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REVIEW ARTICLE

Mutagenic Effects of Sodium Azide and its Application in Crop Improvement

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Abstract: In recent times, chemical mutagens have become important tools in crop improvement. These mutagens are being used to produce resistance in various susceptible crops to improve their yield and quality traits against harmful pathogens. There are several mutagens available for crop improvement and each mutagen has its important role as positive or negative effect on crops. Sodium azide is a chemical mutagen that creates point mutation in the genome of plants by producing metabolite and thus produced protein in mutant plant has different function from the normal plant. The mutant plants produced by the treatment of sodium azide are capable to survive under various adverse conditions and have improved yields, increased stress tolerance, longer shelf life and reduced agronomic input in comparison to normal plants. The selection of plant mutants is based on morphological, biochemical and DNA based markers. The DNA based markers are reliable and reproducible for mutant selection for any crops used in the study. The few DNA based marker are available for plant researcher for point mutation detection which is caused by sodium azide. Since, sodium azide creates point mutation, A.T-->G.C base pair transition and transversion and hence all DNA based markers cannot used for point mutation detection. In this review, we are focusing the mutagenic effects of NaN_3 and its application in crop improvement.

Key words: Crop improvement · Molecular markers · Mutagenesis · Sodium azide

INTRODUCTION

The genetic information must be the same in all the cells of the living creatures. However, mutations may take place in the genetic information causing a cell or living creature to be different from the other. Mutations are the tools used to study the nature and function of genes which are the building blocks and basis of plant growth and development, thereby producing raw materials for genetic improvement of economic crops [1]. Mutation methodology has been used to produce many cultivars with improved economic value and study of genetics and plant developmental phenomena [2, 3]. It has been demonstrated that genetic variability for several desired characters can be induced successfully through mutations and its practical value in plant improvement programs has been well established. The main advantage of mutation breeding is the possibility of improving one or two characters without changing the rest of the genotype. Induced mutations have great potentials and serve as a complimentary approach in genetic improvement of crops [4]. Induced mutations have been used to improve major crops such as wheat, rice, barley, cotton, peanut and cowpea, which are seed propagated. Various mutagenic agents are used to induce favorable mutations at high frequency that include ionizing radiation and chemical mutagens [5]. Chemical mutagens are the one cause of mutations in living organism. It is known that various chemicals have positive or negative effects on living organisms. Many of these chemicals have clastogenic (chromosome damaging) effects on plants via reactive oxygen-derived radicals [6]. These effects can occur both spontaneously and artificially following induction by mutagens. Chemical mutagen generally produce induced mutations which 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

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mutations mostly do [7]. These chemomutagens induce a broad variation of morphological and yield structure parameters in comparison to normal plants. Many researchers compared the mutagenic efficiencies of different mutagens on different crops and their results seem to be entirely specific for particular species and even varieties. While many researchers found chemical mutagens are to be more effective than physical ones [8-11] and many others researchers found the reverse case [12, 13]. A number of workers [14-16] have reported the role of chemical mutagens in enhancing genetic variability in higher plants. Genetic variability is the fundamental to successful breeding programs in vegetatively and sexually propagated plants [17]. 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. The mutants so produced facilitate the isolation, identification and cloning of genes used in designing crops with improved yield and quality traits [5].

Sodium azide (NaN_3) is a mutagen and has been one of the most powerful mutagens in crop plants. It has been reported that sodium azide affects plant physiology and decrease cyanide resistant respiration in tobacco callus [18]. It is known to be highly mutagenic in several organisms, including plants and animals [19-23] and its mutagenic potential has been reported in several screening assays. Sodium azide is marginally mutagenic in different organisms [24, 25] and it is not in several organisms mutagenic in others such as *Drosophila* [26] and *Arabidopsis* [27]. The mutagenicity is mediated through the production of an organic metabolite of azide compound [21]. This metabolite enters into the nucleus, interacts to DNA and creates point mutation in the genome. In order to understand its mutagenic mechanism, many studies in barley and bacteria have been performed in recent years [17, 28]. Being a strong mutagen in plant, it affects the different parts of the plants and their growth developmental phenomena by disturbing the metabolic activities.

Sensitivity of Plant Organs for Sodium Azide: Sodium azide is an ionic compound and N_3-group is centrosymmetric with N-N distances of 1.18 Å. It is highly soluble in water and such solutions contain minute amounts of hydrogen azide, as described by the following equilibrium:

$$N_3 + H_2O \leftrightarrow HN_3 + OH^- K = 10^{-4.6}$$

At low pH or with strong acid, it gives the corresponding acid, hydrazoic acid as described here.

$$H_2SO_4 + NaN_3 \rightarrow HN_3 + NaHSO_4$$

Seeds have high regenerative potential and are advantageous for use in mutagenesis. *In vitro* technique can be used for both seed and vegetatively propagated species. The promutagen NaN_3 is highly used with seeds to create mutation, which must be metabolized by plant cells to the mutagenic agent, presumably azidoalanine [29]. 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-acetylserine sulfhydrylase (E.C. 4.2.99.8). This enzyme catalyses the condensation of azide (N^-_3) or sulfide (S_2^-) with O-acetylserine to produce azidoalanine or L-cysteine respectively [30, 31]. The seeds of barley germinated in the presence of azide confirmed that this compound may act as point mutagen during DNA replication [32]. Tissue culture technique is an important way of mutant plant production with mutagen and thus speeds up the breeding program. There is some evidence that not all species in tissue culture are able to metabolize NaN_3 to the mutagenic agent [33, 34], which is a potent mutant in barley, pea and rice [35, 36], but it is hardly effective in *Arabidopsis* [37].

Conditions for Seed Treatment with Sodium Azide and Germination: The effect of chemical mutagens depends on the permeability of seed coat and nature of the mutagens. To enhance the mutagenic effectiveness and efficiency of NaN_3 and especially the metabolite, more knowledge about the effect of time, temperature of seed soaking and various concentration of NaN_3 are required. The pH value affects the rate of mutation with NaN_3. At low pH values, a considerable reduction in plant height was found [38-41]. At low pH value, the quantity of sodium azide dissociated to hydrazoic acid which are theoretically many times greater (at pH 3 there is approximately 19 times more hydrazoic acid than at pH 6, for the same concentration of sodium azide) and that would be the condition for better penetration through the cell membrane [42, 43]. The mutagenic effects of the sodium azide at various concentrations are contradictory in barley reports probably due to the different treatment

conditions (presoaking, pH, temperature and time of exposure) and different varieties used. At the lowest concentration of sodium azide from 5 to 100×10^{-4} M, was found a higher frequency of chlorophyll-deficient mutants [44]. Sodium azide affects the % seed germination, shoot length, root length and also affects delay in seed germination. Some authors made the measurements after 5-7 days from sowing, when the length of the first leaf had not reached its maximum, render it impossible to distinguish between delay in germination and a real length reduction [45]. Reduction in seed germination in mutagenic treatments has been explained due to delayed or inhibition in physiological and biological processes necessary for seed germination which include enzyme activity [46], hormonal imbalance [47] and inhibition of mitotic process [48]. Azide ion plays an important role in causing of mutation by interacting with enzymes and DNA in the cell. These azide anions are strong inhibitors of cytochrome oxidase, which in turn inhibits oxidative phosphorylation process. In addition, it is a potent inhibitor of the proton pump [17] and alters the mitochondrial membrane potential [49]. These effects caused by NaN_3 together may hamper ATP biosynthesis resulting in decreased availability of ATP molecule which may slow the germination rate and reduce the germination percentage. The another reason behind this is that seeds have probably developed tolerance to the inhibitory effect of NaN_3 on germination and had improved their physiological conditions on additional days with respect to seed germination. All living cells require energy in the form ATP molecules to carry all biological reactions. At low energy level, the rate of biological reactions inside the cell decreases. Cheng and Gao [50] treated barley seeds with sodium azide and found a significant decrease in the percentage germination. It is important to stress the fact that treatments with sodium azide, under the same conditions, produce a delay in the initiation of plant growth, as can be observed and mentioned by Pearson *et al.* [51, 52]. The various concentration of sodium azide showed different effects of mutagenesis as mentioned by many authors in literature. Kleinhofs *et al.* [35] suggested that 0.003 M NaN_3 dose increases mutations in pea. The higher dose of sodium azide also cause disturbance in genetical and physiological activities leading to the death of the cells.

The toxicity of sodium azide and most of its physiological effects can be traced to its reversible inhibitory effect on enzymes containing a coordinated divalent ion, such as those of cellular respiration [17]. It binds to metal sites in enzymes, either as the free acid HN_3 or as the ionic compound N_3-. When the ionic form is involved, there is little effect on pH, but when the free undissociated acid is involved, the pH effect may be large and inhibition is increased by lowering pH [53]. A decrease in intracellular pH was reported in *D. discoideum* cells treated with sodium azide [54] and is expected to be related to its toxicity through energy deprivation, as a result of general inhibition of cellular respiration and oxidative phosphorylation processes. Both the processes are necessary for the cell to derive energy in the form of ATP molecule and to complete biological reactions. Cell recovery can be related to the reversible effects of sodium azide on proteins and/or possibly related to its degradation into less toxic metabolites. The enzyme *O*-acetylserine sulphydrylase, modifies sodium azide in *D. discoideum* cells into a mutagenic metabolite, azidoalanine, which is highly mutagenic to *Salmonella typhimurium*, but its toxicity was not investigated [55].

Mutagenic Effects of Sodium Azide on Mitotic Index: The mitotic index is a reliable predictor of cell proliferation in the tissue. The MI assay is used to characterize proliferating cells and to identify the compounds that inhibit mitotic progression resulting in a decrease in the MI of that population. It is defined as the ratio between the number of cells in mitosis and the total number of cells. The mitotic index can be worked out from a slide, even with light microscopy. The mitotic index (MI) is a cytogenetic test that is used *in vivo* and *in vitro* for the examination of genotoxic effects of a compound over a short period and there are considerable variations in the mutagenic effect of agents in different environments. Increasing concentrations of NaN_3 and treatment duration decreased the mitotic index compared with the untreated plant. The inhibitory effects of NaN_3 on the mitotic index indicate that NaN_3 can have genotoxic and mutagenic effects. The similar effect of NaN_3 was observed on barley seedlings [56]. A similar observation of sodium azide on *Hordeum vulgare* seeds was made by other workers [52]. At the highest dose of sodium azide, 50% reduction in mitotic cells was observed which clearly indicates the inhibitory effect of sodium azide on the cell cycle. The ATP demands of dividing cells are much higher as compared to nonproliferating cells and ATP deficiency caused by sodium azide may be one of the reasons for the decrease in the mitotic index. Besides, sodium azide is expected to induce changes in the free cytosolic Ca^{2+} through the inhibition of the cAMP-induced

Ca^{2+} uptake and the inhibition of calmodulin respectively. The changes in the cytosolic Ca^{2+} is important for certain cellular processes such as growth development phenomenon in *D. discoideum* as suggested by [57] and is expected to induce changes in the structure, activity and interaction of intracellular elements. Sodium azide is found to decrease the cellular calmodulin level [58], which is a calcium binding protein for signal transduction and cell division and it is a proton pump inhibitor [17] that blocks secretion and accumulation of cAMP [59].

Mutagenic Effects of Sodium Azide on Plant Parts: Sodium azide is a strong mutagen and growth of plant parts are strongly inhibited with increasing its concentration and treatment duration. The impact of sodium azide has been observed on tomato and it was very effective in inducing mutations with respect to germination percentage, root length, seedling height, seedling survival, number of branches per plant and yield per plant respectively [1]. The effect of different concentration of NaN_3 treatment on root length was clearly observed in different crops. The lowest root length (9.10 cm) was found on day 14 for the group exposed to 2.5 mM NaN_3 for 3 h in barley except for groups exposed to NaN_3 for 3 h and measured on day 7 and its treatment had a statistically significant effect on leaf length and this effect appeared to be particularly evident on day 14 [56]. The NaN_3 treatment affects coleoptile length in groups exposed to NaN_3 for both 3 and 4 h and the lowest coleoptiles lengths were found in barley in the groups exposed to 3 mM NaN_3 for 3 h (2.54 cm) and to 2 mM NaN_3 for 4 h (1.59 cm) respectively [56]. The reduction in seedling survival is attributed to cytogenetic damage and physiological disturbances [60, 48]. The greater sensitivity at higher mutagenic level has been attributed to various factors such as changes in the metabolic activity of the cells, inhibitory effects of mutagens [61] and to disturbance of balance between promoter and inhibitors of growth regulators [62].

Effect of Sodium Azide on Chromosome: Cytological analysis with respect to either mitotic or meiotic behavior is considered one of the most dependable indexes to estimate the potency of mutagen. Therefore, investigations on meiotic aberrations and their genetic consequences form an integral part of most of the mutation studies. It also provides a considerable clue to assess sensitivity of plants for different mutagens. The mechanism of action and the nature of mutations created by sodium azide are becoming understood and it has been accelerated by the discovery of a mutagenic metabolite formed by sodium azide. The chromosomes are damaged by sodium azide in mitosis, as seen in barley [63, 64, 43, 65, 28, 32], in bean [66] and in human leucocytes [65, 32] respectively. Thus, sodium azide induced chromosome aberration frequencies, which were similar or slightly superior to those of the untreated controls. The most predominant aberrations induced by sodium azide are translocations, lagging chromosome, bridges and sticky chromosomes. Chromosome stickiness arises from improper folding of the chromosomes into single chromatids and chromosomes as a result of which chromatin fibers intermingle and chromosomes become attached to each other by means of subchromatid bridges and incidence of lagging chromosomes also increased with increasing sodium azide dose [67]. The spindle fiber organization and movement during the cell division is ATP dependent process. Due to the reduced synthesis and less availability of ATP, the spindle fibers organization in sodium azide-treated root tip cells may get affected which in turn may affect the organization of chromosomes at the metaphase plate and migration of chromosomes towards the respective poles during anaphase [67]. The deformity in spindle formation and chromosome segregation during mitosis will result in chromosomal aberrations like lagging chromosome, sticky chromosomes and bridge formation. The mutagenic agent, sodium azide decreases the germination percentage and increases the chromosomal aberrations in root tip mitotic cells of plant which was a dose-dependent manner [68]. Mutagens induce structural changes in chromosomes and create mutations, which might be responsible for the failure of pairing among homologous chromosome. Adegoke [69] reported that sodium azide induces chromosomal damages leading to bridge formation during mitotic division and hence increased phenotypic aberration. It also plays important role in genetic sterility as shown in rice without changes in vigour [70]. Sodium azide and colchicine both are polyploidising and mutagenic agents [71] and have been used for a long time to produce polyploid plants. The mutagenic effects of sodium azide on plant morphology, chlorophyll, sterility and yield had been earlier confirmed by Ahoowalia [72] and Castro *et al.* [73] respectively.

Application of Sodium Azide and Crop Improvement: The presence of genetic variability is necessary for the crop improvement. The variability available to the

breeders comes from spontaneous or artificially induced mutations. Plant breeding involves procedures that increase genetic variation, select desirable genotypes, evaluate selected genotypes and finally multiply and release new cultivars. Mutation breeding generates a knowledge base that guides future users of mutation technology for crop improvement. In mutation breeding, the enhancement of the genetic variation is made through the influence of different mutagens. Despite the advantages and limitations of this method, it has been applied in the development of numerous improved cultivars and in different crops, such as wheat, rice, barley, soybean, lupines, vegetables, ornamentals, etc. Several traits have been subjected to mutation breeding such as yield, lodging resistance, disease resistance, maturity, culm length, etc. The artificial mutation is a practical mean to achieve genetic improvement in crop species and it is done with physical and/or chemical mutagens that enlarge the mutation frequency, when compared to the spontaneous occurrence. However, for extensive use of these mutants in plant breeding, high production efficiency is essential. This means that the utility of any mutagen depends not only on its effectiveness (mutation factor/dose) but also on its efficiency. The effectiveness of a mutagen has no practical implications since radiations and chemical mutagens are relatively inexpensive. On the other hand, lower levels of mutagens efficiency can limit their uses. Mutagenic efficiency is the production of desirable changes that are free from associations with undesirable genetic alterations. This is generally measured by the proportion of the mutation frequency in relation to damages associated to mutagenic treatments such as height reduction, chromosome breakages, sterility, lethality, etc. The use of mutagens in crop improvement helps to understand the mechanism of mutation induction and quantify the frequency as well as the pattern of changes in different selected plants by mutagens. The ability of these mutagens to enter the cell of living organisms to interact with the DNA produces the general toxic effects associated with their mutagenic properties. Thus, their effects are mainly due to the direct interaction between the mutagen and the DNA molecules.

A single diploid oat species, *Avena longiglumis*, has been identified that lacks triterpenoid saponin, avenacins. Significantly, *A. longiglumis* is susceptible to *G. graminis* var. *tritici*. However, genetic analysis of the association between avenacins and disease resistance has not been carried out because *A. longiglumis* does not hybridize readily with other avenacin-producing oat species. The avenacin-deficient mutants of the diploid oat species *Avena strigosa* has been developed to assess the contribution of the saponins to the resistance of oats to *G. graminis* var. *tritici* and other fungal pathogens. The seed of the diploid oat species *A. strigosa* was mutagenized with 10 mM sodium azide in 0.1M sodium phosphate buffer (pH 3.2) as described and M_2 seed from individual M_1 plants was kept separate to avoid the isolation of siblings [74]. The efficiency of the mutagenesis was assessed by monitoring the frequency of chlorophyll-deficient seedlings, which was 4.6% consistent with data previously reported for sodium azide mutagenesis of *A. strigosa* [74]. Amplified fragment length polymorphism (AFLP) analysis was used to confirm that the saponin-deficient mutants identified in this study originated from the seed used for the mutagenesis. For genetic analysis, F_1 progeny generated by crossing and F_2 plants generated by selfing were grown to maturity in the greenhouse. Sodium azide mutagenesis can not only generate diverse resistance but also provide an efficient method for breeding disease resistant varieties.

The genus *Striga* in the family Scrophulariaceae is composed of some 50 species, all of which are holoparasites of tropical cereals or legumes. *Striga hermonthica* (Del.) Benth and *Striga asiatica* (L.) Kuntze are the species that cause the most economically significant damage to cereals. The *Striga* seed germination and the production of germination chemical stimulant were adversely affected by the azide-based mutagenesis. On average, the mutagenesis reduced the release of chemical stimulants by the host plants to zero in some maize grains depending on the effect of the mutagen. Mutant lines of maize were created in the laboratory using NaN_3 and their performance in respect to the degree of *Striga* resistance screened [75]. These varieties could be used in breeding programs for the improvement of maize for resistant to *S. hermonthica*. Among them, the developed varieties, K9908, K9910 and K9911 showed a particularly high degree of resistance and their use as resistant gene source in maize improvement programs should be considered.

A stable pyridoxine-deficient pea mutant was obtained by screening the M_2 progeny of azide-treated *Pisum sativum* cv. Pusa Harbhajan [9]. The mutation was visible and lethal. The isolation of pyridoxine-deficient mutant demonstrates directly that pea plants synthesize their own pyridoxine and that pyridoxine is an essential growth factor for pea plants. The mutant character is

determined by homozygous recessive alleles, designated *pdx-1*, at a single locus. Pyridoxine-deficient plants are fertile and indistinguishable from the wild type if supplied exogenously with 2 mg of pyridoxine. In *Arachis hypogaea* L. induction of genetic variability was undertaken by treating seeds of TFDRG 5 with 200 and 300 Gy of gamma rays and with 1, 2 and 3 mM of sodium azide singly and in combination [75]. In newly formed leaves of disease lesion mimic mutant, catalase activity was reduced as compared to the parent. In large seed mutants, seed size ranged from 52 to 69 g/100 seeds as compared to 45 g in parent, with 15 to 53% superiority along with resistance to rust.

The various mutagens such as sodium azide, diethyl sulphate and ethyl methanesulphonate were used to evaluate their effectiveness in inducing mutations and also with the aim of producing variants tolerant to the fungus *Fusarium oxysporum* f. sp. cubense fungus which causes fusarial wilt or Panama disease in banana and plantain [76]. Based on phenotypic variations in regenerated plants, factors of effectiveness were calculated for each mutagen treatment. There were no significant differences between the numbers of variations induced by the mutagens and between different mutagen treatment durations. Regenerated plants were screened for tolerance to the fungus under greenhouse conditions. After twelve weeks of inoculation, 4.6, 1.9 and 6.1% of plants regenerated after sodium azide, diethyl sulphate and ethyl methane sulphonate mutagenesis respectively and had less than 10% vascular invasion of their corms with no external symptoms of the disease. These plants were considered tolerant and were multiplied, *ex vitro*, for field screening.

Mildew-resistant mutants were induced with sodium azide in three North American malting barley cultivars, two in the six-rowed Ursula (URS I and URS2), one in the six-rowed Gertrud (GER1) and one in the two-rowed Prudentia (PRU1) [77]. Two of the mutants, URS1 and PRU1, showed complete resistance and were shown to have two new alleles at the mlo locus; these were designated as mlo31 and mlo32 respectively. Mutant URS2, showing partial resistance, was inherited as a dominant gene, but was not an allele at the Mla locus. The mean yield of each mutant was higher than that of its parental line, but yield levels varied across environments, although this was independent of the severity of the mildew attack. Similarly, the yield parameters in *Arachis hypogaea* L. cv. 'SS1145B' and 'RMP 91' were investigated by treatment of sodium azide [79]. Azide treated *Rhizobium* increased the nodulation in lotus plants and mutants of *Rhizobium loti* strain NZP2037 were isolated [78]. AzR are the azide resistant mutant of *R. loti* strain and azi are mutation confirming azide resistance. Mutations conferring azide resistance (azi) appeared at a frequency of 0.5×10^{-7}. The AzR mutants of *R. loti* were characterized for their symbiotic behavior with *Lotus pedunculatus* plants. In comparison to the wild type parent strain, AzR mutants exhibited either similar or higher symbiotic effectiveness. The azi mutations, which enhanced nitrogen fixation as well as improving, shoot dry weight of the inoculated plants also increased nodulation.

Spathoglottis plicata Blume, a terrestrial orchid having prolonged inflorescence with beautiful lilac (pinkish purple) coloured flowers, is commercially important. Treatment of its seeds with sodium azide induced strikingly attractive flower colour modification exploiting seed culture protocols [80]. A treated plant with deep green leaves showed conspicuous variation from the mother strain by its charming milk-white flower color and green floral bracts in contrast to lilac colored flower and floral bracts in the latter. The mutant having completely different flower color modification with milky whiteness possesses special floricultural significance and may be treated as a new horticultural variety.

The two mungbean varieties, K-851 and Pusa Baisakhi were treated with sodium azide and the immediate effects of mutagenic treatments were measured in terms of biological damage caused in M1 generation [81]. All the mutagenic treatments brought reduction in seed germination, pollen fertility and survival at maturity. Such reduction, with an exception of survival, was found to be dose dependent. Significant shift in mean values for quantitative characters was observed in M_2 and M_3 generations and genetic parameters were recorded higher for all the treatments in both the generations. Two recessive auxin-resistant lines of rice (*Oryza sativa* L. ssp. indica cv. 'IR8'), arm1 and arm2, have been isolated by screening for resistance to 2, 4-dichlorophenoxyacetic acid (2, 4-D) with treatment of sodium azide [82]. The calli of *Saccharum officinarum* were either treated with different concentrations of sodium azide ranging from 1.0 to 5.0 mg/L or irradiated with 10, 20, 30, 40 and 50 Gy doses of γ-rays for induction of mutation [83]. Partially purified toxin of *Colletotrichum falcatum* ranging from 0.05 to 0.5% was added in callus regenerating medium and minimum plants were regenerated at 0.5% toxin with maximum callus death. These plants regenerated from callus, which was insensitive to red rot toxin were supposed to be disease resistant against red rot.

Table 1 Various mutant crops produced by sodium azide (NaN3) treatment

Name of plant species	Traits	Authors
Zea mays (maize)	Resistant against pathogen Striga	Kiruki *et al*., 2006
Pisum sativum (pea)	Pyridoxin deficient	Kumar 1988
Hordeum vulgare (barley)	Chlorophyll mutant	Prina and Fevret 1983
Musa spp. AAA	Resistant against Fusarium oxysporum f. sp. cubense	Bhagwat and Duncane 1988
Hordeum vulgare (barley)	Mildew resistant	Molina-Cano *et al*., 2003
Avena strigosa (oat)	Disease resistant	Papadopoulou *et al*., 1999
Arachis hypogaea L. (groundnut)	Yield traits	Menash and Obadoni 2007
Vigna radiate L. (mungbean)	Quantitative traits	Samiullah *et al*., 2004
Saccharum officinarum (sugarcane)	Red rot (Colletotricum falcatum) resistant	Ali *et al*., 2007
Araches hypogea (groundnut)	Disease resistant	Mondal *et al*., 2007
Lagerstroemia indica (crape myrtle)	Resistant to powdery mildew and leathery foliage	White and Carl 2004
Spathoglottis plicata Blume	Improved floricultural significance	Roy and Biswas 2005
Oryza sativa L. (rice)	Auxin resistant mutant	Chhun *et al*., 2003
Lactuca sativa (lettuce)	Down mildew resistant	Okubara *et al*., 1994
Hordeum vulgare (barley)	Anthocyanins and proanthocynidins deficient	Olsen *et al*., 1993
Oryza sativa L. (rice)	Enhanced amylase content	Suzuki *et al*., 2008
Halianthusannuus (sunflower)	Enhanced stearic acid content	Scoric *et al*., 2008

The *Halianthus annus* (sunflower) is one of the four most important oilseed crops in the world and the nutritional quality of its edible oil ranks among the best vegetable oils in cultivation. Typically up to 90% of the fatty acids in conventional sunflower oil are unsaturated, namely oleic (C 18:1, 16%-19%) and linoleic (C 18:2, 68%-72%) fatty acids. Palmitic (C 16:0, 6%), stearic (C 18:0, 5%) and minor amounts of myristic (C 14:0), myristoleic (C 14:1), palmitoleic (C 16:1), arachidic (C 20:0), behenic (C 22:0) and other fatty acids account for the remaining 10%. Advances in modern genetics, most importantly induced mutations, have altered the fatty acid composition of sunflower oil to a significant extent. The stearic acid was increased in sunflower up to 35 % by using sodium azide mutagen [84]. Sodium azide has been used in various crops to develop resistance against harmful pathogens and to improve their yield and quality traits (Table 1).

Detection Methods for Mutation Caused by Sodium Azide: Traditional methods for mutant plant selection based on morphological and biochemical markers, but these markers are less reproducible due to influenced by environmental conditions. The mutation detection based on polymerase chain reaction (PCR) and non PCR techniques are reliable and reproducible and have been used in various mutant crops for screening. The development of PCR has revolutionized the methods available for the detection of mutations at the molecular level. The simplest use of the PCR in mutation analysis determines the presence or absence of a particular region of DNA. Multiplex PCR used to extend the single PCR and can be amplified several DNA regions simultaneously. Identifying these mutations require novel detection methods. Intensive research over the years has led to several new detection techniques and some reproducible techniques are given here for detection of mutation caused by sodium azide, which are reproducible, inexpensive and easy to execute in a laboratory.

Amplified Fragment Length Polymorphism: It has quickly become one of the widely used methods of DNA fingerprinting for crops and wild plant species. This technique differentiates the normal and mutant plants easily based on modified restriction endonucleases sites caused by sodium azide. Amplified fragment length polymorphism (AFLP) analysis was used to confirm the saponin-deficient mutants produced by sodium azide treated seeds [85].

Allele Specific Oligonucleotides: It has been applied to detection of specific point mutations. The method is based on the differences in the melting temperature of short DNA fragments differing by a single nucleotide. This method can be used to detect one or more known specific mutations in the population caused by sodium azide. This is a simpler and less expensive approach than searching for any new mutation in the population.

Protein Truncation Test (PTT): This method detects mutations arising from termination of mRNA translation process. Because of this, the protein product is truncated in mutant plants. New mutant alleles were identified at four agronomic important genes (HvCO1, Rpg1, eIF4E and NR) of barley caused by sodium azide. All mutations except one were G/C to A/T transitions and several (approximately 68%) implied a change in protein amino acid sequence and therefore a possible effect on phenotype [86].

Southern Hybridization: This method readily detects large gene alterations such as deletions, insertions and rearrangements. In rare instances, a point mutation (caused by sodium azide that changes the cleavage site of enzyme) can also be detected if the mutation happens to lie within a restriction endonuclease recognition sequence.

Enzymatic Cleavage of Nucleic Acid Heteroduplexes: If a mutant mRNA molecule is produced by mutant plant produced by sodium azide, then it is possible to detect some point mutations by means of the RNase A cleavage method. It is a simple and inexpensive method for researcher to detect mutation.

Chemical Cleavage of Nucleic Acid Heteroduplexes: This method is based on cleavage of nucleic acid heteroduplexes (mutation bearing) by hydroxylamine and osmium tetraoxide (HOT). Hybrid duplexes are formed from wild-type and mutant DNAs (caused by sodium azide) and treated such that cleavage occurs if the appropriate nucleotide is mismatched. Since the two chemicals detect mismatched C or T nucleotides and because the original DNA duplex can be labeled on either strand, there is the potential to detect all point mutations caused by sodium azide by this method.

Chemical Modification of Nucleic Acid Heteroduplexes: This method is used to detect mismatches in DNA heteroduplexes (having mutation) sized up to several kilobases by the binding of l-cyclohexyl-3-[2-(4-morpholinyl)-ethyl] carboiimide (CMC) to the point of mismatch. The complex is then treated with a second anti-CMC antibody conjugated to an electron-dense marker and visualized by electron microscopy in order to estimate the approximate location of the mutation. This technique is very simple and easy to perform and can be applied for detection of mutation caused by sodium azide.

Altered Electrophoretic Mobility of Heteroduplexes: The method of denaturing gradient gel electrophoresis (DGGE) relies on differences in behavior of heteroduplexes (having point mutation in a gene) compared with homoduplexes (no mutation in a gene). In the natural form, a duplex of complementary DNA strands exists as a tightly associated double helix. As the environment becomes more denaturing (e.g. in the presence of formamide or urea), the two strands begin to dissociate (melt) starting with the region (s) most loosely held together. If a DNA duplex is being subjected to electrophoresis in a gradient of denaturant, at the point where the strands begin to separate, the rate of migration is abruptly reduced because of the more relaxed configuration of the duplex. If that same fragment of DNA is instead a heteroduplex (bearing point mutation caused by sodium azide) of almost complementary strands with a mismatch in a region of the molecule that denatures early, the electrophoresis profile will be altered. Thus, the mutant plant produced by sodium azide can be differentiated from wild plants based on altered electrophoretic mobility.

Single Strand Conformational Polymorphism and Enzymatic Cleavage: This method can detect single base pair mutations like frameshift mutations, nonsense mutations and missense mutations. The basic principle of this test is that double stranded DNA when denatured, assumes a special conformation. This conformation is unique and depends on primary nucleotide sequence. This method is quite sensitive to detect even single nucleotide difference that occupies a different conformation and when subjected to electrophoresis, the variant nucleotide occupies a different position. When mixmatched heteroduplex is restricted by CEL I nuclease enzyme and resulting products are electrophoresed to see the mutation. The two mutant rice populations were produced by treatment with chemical mutagen ethyl methanesulphonate (EMS) and the other with a combination of sodium azide plus methyl-nitrosourea (Az-MNU) [87]. Heteroduplexes were formed through denaturation and annealing of PCR products, mismatches digested with a crude preparation of CEL I nuclease and cleaved fragments visualized using denaturing polyacrylamide gel electrophoresis to see the mutation.

Nucleotide Sequencing: In this method, the complete gene is sequenced to identify the presence of any mutation caused by mutagen. Till date, complete gene

sequencing is considered as a gold standard method because, any type of mutation can be identified. The mutant plants of barley produced by sodium azide were characterized in (ant 18 gene) for anthocyanins and proanthocyanidins deficiency by sequencing [88]. This gene produces dihydroflavonol 4-reductase in mutant plants. Sodium azide generated 21 base substitutions, which corresponds to 0.17 % of the 12, 704 nucleotides sequenced. Of the substitutions, 86% were nucleotide transitions and 14 % were transversions. A.T-->G.C base pair transitions were about 3 times more frequent than G.C-->A.T transitions. No deletions or hot spots mutation were found. The absence of dihydroflavonol 4-reductase activity in ant18-159, ant18-162 and ant18-164 mutant plants is caused by missense mutations in the respective genes. A rice mutant with enhanced amylose content in endosperm without affecting amylopectin structure was developed by treatment with sodium azide and mutant was screened by amplifying the Wx gene and its sequencing [89]. Sequencing is done by two methods:

Sanger's Dideoxy Nucleotide Method: Dideoxynucleotide sequencing represents only one method of sequencing DNA. This technique utilizes 2', 3'-dideoxynucleotide triphosphates (ddNTPs), molecules that differ from deoxynucleotides by having a hydrogen atom attached to the 3' carbon rather than an OH group. These molecules terminate DNA chain elongation because they cannot form a phosphodiester bond with the next deoxynucleotide.

Shotgun Method: DNA can be sequenced by a chemical procedure that breaks a terminally labelled DNA molecule partially at each repetition of a base. The lengths of the labelled fragments then identify the positions of that base.

DNA Microarray Technology: To date, the main applications of microarrays are in comprehensive, simultaneous gene expression monitoring and in DNA variation analyses for the identification and genotyping of mutations and polymorphisms. In plant research, effective insertional mutagenesis and transgenic methods are limited to relatively few species or are inefficient. Fortunately, single-nucleotide changes can be induced in any plant by using traditional chemical mutagens (for example, sodium azide) and progress has been made in efficiently detecting changes. Because base substitutions in proteins, provide allelic series and not just knockouts, this strategy can yield refined insights into protein function.

Future Prospects: Development of pathogen resistant crops is necessary to reduce the environmental pollution caused by pesticides. The conventional breeding method takes several years to develop new cultivars from wild species. Chemical mutagens are important tools and being highly used in crops to improve their quality and yield traits. These mutagens are easy to apply on crops and inexpensive to develop resistant varieties. The sodium azide, a potent mutagen in plants and animals and create point mutation easily. The mutant plant species can be easily selected from wild by PCR and non PCR based techniques. These markers are reproducible and consistent as compared to morphological and biochemical markers. The mutant plants produced by sodium azide are capable to tolerate various biotic stress conditions and avoid application of hazardous pesticides against harmful pathogens. Therefore, it should be apply on various crops, which are susceptible to harmful pathogens and create resistance to them against these pathogens.

CONCLUSION

Current protective measures rely heavily on chemical control measures for pathogen vectors, which have undesirable environmental consequences. Attack by pathogens, such as viruses, bacteria, fungi, nematodes and insects and is a severe economic problem, which influences all economically important crops. A more effective approach to protecting plants from pathogen attack is to create plants that are endogenously resistant to pathogens. However, plant breeders have limited sources of resistance genes against plant diseases. This can now be achieved using mutation tools, by providing the plant with genetic information required for affecting the pathogens and for being resistant to the disease caused by the pathogen. The application of sodium azide on crop is easy and inexpensive and creates mutation to them to improve their traits. The efficiency of mutant production depends on the conditions such as pH, soaking into water, azide solution, temperature, concentration of azide and treatment duration. The mutagenic effects of sodium azide appear after sowing the seeds observed by naked eyes. However, sodium azide has been being used in various crops to improve their yield and quality traits and create resistance to them against harmful pathogens. It creates point mutation, damages the chromosomes and thus produces tolerance in the plants for numerous adverse conditions. DNA based molecular markers proved the potent markers for mutant selection and highly used in crops. These markers

are reproducible and reliable for any crops used in the study. There are quite a few techniques capable of recognizing the presence of single base changes caused by sodium azide in plant genes. The PCR and non PCR based mutation detection techniques viz., AFLP, ASO, DGGE, SSCP, sequecncing and RNase treatment of mismatch in RNA-RNA or RNA-DNA heteroduplex are proved for point mutation detection. The point mutation caused by sodium azide in any plant species can be detected by these PCR and non PCR based techniques. Therefore, sodium azide (NaN_3) should be used to create mutation in those crops which are highly susceptible for harmful pathogens and made them economically inexpensive and beneficial for farmers.

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