatments with sodium azide and MNU on reduction in seedlings emergence, plant height and fertility of M_1 plants.

method of mutagenic treatment in barley is given in **Box 13.1**. In treatment of dormant rice seeds, the germination rate and seedling growth were reduced in proportion to both 1) the increase in sodium azide concentration and 2) the duration of treatment. SA alone also delayed seed germination. Given that the physiological effect of sodium azide is inhibition of catalase, peroxidase and cytochrome oxidase, delay in germination may be explained by inhibition of metabolic activity necessary for germination. The fact that SA has a limited effect on the fertility reduction of the M_1 supports the observation that somatic damages and mutation induced by this mutagen are not accompanied by chromosomal aberrations. The percentage sterility of barley M_1 ovules after sodium azide treatment can be described by a second degree parabola in which higher values of sterility are obtained at intermediate concentration of the mutagen (Prina and Favret, 1983). Sodium azide has been used successfully in the induction of mutations in plants, including numerous crop species (**Table 13.1**). The mutagenicity of SA can be affected by cellular metabolism during germination. Sodium azide, when applied at the DNA synthesis stage of the first cell division of germination process, is more effective in the induction of mutation. In rice, when applied at this stage at a dose of 10 mM for six hours, sodium azide yielded the maximum percentage of mutations 11.1% and 1.22% based on M_1 panicles and M_2 seedlings, respectively (Hasegawa and Inoue, 1980).

Sodium azide proved to delay the *in vitro* differentiation of callus structures derived from leaf segments of sugar cane in a concentration-dependent manner (0.07 to 0.7 mM). The number of regenerated plantlets in sodium azide treated callus was reduced. Additionally, at a concentration of 0.9 mM necrosis in meristematic sugar cane callus were observed (Gonzalez *et al.* 1990).

Box 13.1: Protocol for highly efficient method of barley seeds treatment with sodium azide and MNU

Pre-soaking of seeds in distilled water
Duration: 8 hours

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Treatment with 1.5 mM sodium azide (pH 3, phosphate buffer)
Duration: 3 hours

↓

Inter-incubation germination period (seeds on trays with wet filter paper)
Duration: 6 hours

↓

Treatment with 0.7 mM MNU
Duration: 3 hours

↓

Washing the seeds under running tap water, 3 times for 10 min.

Table 13.1: The use of sodium azide for mutation induction in plant species

Species	Common name	Recommended treatment method (and outcomes)	Material
Brachypodium distachyon	Purple false brome	1.5 mM for 2 hours (estimation of mutagenic effects)	Seed
Corchorus capsularis	Jute mallow	20 mM (treatment period not available; development of new varieties)	Seed
Hordeum vulgare	Barley	1.5 mM for 3 hours (estimation of cytotoxic and mutagenic effects; in combination with MNU)	Seed
		1 mM for 3 hours (estimation of cytotoxic and mutagenic effects)	Seed
		1 mM for 2 hours (determination of mutations types)	Seed
		0.1; 1 mM for 6 hours (production of androgenic doubled haploid mutants)	Anther
		0.1; 0,5; 1; 5 mM for 20 hours (estimation of concentrations' influence on mutagenic effect)	Seed
Oryza sativa	Rice	1 mM for 3 hours (determination of mutations frequencies; in combination with 15 mM MNU)	Seed
		0.1; 1; 5; 10; 50 mM (estimation of cytotoxic and mutagenic effects)	Seed
Phaseolus vulgaris	Bean	0.04; 0.12; 0.36; 1.08 mM for 3 hours (estimation of mutagenic effects)	Seed
Pisum sativum	Pea	2 mM for 3 hours (development of nitrogen fixation mutants)	Seed
Saccharum officinarum	Sugarcane	0.07; 0.15; 0.46; 0.77 mM for 10 min. (estimation of mutagenic effects)	Leaf callus
Solanum nigrum	Black nightshade	10; 20 mM for 24 hours (increase in the efficiency of androgenic production)	Seed
Sorghum bicolor	Sorghum	0.5; 1; 2; 4 mM for 4 hours (estimation of mutagenic effects)	Seed
Tradescantia hirsutiflora	Spiderwort	0.2 mM for 6 hours (estimation of cytological effect)	Inflorescence
Trigonella foenum-graecum	Fenugreek	0.1; 0.2; 0.3; 0.4; 0.5% for 6 hours (estimation of the rate of cytogenetic changes)	Seed
Triticum aestivum	Wheat	5 mM for 6 hours (development of thermo-tolerant mutants)	Seed
Zea mays	Maize	0.1; 1; 10 mM for 1 hour (mutation induction)	Immature embryo

3.3. Types and Frequencies of Induced Mutation

The frequency and type of point mutations induced by this mutagen at a concentration of 1 mM were determined by sequence analysis of the barley gene *Ant18* encoding dihydroflavonol 4-reductase, which catalyzes the last step in flavonoid synthesis pathway. Sodium azide, when applied at the above concentration, generated 21 base substitutions within the analysed sequence, which corresponds to 0.17% of the 12,704 nucleotides sequenced. Transitions made up 86%, whereas transversions constituted 14% of identified substitutions. The frequency of A-T → G-C transitions was about three times higher than G-C → A-T. Deletions and mutation hot spots were not found. Mutations induced by sodium azide do not show a tendency for clustering. The absence of G-C → C-G transversions was observed in the analyzed sequences. This kind of transversions is induced by ^{60}Co γ-rays or Fe^{2+} ions in the DNA through the action of oxygen free radicals, and absence of these transversions rules out the option that oxygen free radicals from azide-derived peroxide accumulation are

the secondary mutagens, responsible for the identified substitutions (Olsen *et al.* 1993).

4. Interactions with other Mutagenic Agents

Apart from being an independent mutagen, sodium azide proved to display interesting interactions in terms of cytotoxic and mutagenic activity with other chemical and physical mutagens. A beneficial influence of simultaneous treatment of rice seed with sodium azide and MNU was noted in terms of obtaining a high density of mutations with relatively low toxicity to treated seed. Concentrations of MNU above 15 mM cause a large drop in germination and viability of germinated plants. Therefore, a combination of 1 mM sodium azide plus 15 mM MNU was applied and the density of induced mutations was determined as 1/265 kb. This observed mutation frequency is satisfactory for high throughput TILLING strategy and proved to be about three times higher than have been determined for treatments with lower concentrations (Till *et al.* 2007).

When applied at concentration of 10 mM to barley seed, after γ-irradiation (160 Gy) in anaerobic conditions with a four-hour treatment, SA considerably raises the frequency of chromosomal aberrations from 26 to 64%. Sodium azide is an inhibitor of catalase, peroxidase and terminal cytochrome oxidase. Catalase and peroxidase are important in the elimination of the peroxides, which are induced by radiation, whereas the inhibition of cytochrome oxidase interferes with the efficiency of DNA repair process at the chromosome level, especially by reducing ATP production, which is required during the damage repair process. The synergistic relationship between these two mutagenic agents was noted only at acidic (pH 3) treatment. The interaction between sodium azide and ionizing radiation and its association with a decreased catalase, proxidase and respiratory activity is explained by enhanced formation of peroxy radicals in irradiated tissues and the demand for ATP during the repair of the induced lesions. Additional corroboration comes from the fact that the induction of sodium azide activity was also observed by pre-soaking of the seeds in either O_2- or N_2-bubbled water prior to and during the treatment.

SA treatment modifies the extent of the lesions and efficiency of the repair processes. The strong inhibition of catalase and peroxidase activity, induced by sodium azide, results in the reduced scavenging of peroxide, leading to an increase in radiation-induced damage. Inhibition of respiratory activity brings about reduction of the rate of oxidative phosphorylation and thus a reduced supply of ATP to the affected cells. Sodium azide disrupts the cascade of oxidative phosphorylation re-directing the electron stream to flavoproteins and increasing the production of peroxide, furthermore accumulation of this compound is accelerated by the synchronous inhibition of catalase.

Taking into account that sodium azide mutagenesis (also in combination with other mutagens) produces relatively high frequencies of mutation with mild effect on plants fertility the use of this mutagen in the future may be of significant importance in functional genomics when applied along with high throughput technologies, like TILLING, especially for crop species.

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