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Evaluation of Critical Dose for Mutagenic Treatments of Barley Varieties with N-nitroso-N-methyl Urea (NMU)

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Abstract

Induced mutations have been extensively used to improve main crop species, particularly cereals including barley (*Hordeum vulgare* L). Two laboratory studies have been conducted to estimate the critical dose of N-nitroso-N-methyl urea (NMU) on seedling emergence and growth reduction of three barley varieties. Seeds of barley varieties were treated with five different doses of N-nitroso-N-methyl urea (NMU) in addition to control. The doses were 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM for 3 hours and the double treatment with 0.5 mM/ for 3h and 5 hrs of the inter incubation germination period (iig). The pot test was applied for estimation of seedling emergence and seedling growth reduction, while for seminal root length, and first leaf length reduction, the paper roller test was used. Mutagenic treatments significantly reduced shoot and root growth and the effects where highly pronounced on roots more than on shoots. The reduction percentage reached 80 % in seminal roots and more than 50% in shoots of ACSAD-176 and Rum varieties. The results showed that the dose of 2.0 mM MNU was critical for most of analyzed varieties and suggests that lower doses should be applied for field experiments to avoid elevated levels of mutated phenotypes in barely fields.

Key words: N-nitroso-N-methyl Urea (NMU), Mutation, Barley, Seminal roots, Seedling emergence, Shoot growth

Introduction

Sustainable crop yield and nutritional value improvement through plant breeding programs is well recognized target worldwide. Increased crop yields, based on the better efficiency in using agricultural inputs (fertilizers, pesticides, herbicides, crop rotation and use of agricultural machinery) would not be possible without

varieties designed to meet the specific agro-climatic conditions. After the discovery of the role of X-rays in induced mutagenesis, which later became the most important tool in locating genes on chromosomes, studying gene structure, expression and regulation, and for exploring genomes, many plant breeders and geneticists started to investigate the use of radiation-induced mutations for changing plant important traits.

The prime strategy in mutation-based breeding has been to upgrade the well-adapted plant varieties by altering one or two major traits. These include characters such as plant height, maturity, seed shattering, and disease resistance, which significantly contribute to increased yield potential and quality traits. However, in many cases, the changed traits had a synergistic effect on the cultivation of the crop, agronomic inputs, crop rotation and utilization (Ahloowalia *et al.*, 2004).

Worldwide, more than 2252 varieties have been released as direct mutants or from their progenies during the past seventy years (Ahloowalia *et al.*, 2004). Most mutant varieties (1,603) were released in seed-propagated species, which include 1,072 cereals and 311 legumes (Maluszynski *et al.*, 2000). Radiation has been the most frequently used method for directly developed mutant varieties, while the use of chemical mutagens was relatively infrequent. In several mutation-derived varieties, the changed traits have resulted even in synergistic effect on increasing the yield and quality of the crop and consumer acceptance (Ahloowalia *et al.*, 2004).

In barley, the high-yielding and short stature mutant cultivars of barley cultivars 'Diamant' and 'Golden Promise' have added billions of dollars to the value of the brewing and malting industry in Europe. The mutants have also been used as parents of many leading barley cultivars. Recent studies have shown that Golden Promise variety is also salt-tolerant whereas the parent cultivar Maythorpe is salt-sensitive. Several genes related to salt tolerance were identified in this variety (Forster, 2001; Wei *et al.*, 2001). Ramesh *et al.*, (2001) have developed semi-dwarf (76 cm) barley mutant characterized by erect, compact tillers with bright green leaves along with good lodging resistance. The mutant also recorded improvement in biological and grain yields per plant over its parent with slightly delayed maturity (by 4 days) and medium sized seed. The most significant feature of this mutant is its remarkably high seed protein content (15.46%). N-nitroso-N-methylurea, N-nitroso-N-ethylurea and ethyleneimine were used to produce a new varieties and mutation lines of winter barley (Zayats, 2001).

Mutations that reduce phytic acid content of grains, have been isolated by chemical mutagenesis from maize and barley (Raboy and Gerbasi 1996). The inheritance and linkage map positions of two low phytic acid barley mutations, *lpa1-1* and *lpa2-1*, that dramatically reduce grain phytic acid content and increase inorganic seed phosphorus was described (Larson *et al.*, 1998).

Mutation breeding program in durum wheat (*T. durum*) in Italy which began in 1956, has yielded around 1000 mutants, 292 of which are considered useful for breeding purposes. Six were released as new varieties, while a further five varieties were produced by selection and hybridization among mutants (Scarascia *et al.*, 1993). In former USSR Salpikova and Rapopout, (1993), reported that, mutation breeding program since 1965 in using chemical mutagenesis led to the development of 499 mutant varieties of crop plants, of which 134 were released during the period 1977-

1992. Mutant varieties of winter wheat, spring and winter barley and maize account for over 25% of these released varieties. In Pakistan, three wheat varieties were released. The mutant variety, 'Jauhar 78' derived from 'Nayab' after neutrons treatment had high yield, wide adaptability, amber grain, and resistance to shattering. Both varieties 'Soghat 90' and 'Kiran 95', derived with sodium azide treatment had high grain and biomass yield, high protein and lysine content and tolerance to leaf-rust (Arain *et al.*, 2000).

Zhagin (1995), found that bulbous- leaf and reduced-leaf mutants, which is obtained by treating winter wheat varieties by chemicals, were temperature sensitive. The reduced-leaf mutants were adaptive to low-temperatures (8-15°C), whereas, the bulbous-leaf mutants adapted better to high temperatures (18-20°C). Sartaer, (1991) found that chemical mutagens and pesticides produced shorter-strawed, compacted ears, awned, semi-awned, early maturing, late-maturing and resistant to rust mutant lines in the M2 and M3 generation. Induced mutation in hexaploid wheat by treating seeds with ethylmerthane Sulfonate (EMS) produced mutant lines with resistance to 13 races of stem rust out of 15 races (Williams *et al.*, 1992).

Vasil and Knyazyuk, (1988) found that treated grains of wheat variety with different chemical mutagens, showed that mutation frequency depends on the genotype, mutagens and concentration of mutagens and the duration of the treatment. Among the chemical mutagens, ethyl methane sulphonate (EMS), diethyl sulphate (DES), methyl nitroso urea (MNH), ethyl nitroso urea (ENH), ethyleneimine (ED) are among the most commonly used mutagens, all of which belong to a special class of alkylating agents. Avemenko *et al.*, (1989 and 1991) reported that laser irradiation and treatment with NAD and NADH increased the frequency of mutation in barley and wheat cultivars up to 6.6% in the M2 generation in barley after treatment with oxidized NAD. However, combined chemical and laser treatment gave the lower mutation frequency than treatment of each factor separately with relatively stable phenotype.

Reddy and Revathi, 1992, results showed elevated mutation frequency in barley and wheat with duration, concentration of the mutagen treatment, and was higher in the combination of treatments of gamma rays, ethyl methane sulphonate (EMS) sodiomazide (SD). Kazoanzki *et al.*, (1990), found that combination of hybridization and irradiation increased induced mutations and widened variation in the hybrid progeny and increased the efficiency of selection.

Because of the release of semi-dwarf, high yield potential varieties, the average yield of rice in Egypt increased to 10 t/ha compared with 3.8 t/ha of the world. Shehata *et al.*, (2009) developed M5 mutant rice lines from Egyptian Yasmine with early duration (one month earlier), short stature, salinity tolerance and better yield potential using Gamma irradiation. Also, Abdullah *et al.*, (2009) . Used Gamma rays to develop elite lines from five Egyptian varieties. Morphological variations at vegetative and reproductive stages of mutant lines and their respective parents revealed better resistance to lodging, blast disease, high yield potential, as well as, early maturity. The results obtained from field evaluation over three years, and, also, through PCR analysis, indicated that mutants were differed genetically from their

parents. Therefore, these mutants could be used as a donor parents in the rice breeding program and some of them could be recommended to be new rice varieties.

Elayaraja *et al.* (2005) Concluded that irradiation not only induces higher proportion of chromosomal and physiological changes but also brings about a high frequency of gene mutations. It is evident that gene mutations using irradiation could generate a considerable amount of genetic variability and opens a new avenue for crop improvement and diversification.

Using Methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), N-methyl-N-nitrosourea (MNU) and N-ethyl-N-nitrosourea (MNU) linear concentration response characteristics for tail moment values and somatic mutations were observed when tobacco seedlings were treated under identical conditions (Menke *et al.*, 2000). 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 (Khan *et al.*, 2009).

This study is amide at estimating the critical dose of N-nitroso-N-methyl urea (NMU) on seedling emergence and growth reduction of three barley varieties.

Materials and methods

Three barley varieties, viz, Acsad-176, Rum and Start were used for the current investigation. Seeds of each variety were presoaked for 8 hours in distilled water, then were exposed to one of six treatments which were; control (no mutagenic solution, soaked in distilled water), 0.5, 1.0, 1.5, 2.0 mM and 0.5 mM N-nitroso-N-methyl urea (NMU) treated two times with 5 hours inter-incubation germination brake. After 3 hours of treatment, the seeds were rinsed in tap water three times. In the double treatment combination, during the iig period, the seeds were placed in the container with wet filter paper, covered and stored in the room temperature.

The emergence and seedling growth reduction were measured using pots filled with the soil mixed with the sand in 3:1 ratio, respectively. Hundred seeds from each treatment were sown into one pot, and covered with 2 cm layer of sand. The pots were watered and placed in Svalöf Weibull Growth Chamber at 20°C with 16 light hours per day for ten days. All observations were taken in three replications for each treatment. After 10 days, the number of seedlings per pot and per treatment was counted, and the seedlings heights were measured. The percent of reduction in seedling number and seedling growth in comparison to the non-treated control was calculated with following formula (1):

$$\% \text{ Reduction} = (1 - x_{\text{treatment}}/x_{\text{control}}) * 100$$

where $x_{\text{treatment}}$ is the measured trait: number of seedlings/treatment, or seedling height.

The average root and shoot length reduction was measured using paper rollers test. For this evaluation, a 10 seeds from each variety and treatment combination were placed embryo down into the two sides of 5 wires rolled by the wet filter paper, and covered by black sheath. The paper rollers were kept in glass jars with the equal level

of distilled water, in Svalöf Weibull Growth Chamber at 20 °C with 16 light hours per day for 7 days.

The percent of reduction in root and first leaf were calculated with the use formula (1), where \bar{X}_0 is measured trait (average length of the longest seminal root or average length of the first leaf).

Means and standard deviation have been calculated for each replication of both tests for treatments and varieties. The analysis of variance using *MSTATC* program using 0.05 probability level.

Results and discussion

The results for germination test under different treatments are presented in Table (1). The effect of mutagen on total number of seedling emerged was not strongly pronounced. However, there was stimulation in emergence in most treatments. The relatively lower number of seedling emerged in Start genotype was not due to treatment effect *per se* but due to the lower percentage of germination in this genotype compared with the other varieties under normal conditions. Seedling emergence reduction of barley genotypes in response to MNH mutagen treatments is presented in Figure (1).

For first leaf length, the results presented in Table (2), showed that MNH treatments significantly reduced first leaf length in all tested varieties. The 2.0mM of MNH treatment produced the shortest leaves compared with other treatments (Fig. 5 a-c). Except for Start genotype, about 50% reduction in leaf length due to high concentration treatment (2.0mM) was observed compared with the control. The pronounced reduction in the length of the first leaf is presented in Figure (2).

The seminal root and first leaf length results are presented in Table (3). There were pronounced effects of mutagen on roots and first leaf length in all tested varieties. However, the effects in general were higher on roots than shoots. The percent of reduction reached 80% using 2.0mM compared with the control while the reduction reached 53% in leaf length in both varieties Acsad-176 and Rum while 48% and 30% for roots and leaf length reduction in Start genotype (Figures 4 and 5).

Radiation has been the most frequently used method for varietal improvement while the use of chemical mutagens was relatively infrequent. In several mutation-derived varieties, the changed traits have resulted in synergistic effect on increasing the yield and quality of the crop and better consumer acceptance (Ahloowalia *et al.*, 2004).

N-nitroso-N-methylurea, N-nitroso-N-ethylurea and ethyleneimine were used to produce new varieties and mutation lines of winter barley (Zayats, 2001). Our results showed that N-nitroso-N-methyl urea (NMU) treatments significantly reduced leaf length in all tested varieties which coherent with the results of Ramesh *et al.*, (2001), who developed a semi-dwarf (76 cm) and erect barley mutant with compact tillers and good lodging resistance. The mutant also recorded improvement in biological and grain yields over its parent and had remarkably high seed protein content (15.46%). Our results also support that of Sartaer, (1991) who found that

chemical mutagenes and pesticides produced shorter-strawed, compacted ears, awned, semi-awned, early maturing, late-maturing and resistant to rust mutant lines. Induced mutation in hexaploid wheat by treating seeds with ethylmerthane Sulfonate (EMS) produced mutant lines with resistance to 13 races of stem rust out of 15 races (Williams *et al.*, 1992).

Our results also were in agreement with that of Vasil and Knyazyuk, (1988) who found that mutation frequency was largely depending on the genotype, mutagenes and concentration of mutagenes as well as the duration of the treatment.

The 2.0 mM of MNH treatment produced the shortest leaves compared with other treatments agreed with those of Reddy and Revathi (1992) who showed that the mutation frequency increased with duration, concentration of the mutagene treatment, and was higher in the combination of treatments treating seeds of barley and wheat individually and in combination with gamma rays, 0.5% ethyl methane sulphonate (EMS) and sodiomazide. Kazoanzki *et al.*, (1990), found that combination of hybridization and irradiation increased induced mutations and widened variation in the hybrid progeny and increased the efficiency of selection.

The obtained results are also in full agreement with that of Shehata *et.al.*, (2009) who produced rice lines with early duration, better agronomic performance compared with the original variety Egyptian Yasmin using various doses of gamma rays. AbdAllah *et al.*, (2009) also used gamma rays to produce elite mutants from five Egyptian rice varieties. They highlighted the usefulness of mutation as breeding method to enhance genetic variability for development of elite rice lines with better agronomic performance.

Gichner *et al.*, (2000) have found that Maleic hydrazide (MH) induced a high frequency of somatic mutations in leaves of tobacco and a high yield of chromosome aberrations in roots of field bean. Using Methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), N-methyl-N-nitrosourea (MNU) and N-ethyl-N-nitrosourea (MNU) linear concentration response characteristics for tail moment values and somatic mutations were observed when tobacco seedlings were treated under identical conditions (Menke *et al.*, 2000).

Our results showed also that there were pronounced effects of mutagen on roots and first leaf length in all tested varieties which in full agreement with Ilbas *et al.* (2005) who found that the lowest root length (9.10 cm) was found on day 14 for the group exposed to 2.5 mM NaN₃ for 3 h in barley and its treatment had a significant effect on leaf length and this effect appeared to be particularly evident on day 14 (Ilbas *et al.*, 2005).

In conclusion in both laboratory tests, mutagenic treatment showed significant effect on seedling characteristics and growth reduction on barley seedlings and the effects were highly pronounced on roots more than on shoots. The percent of reduction reached 80% in seminal roots and more than 50% in shoots of Acsad- 176 and Rum varieties. The dose of 2.0 mM/3h MNU seems to be critical for most of analyzed varieties. It could be concluded that using chemical mutagen MNH, it is possible to diversify the genetic resources with high possibility of getting elite lines with even better performance than original parents. This also emphasizes the usefulness of

mutation breeding with chemical mutagen or irradiation as a breeding method for crop improvement strategies.

Table 1. Pot test Mean values of number of germinated seedlings in the M₁ generation of three barley varieties after various mutation treatments.

Genotype	MNH dose (mM/3h)					
	0 (control)	0.5	1.0	1.5	2.0	0.5 x 0.5
Acsad 176	79.7±7.1 ^{BC}	85.0±8.8 ^{ABC}	87.0±1.0 ^{AB}	84.0±5.2 ^{ABC}	77.0±3.0 ^C	91.3±0.6 ^A
Rum	91.7±5.7 ^A	87.3±6.5 ^A	90.7±3.5 ^A	86.7±3.5 ^A	75.3±5.7 ^B	85.3±7.4 ^A
Start	46.3±3.2 ^B	60.0±7.5 ^A	48.7±4.0 ^{AB}	51.0±1.0 ^{AB}	51.7±7.4 ^{AB}	59.0±6.0 ^A

* For each genotype, the values followed by the same uppercase letter are not statistically significant (p=0.05)

Table 2. Pot test mean values of first leaf length (cm) of seedlings of the M₁ generation of three barley varieties after various MNH mutation treatments.

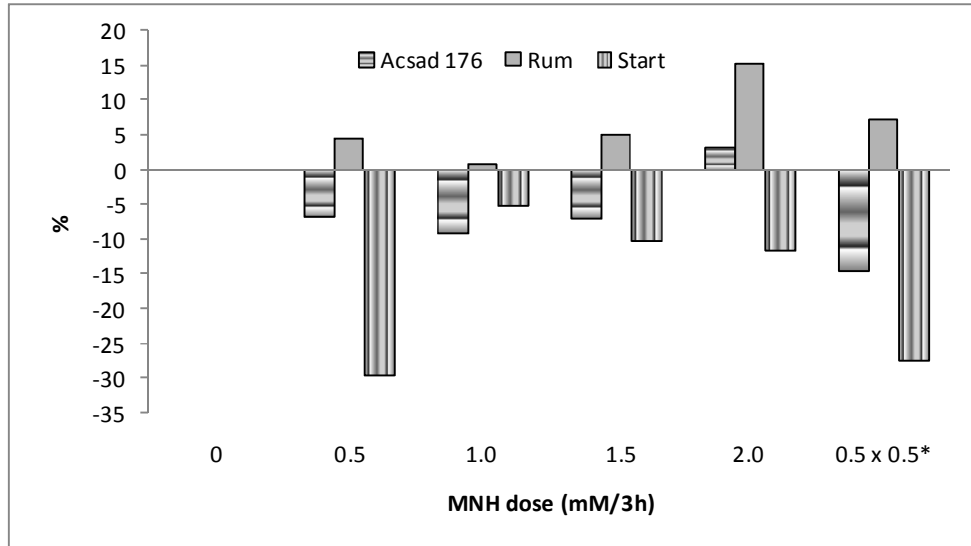
Genotype	MNH dose (mM/3h)					
	0 (control)	0.5	1.0	1.5	2.0	0.5 x 0.5
Acsad 176	17.3±1.0 ^{A*}	18.4±0.9 ^A	15.5±0.7 ^B	11.7±0.5 ^D	9.9±0.4 ^E	13.4±0.8 ^C
Rum	19.8±0.3 ^A	15.6±2.1 ^B	16.2±0.6 ^B	12.2±0.7 ^C	9.0±0.3 ^D	14.6±3.4 ^{BC}
Start	12.7±0.0 ^A	10.9±0.7 ^B	10.8±0.6 ^B	10.7±1.7 ^B	10.0±0.5 ^B	9.5±0.3 ^B

* For each genotype the values followed by the same uppercase letter are not statistically significant (p=0.05).

Table 3. Seminal root and first leaf length mean values (cm±SD) in paper rollers test for M₁ generation of three barely varieties after various MNH mutation treatments.

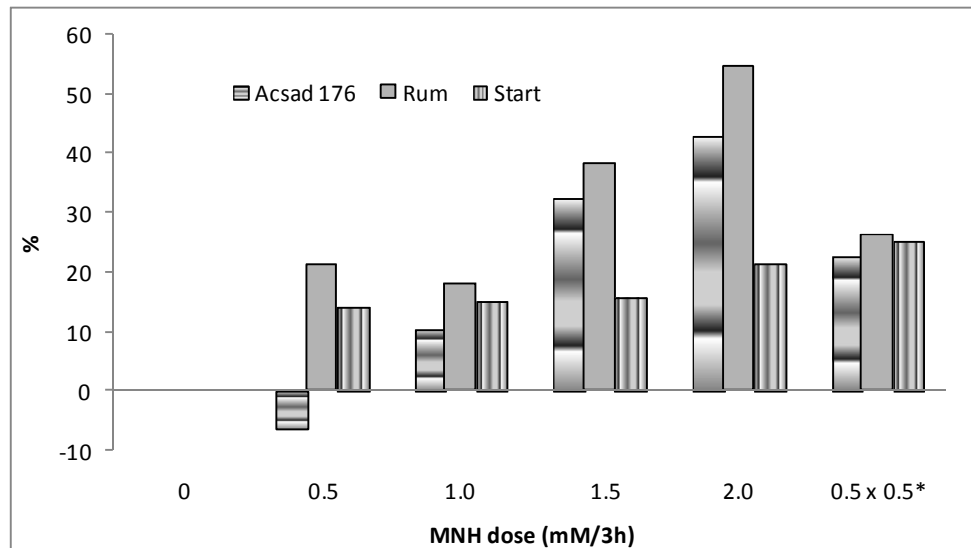
MNH Dose (mM/3h)	Acsad 176		Rum		Start	
	Root	leaf	root	leaf	root	leaf
0	25,0±0,8 ^{A*}	17,0±0,6 ^A	23,4±2,0 ^A	15,7±0,3 ^A	17,3±0,2 ^A	11,5±1,0 ^A
0.5	21,8±1,4 ^B	15,3±0,1 ^B	19,1±1,6 ^B	14,4±1,6 ^A	16,4±1,6 ^A	12,2±2,7 ^A
1.0	13,2±0,5 ^C	13,5±0,9 ^C	10,5±0,5 ^C	12,0±0,7 ^B	13,5±1,1 ^B	10,6±0,6 ^{AB}
1.5	10,4±1,2 ^D	12,6±0,6 ^{CD}	7,1±0,9 ^D	10,3±1,4 ^B	12,1±0,1 ^B	11,7±1,1 ^A
2.0	4,8±0,3 ^E	8,0±0,2 ^E	4,2±0,8 ^E	7,3±0,8 ^C	9,0±1,3 ^C	8,1±1,6 ^B
2.5	9,6±1,2 ^D	11,4±1,4 ^D	8,4±1,5 ^{CD}	10,7±1,0 ^B	12,2±1,2 ^B	10,3±1,0 ^{AB}

* For each genotype the values followed by the same uppercase letter are not statistically significant (p=0.05).



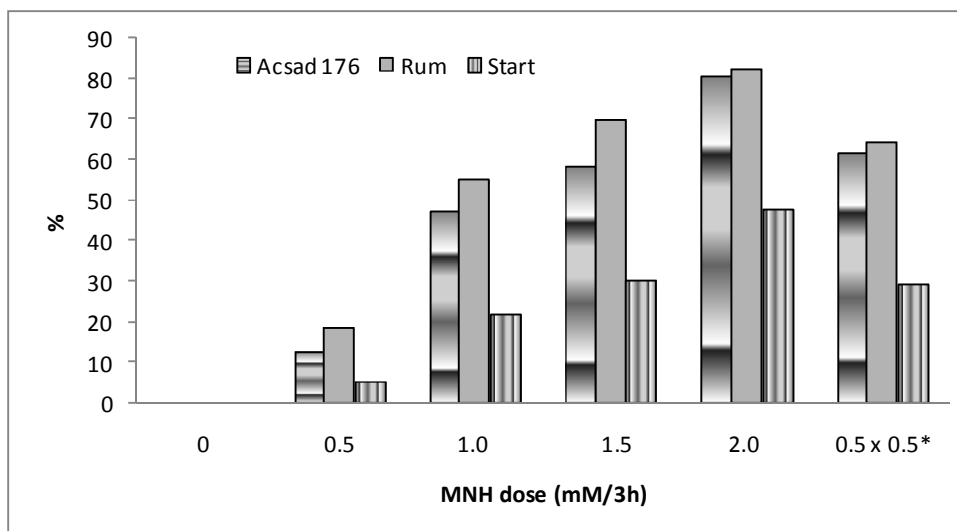
* Treatment with 5h of inter incubation germination

Figure 1. Seedling emergence reduction of barley in response to MNH mutagen treatments



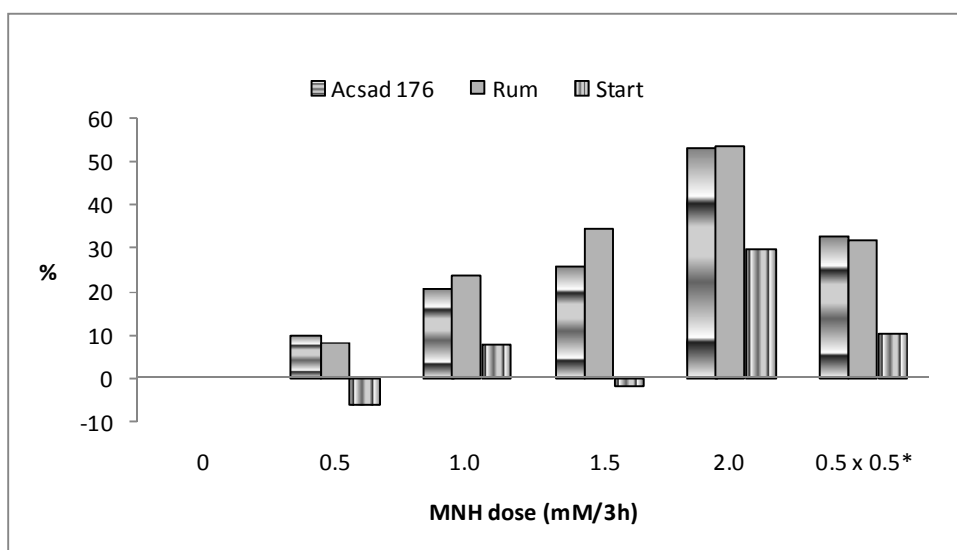
* Treatment with 5h of inter incubation germination

Figure 2. Seedling growth reduction of barley in response to MNH mutagen treatments.



* Treatment with 5h of inter incubation germination

Figure 3. Seminal root length reduction of barley in response to MNH mutagen treatments



* Treatment with 5h of inter incubation germination

Figure 4. First leaf length reduction of barley in response to MNH mutagen treatments

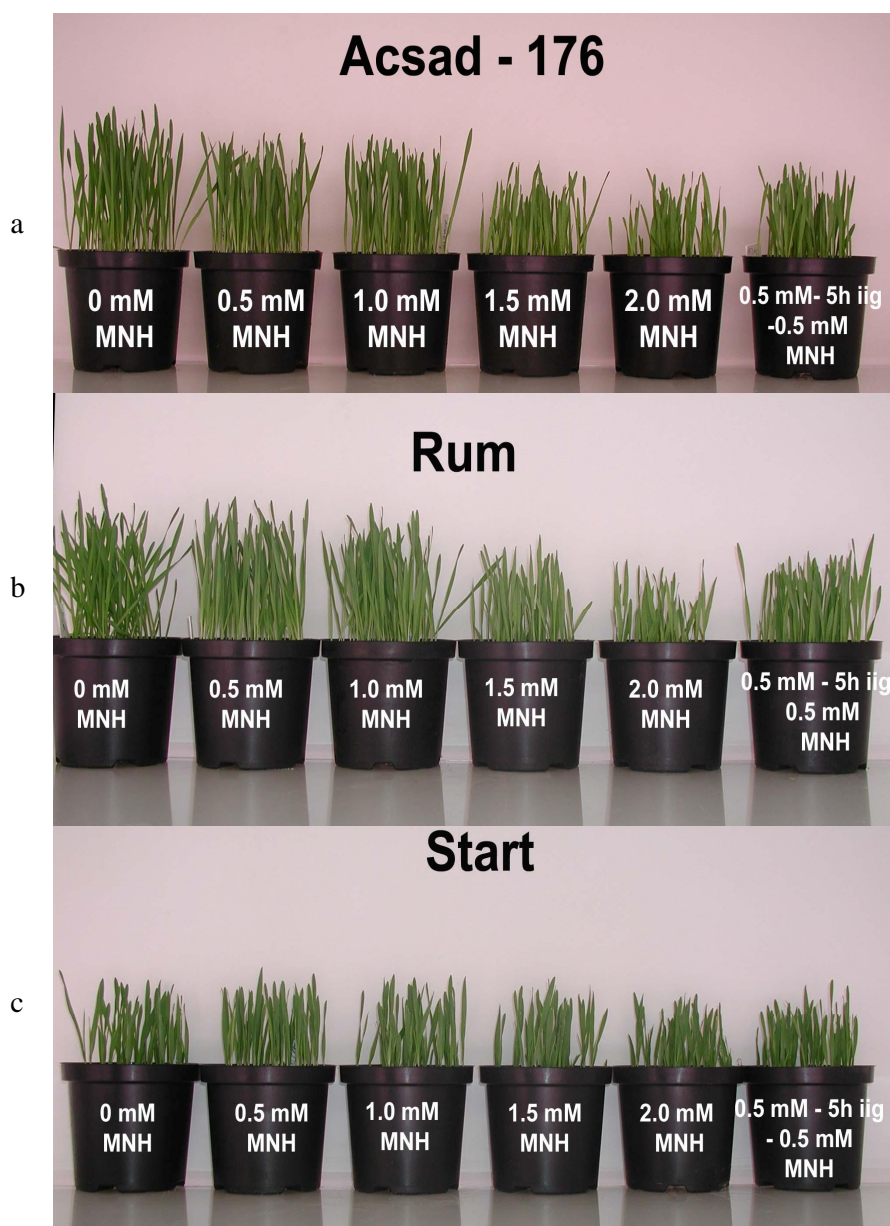


Figure 5. Comparison of first leaf length in the Acsad-176 (a), Rum (b) and in Start (c) varieties after mutation treatment.

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