

Application of sodium azide for chemical induced mutagenesis of proso millet culture (*Panicum miliaceum* L.)

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Abstract

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Present study aimed to identify the effect of different concentrations of sodium azide (NaN_3), depending on seed exposure time on various economically valuable traits of millet plants in M_1 generation and establish the effectiveness of inducing beneficial mutations. From the experiments, it is concluded that the optimal concentration of sodium azide for millet mutagenesis in laboratory conditions is 1% at 4 hours of exposure, while the germination of seeds and the length of seedlings remain at the level of control. In the field in the nursery of the first generation (M_1) mutants, sodium azide reduced the germination of seeds and plants survival, whereas an increase in the mutagen concentration increased the inhibitory effect on seeds and the development of plants. It was noted that the concentration of the mutagen does not significantly affect the period of vegetation, which in all variants was at the control level. Least conservancy of the indicator was observed, when treated with 0.5% concentration of the mutagen. A decrease in vegetation period by 3–6 days was recorded as the time of treatment with a mutagen increased with an exposure of 8 and 12 hours, compared to an exposure of 4 hours, the longer the time the seeds were exposed to the mutagen, the lower the indicator of the vegetation durability. A higher frequency and a wide range of changed forms were registered at 0.1% concentration of mutagen at 12 hours of exposure and varied from 3.2% to 6.3%. The identified promising mutant forms of millet with a complex of economically useful traits are of practical value and can be used in further breeding process of this crop.

Keywords: proso millet; chemically induced mutagenesis; sodium azide; M_1 generation.

Introduction

Millet (*Panicum miliaceum* L.), is a valuable cereal and fodder crop on a global scale. Currently, it is grown mainly in East and Central Asia and to a lesser extent in Eastern Europe, Western Asia, Pakistan, and India. It is an important food item in semi-arid areas, where it is almost impossible to grow grain crops. Millet is a potentially valuable fast maturing crop for drier regions (Kate et al., 2018). Its

cultivation started 10,000 years ago in northern China (Luet al., 2009). The crop was first introduced to Canada, in the 17th century (Baltensperger et al., 1996). For many decades, breeders have created new varieties using chemical mutagens (Dubey et al., 2017). Most millet cultivars have been developed using classical breeding methods. Mutagenesis occupies one of the leading places among those techniques, that can be successfully used to create genetic diversity and new varieties. Chemically induced mutagenesis facilitates

the creation of valuable source material in a short time with a variety of morphological and physiological characteristics, biochemical parameters, increasing the frequency and expanding the range of original mutations (Krotova, 2015; Khan et al., 2009; Bahadur et al., 2015).

The role of mutation in increasing genetic variability and the possibility of selection for valuable traits, such as yield, early maturity, the number of grains per panicle and plant, resistance to biotic and abiotic stresses, and grain quality, have been studied with various crops. Mutants are often of excellent breeding value since they may have new, previously unknown beneficial traits (Aviya and Mullainathan, 2018; Khan et al., 2010; Mullainathan et al., 2012). In addition, with the help of mutagenesis, it is possible to overcome the technical difficulties that arise, when crossing small-flowered crops, such as millet (Yashovskii, 1960). One of the most widely used mutagens in plant breeding is sodium azide (NaN_3), a relatively safe, inexpensive, non-carcinogenic, and very effective chemical mutagen (Salvi et al., 2014).

The effect of this substance through mutational influence on the genetic apparatus can contribute to increasing the resistance of plants to adverse environmental factors and pathogens, which leads to an increase in their yield and improvement of quantitative characteristics (plant weight, number of fruits, etc.). Sodium azide, like other chemical mutagens, causes a much higher frequency of mutations in plants, compared with the level of random mutations under normal conditions, which occur quite rarely (Tillet et al., 2007). The prospects of using sodium azide were shown not only in plant breeding, but also in fundamental research of such important agricultural plants as corn (Eze et al., 2015), rice (Herwibawa et al., 2017), wheat (Srivastava et al., 2011), chickpeas (Kulthe et al., 2011), fenugreek (Sidduqui et al., 2007), sunflower (Elfeky et al., 2014), rapeseed (Hussain et al., 2017), tomato (El-Kaabyet et al., 2015), etc.

When studying the effect of one type of mutagen on the growth and development of plants of any agricultural crop, the dose, and duration of treatment exposure are of paramount importance. In addition, when using mutagens in breeding, it is also necessary to consider that different families, genera, species, and individual varieties of the same species exhibit clearly expressed unequal sensitivity to the types of mutagenic factors acting and to their doses. It is manifested with varying degrees of survival, unequal frequency, and the difference in spectra of induced mutations (Auerbakh, 1978; Zoz, 1968; Rapoport, 1978; Strelchuk, 1981).

It has been established that as the concentration of the mutagen increases to a certain level, the frequency of viable mutations also increases, then drops, and the changes resulting from treatment with mutagens over the optimal rate

cause the death of plants. Therefore, applying high concentrations of mutagens is not advisable in the breeding process. Contrary, the concentrations of mutagens should not be too low; otherwise, the effect of the mutagen will be ineffective. In this regard, when creating the initial breeding material for a particular agricultural crop using induced mutagenesis, it is advisable to specify the concentrations of mutagens for each specific variety, based on preliminary studies (Rapoport, 1980). Methods and approaches for chemically induced mutagenesis using sodium azide of many species of cultivated plants, have been developed. However, for millet, such works have not been sufficiently studied and have not been practically used in Kazakhstan (Rysbekova et al., 2020). Therefore, the current study aimed to identify the effect of different concentrations of sodium azide depending on seed exposure time on various economically valuable traits of millet plants in M_1 generation and establish the effectiveness of inducing beneficial mutations.

Materials and Methods

Plant material

In the current work, varieties of millet (*Panicum miliaceum* L.) not previously involved in the research were used: Pavlodarskoe 4 (Kazakhstan), K-10275-Kvartet (Russia), PI289324 (Hungary).

Treatment of millet seeds with sodium azide.

Treatment with a chemical mutagen was performed under laboratory conditions with original seeds, according to the procedure reported by Esson et al. (2018). The initial stage of the research consisted of selecting mutagenic factors and their effective doses to establish the mutability of genotypically heterogeneous varieties. Before the treatment, 150 millet seeds were first immersed in a 12% hydrogen peroxide solution for 15 minutes to destroy harmful microflora on the grains and then washed twice with distilled water. The seeds were pre-soaked in distilled water for 4 hours before mutagenic treatment allowing the mutagen to diffuse faster into the tissues of interest (Rajani et al., 2011). The scheme of the experiment included the treatment of seeds of millet samples with mutagen NaN_3 in concentration 0.1%; 0.2%; 0.3%; 0.4%; 0.5%, with 4, 8 and 12 hours of exposure. Sodium azide was previously dissolved to the desired concentration in distilled water to obtain an aqueous solution. After the treatment, the seeds were washed for 1 hour under running tap water and placed at a temperature of 27°C for 7 days in a thermostat. In each variant, 50 seeds were processed for each sample in triplicate. The control variant was germinated in distilled water. On day 7, the indicators, such as seed ger-

mination, coleoptile length, and roots were determined. The total percentage of seed germination was calculated depending on the concentrations of sodium azide used for treatment, and the percentage of seed germination in relation to the control was determined.

For field assessment of the mutagen effect on the plants, the seeds of the studied genotypes were treated with NaN_3 , according to the procedure described. The sowing of the nursery of M_1 mutants was carried out manually by 250 pieces of seeds, treated with chemical mutagens per 1 linear meter according to the experimental scheme in three repetitions. Seeds of initial samples treated in distilled water served as control. The experiment was performed according to the All-Russian Institute of Plant Growing guidelines and the Field Experiment Methodology (Agafonov et al., 1988; Dospikhov, 2011). During the growing season in the first mutant generation (M_1), seed germination was considered, phenological observations of plant growth and development were conducted, and the plants that survived to harvest were counted. Its onset in 10% of plants in the entire plot was taken as the date of the beginning of the phase, and the full phase was noted when it occurred in at least 75% of plants. Morphological and physiological deviations from control were noted.

Results and discussion

Influence of NaN_3 on the spectrum of changes in morphological traits of millet seedlings.

Model experiments were carried out to study the effect of various concentrations on the spectrum of morpho-physiological changes at the stage of germination of seeds of three genotypes differing in ecological and geographical origin. The experiment started with laboratory studies on the effect of the mutagen. With an exposure of 4 hours, seeds treated with sodium azide at concentrations of 0.1 to 0.3% sprouted on days 4-5, and seeds, treated with 0.4 and 0.5% concentrations of sodium azide on day 6. The appearance of millet

seedlings in the control variant of plants was observed on the second day in all three-time exposures. The studies revealed significant morphometric parameter differences depending on sodium azide concentration. The obtained data on seed germination percentage showed that the best germination is found at the lowest concentration of sodium azide at an exposure time of 4 and 8 hours (Figure 1).

For seeds, treated with a 0.5% sodium azide concentration, the germination percentage noticeably decreases – up to 24.4% at 4 hours of exposure time, 20.9% at 8 hours, and 8.5% at 12 hours, respectively. The average laboratory germination of the studied millet seeds at 4 hours of exposure time in the control variant was 91.2%, while in the experimental variants: 0.1% – 92.8; 0.2% – 84.8; 0.3% – 72.1; 0.4% – 56.6; 0.5% – 30.9; at 8 h exposure time at 0% – 83.1; 0.1% – 82.8; 0.2% – 74.5; 0.3% – 58.8; 0.4% – 47.4; 0.5% – 27.3; at 12 hours exposure time 0% – 86.7; 0.1% – 68.3; 0.2% – 44.8; 0.3% – 24.8; 0.4% – 16.2; 0.5% – 10.1. The results show that NaN_3 differently affected germination; the best seed germination is found at the lowest concentration at 4 and 8 hours of exposure time. Seed exposure at 12 hours at concentrations of 0.4% and 0.5% inhibited seed germination by up to 85 and 90% in all genotypes, respectively.

Determination of the morphometric parameters of seedlings after treatment with sodium azide is of interest since identifying any changes may indicate the influence of a mutagen. Estimation of the indicator coleoptile length showed that in the treatments of 4 and 8 hours, with a concentration of 0.1%, the average value remained similar to the control, ranging from 5.2 to 6.2 cm. In other concentrations, the dynamics of seedling growth goes to a sharp decrease, for example, at 0.2% – 3.8 cm, 0.3% – 3.4 cm, 0.4% – 2.63 cm, 0.5% – 2.42 cm. Particularly strong inhibition of coleoptile growth was noted from 0.3% to 0.5% at exposure times of 8 and 12 hours (Figure 2)

The same tendency was observed when measuring the length of the roots. The roots in the experimental variants at 4 hours of exposure time were, on average, five times lower

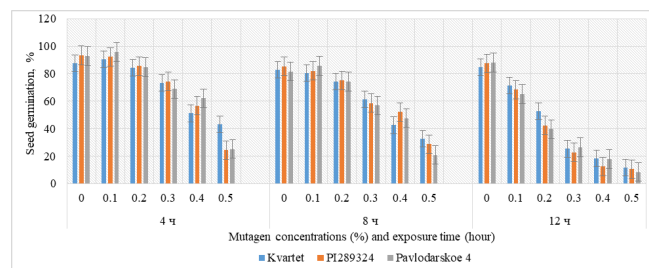


Fig. 1. Influence of NaN_3 on the biometric parameters of millet samples at the stage of seed germination

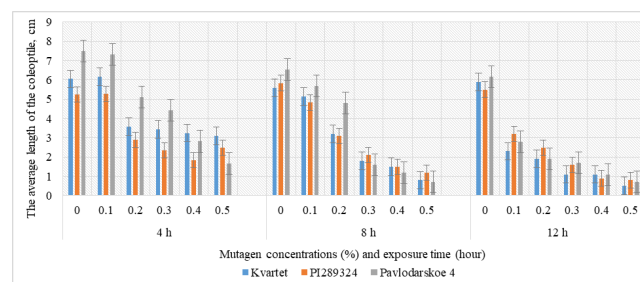


Fig. 2. Influence of NaN_3 on the coleoptile length of millet accessions at the stage of seed germination

than the control at concentrations of 0.2 and 0.3% and 7-8 times lower at concentrations of 0.4% and 0.5% (Figure 3).

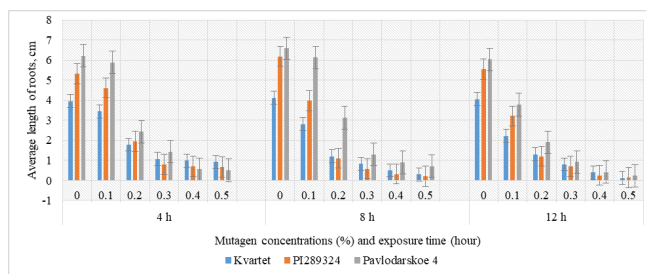


Fig. 3. Influence of NaN_3 on the root length of millet samples at the stage of seed germination

NaN_3 at a concentration of 0.3-0.5% had a solid mutagenic effect on the growth of roots. In all exposures of the processing time, this indicator decreased on average to 90% compared to the control. The effectiveness of the mutagen at high concentrations has been shown in similar studies with chickpea (Wani, 2009) and sunflower (Kumar and Ratnam, 2010) plants. Growth parameters of plants decreased with increasing concentration, and seed germination and plant survival decreased in oil flax plants (Tigova and Soroka, 2018).

High concentrations of sodium azide harmed seed germination. Other studies (Esson, 2016) explain that this is due to the toxic effect of sodium azide. A decreased seed germination under the mutagen action is also demonstrated by a delay or inhibition of physiological processes in the cell, a violation of the synthesis of phytohormones, and inhibition of the cell cycle (Dyulgerova B., 2012). Thus, it was found that the germination of seeds of millet genotypes largely depends on the mutagen concentration and exposure time. Treatment with sodium azide at a concentration of 0.1% sodium azide solution had a stimulating effect on seed germination in laboratory conditions. Seeds, treated with various concentrations of NaN_3 at doses of 0.3%, 0.4%, and 0.5% at time intervals of 8 and 12 hours, showed a strong mutagenic effect on the length of the coleoptile and root. Based on the obtained results, we assumed that the optimal concentration of sodium azide treatment for millet mutagenesis in laboratory conditions could be 0.1% at 4 hours of exposure since the germination of seeds and the length of seedlings remains at the level of the control.

Field evaluation of the effect of NaN_3 on the growth and development of millet plants.

We conducted field experiments to fully assess the effect of various concentrations and exposures of sodium azide on the growth and development of millet genotypes. The subsequent analysis of plants shows that in the M_1 generation,

the mutagen concentrations from 0.1% to 0.5% significantly affected the severity of such traits as field germination, survival, vegetation period of plants, and a number of morphological characteristics. The studies revealed significant differences in seed germination depending on the concentration of sodium azide and exposure time (Figure 4).

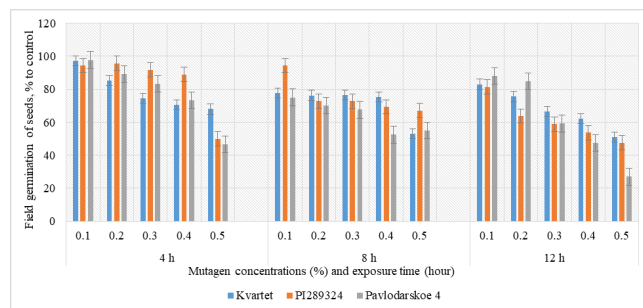


Fig. 4. Influence of the mutagen on seed germination in the field

For instance, 0.1% treatment with a mutagen at an exposure of 4 hours did not affect field germination. This indicator in all samples was at the control level. Increasing the concentration to 0.5% at 4 and 8 hours of exposure decreased seed germination by 1.5-2 times. In the treatments with mutagen concentrations of 0.2-0.3% and 4 and 8 hours of exposure, the average seed germination decreased to 30% for all studied samples. At 12 hours of mutagen exposure, seed germination was from 60 to 75% at concentrations of 0.2-0.3%, whereas at 0.4-0.5% concentrations, seed germination was 2-3 times less than control.

The vegetation period of plants is one of the main indicators for evaluating the action of mutagens. Therefore, phase fixation was systematically carried out during the growing season. Evaluation of the growing season length of millet showed that variety accession PI289324 had a shorter growing season. When the vegetation phases were fixed, this sample showed more intensive maturation of panicles and plants' green mass than Kvartet and Pavlodarskoe 4. It was noted that, depending on the concentration of the mutagen, the growing season of plants did not differ significantly. In all variants, it was at the control level. A shortening of the growing season with 3-6 days was recorded in the samples with exposure of 8 and 12 hours compared to 4 hours (Figure 5).

As shown in Figure 5, at 8 and 12 hours of exposure time, there is a reduction in the growing season, compared with an exposure of 4 hours. Decreasing the vegetation period is of significant breeding importance when creating early maturing forms of millet.

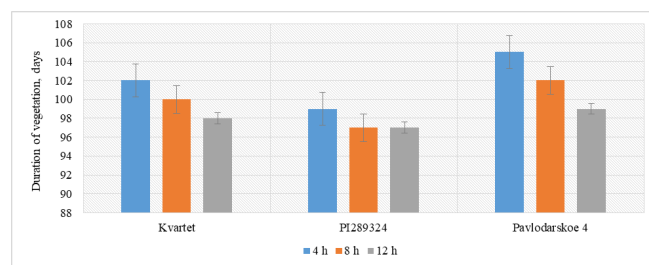


Fig. 5. Influence of a mutagen on the average duration of plant vegetation

During the vegetation period, millet plants treated with sodium azide showed higher resistance to adverse environmental factors, which was reflected in plant survival rates. In all genotypes in all exposures 4, 8, and 12 hours after seed treatment with a mutagen concentration of 0.1 and 0.2%, plant death was insignificant and varied within 5-10% concerning seedlings (Figure 6).

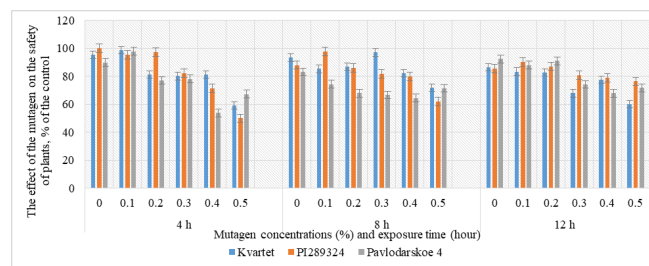


Fig. 6. Influence of different concentrations and exposures in seed treatment with NaN_3 on plant survival

The safety of millet plants in the experiment varied depending on the concentration of the mutagen, the time of exposure, and the treated variety, and it was within the range of 50-100% compared to the control. The lowest safety was established in the variants, where the seeds were treated with a 0.5% mutagen concentration in all three aging exposures. Thus, the obtained data indicate that higher concentrations of the mutagen significantly reduce the safety of plants.

An essential criterion for the effectiveness of mutagens is the change in height and other morphometric parameters of plants in the first mutant generation. The performed subsequent structural analysis of plants shows that an increase in the concentration of a chemical mutagen and different exposure times did not significantly affect plant height (Figure 7) and panicle length (Figure 8).

According to the obtained data, no significant differences in plant and panicle height, depending on the concentration were found in M_1 plants. A slight difference was observed in the Kwartet variety at concentrations of

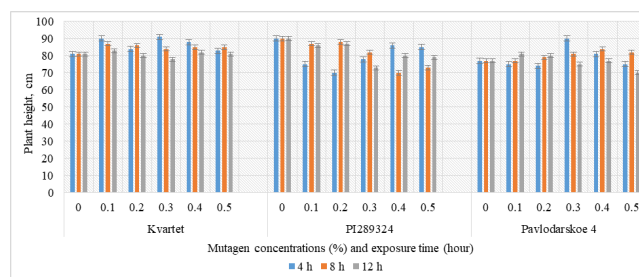


Fig. 7. Influence of different concentrations and different exposures of NaN_3 seed treatment on plant height in M_1 generation

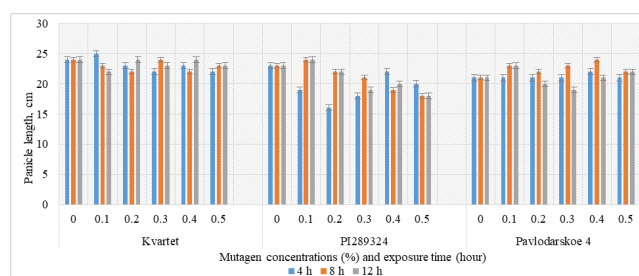


Fig. 8. Influence of different concentrations and different exposures of NaN_3 seed treatment on panicle length in M_1 generation

0.1%-0.5%, at 4 and 8 hours of exposure, compared to the control. For example, at 0.1% concentration, plant height was 90 cm, at 0.2% – 84 cm, 0.3% – 91 cm, 0.4% – 88 cm, 0.5% – 83 cm, which is 9 cm, 3 cm, 10 cm, 7 cm and 2 cm superior to controls, respectively. At 8 hours of exposure time, the average height of plants was 0.1% – 87 cm, 0.2% – 86 cm, 0.3% – 84 cm, 0.4 and 0.5% – 85 cm. In the variety PI289324, the average index was lower than the control in all concentrations and exposure time. In the zoned variety Pavlodarskoe 4, the exception was concentrations of 0.4 and 0.5% at 4 and 8h exposure time. For instance, in the control variant, the average plant height was 77 cm, whereas in the mutagen concentration 0.4% with 4h exposure – 90 cm; 8h – 81 cm, at 0.5% at 4h – 81 cm, 0.5% – 84 cm. The panicle length in the Kwartet variety did not differ from the control in all variants, ranging from 22 to 24 cm, in the PI289324 genotype from 16 to 23 cm, and in the Pavlodarskoe 4 variety from 19 to 23 cm.

In terms of the weight of 1000 seeds, there is a slight decrease in the concentration of 0.5% at 12 hours of exposure in varieties PI289324 and Pavlodarskoe 4, and in the variety Kwartet at 4 hours of exposure of 0.1-0.3%, a decrease in the weight of 1000 seeds from the control was observed (Figure 9).

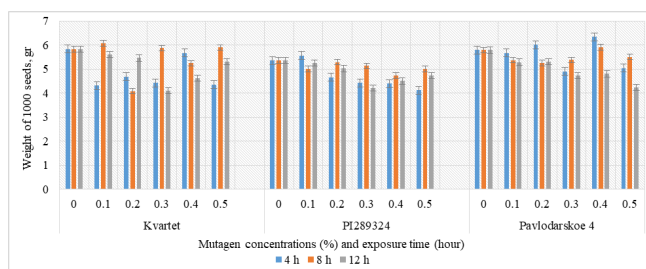


Fig. 9. Influence of different concentrations and different exposures of NaN_3 seed treatment on the mass of 1000 seeds in M_1 generation

In barley, under the influence of sodium azide, such traits as plant height, ear length, frost resistance, and grain weight changed positively (Dyulgerova B., 2012). In our experiments, the alignment in growth parameters in most plants treated with various concentrations of sodium azide is possibly associated with successful germination and the ability for further plant growth, and this did not significantly depend on the concentration of the mutagen.

In plants, induced with chemical mutagen, in accordance with the analysis of the crop structure in general, it can be noted that the variants with the use of sodium azide had a higher yield than the control (Figure 10).

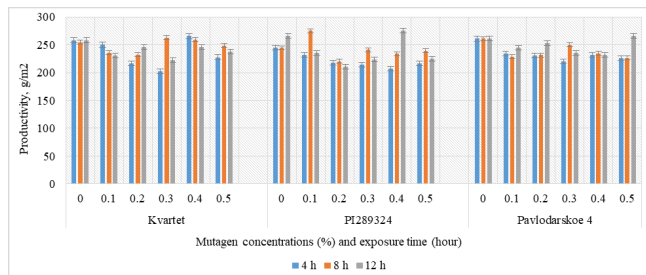


Fig. 10. Influence of different concentrations and different exposures of NaN_3 seed treatment on plant yield in M_1 generations

So, with seed exposures of 4 hours, the highest yield is noticeable in the variant with a concentration of 0.4% – 257.4 g / m², due to the weight of 1000 seeds of 5.67 grams and productive bushiness of 4.04. At exposures of 8 hours, the highest yield is noticeable at concentrations of 0.1% and amounted to 285.3 g/m², and at 12 hours with a concentration of 0.4% – 275.1 g/m².

A correlation analysis was carried out to identify a significant correlation between quantitative traits, depending on the concentration of the mutagen and exposure time. As a result of the analysis of the data obtained, a non di-

rect correlation was revealed between field germination and sodium azide concentration ($r = -0.9231$) (Figure 11), the duration of the growing period and exposure time ($r = -0.7496$) (Figure 12).

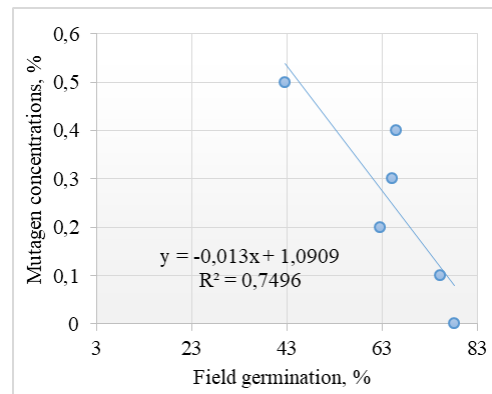


Fig. 11. Correlation between mutagen concentration and field germination of M_1 plants

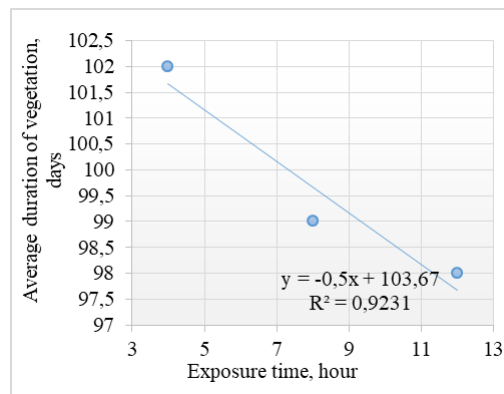


Fig. 12. Correlation between the vegetation period M_1 of plants and the exposure time of exposure to the mutagen

Thus, it was established a non direct correlation dependence of field germination on the mutagen concentration, as well as the duration of the vegetation period on the exposure time of the mutagen, while the correlation coefficient for these traits was $r = -0.7496$ and $r = -0.9231$, respectively. It was noted that the longer the exposure time of the seeds with the mutagen, the lower the indicator of the vegetation period.

Leaf color mutations are one of the most frequently observed mutations in both spontaneous and induced mutant plant populations, and they are often used as an indicator of mutagenic effects and the effectiveness of various mutagens. Chlorophyll mutations are one of the most reliable indicators for assessing the genetic impact of mutagenic influences (Bind et al., 2016; Wani et al., 2011).

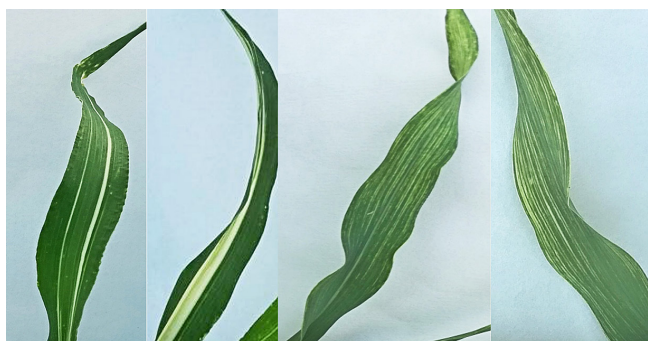


Fig. 13. The appearance of mutant plants in the PI289324 genotype with stripes on the leaves (variegation) under the influence of NaN_3

The results of the morphological analysis of M_1 plants show that sodium azide induced the appearance of changes in the tubing phase, the so-called sign of millet leaf striping (zebra type of mutation) (Figure 13).

In the PI289324 genotype, sodium azide caused changes with a frequency of 2.3% at a concentration of 0.3% and 12 hours of exposure. This type of zebra mutation is associated with mutations in chloroplasts that disrupt the synthesis of chlorophyll and belongs to a special category of plant chimeric. In the first generation, after mutagenic effects, barley plants with stripes on leaves, stems, and other organs with signs of chlorophyll insufficiency appear with a fairly high frequency (Sharma, 1970). Mutants with complete or partial deficiency of chlorophyll were also found in rice, which is usually determined by recessive genes: al (albina), y (yellow

seedling), lu (lutescent), v (virescent), fs, and z (leaf striping (zebra) or variegation) (Reddi and Suneetha, 1971).

During the study, morphological variations (plant heights, growth patterns, leaves, panicles, seeds) were analyzed, and the frequency of morphological mutants with altered traits in the M_1 generation was determined (Table 1).

According to the data obtained, it was revealed by the spectrum of mutations that sodium azide was effective in inducing mutations. A higher frequency and a wide range of altered forms were registered at 0.1% mutagen at 12 hours of exposure and varied from 3.2% to 6.3%.

Conclusion

The morphometric analysis of plants performed under laboratory conditions shows that an increase in the concentration of a chemical mutagen has a negative effect on growth parameters, which indicates the undesirability of using high concentrations of a mutagen for millet breeding. Our observations show that sodium azide in the nursery of the first generation (M_1) mutants reduced field germination, survival, and preservation of plants. It was found that the mutagen's negative effect on plants' survival is directly proportional to its concentration and time of exposure. It was noted that the longer the time of seed exposure to a mutagen, the lower the indicator of the vegetation period.

New traits obtained by treatment with sodium azide are important, both for fundamental and applied research. The created genetic diversity is a basis for further breeding experiments. Isolated mutant genotypes can be used to develop new varieties of millet with various economically valuable traits.

Table 1. Frequency of change (%) of morphological traits of plants in M_1 generation

Exposure time, hour	Concentrations of NaN_3 , %	Proso millet varieties		
		Pavlodarskoe 4	Kvartet	PI289324
4	0,1	0,8	1,2	–
	0,2	1,3	0,3	–
	0,3	–	0,9	1,8
	0,4	1,4	–	2,2
	0,5	3,8	2,1	–
8	0,1	–	0,8	2,4
	0,2	0,9	–	–
	0,3	2,3	3,1	4,5
	0,4	–	0,9	2,9
	0,5	1,6	2,1	1,9
12	0,1	3,2	6,3	4,2
	0,2	2,5	–	0,9
	0,3	–	3,7	2,3
	0,4	4,2	2,5	3,5
	0,5	2,9	1,7	1,5

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