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MUTAGENIC INTERACTIONS IN COMBINED TREATMENTS OF ETHYLMETHANE SULPHONATE, DIETHYL SULPHATE AND SODIUM AZIDE ON BARLEY[1]

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ABSTRACT

Presoaked barley (*Hordeum vulgare* L.) seeds received, as pre- and post-treatment, all possible combinations and sequences of ethylmethane sulphonate of (EMS), diethyl sulphate (dES) and sodium azide (AZ), intermediated by wet and redry inter-treatment. Mutagenic interactions were obtained in combination of different mutagens as well as in repeated treatments with the same mutagen. In combined treatments, the sequence of the mutagens used and the inter-treatment condition both significantly affected the mutagenic interaction obtained. Some of the combined treatments showed synergistic as well as less than additive interactions, depending on the biological criteria measured.

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خلاصه

بذورجوکه قبلا"خیس شده بودندتحت تاءثیرتمامی ترکیبها وترتیب های موادموتاژن سولفانات اتیل متان وسولفات دی اتیل وآزیدسدیم‌تواءم باخشک کردن وخیس ماندن بذردرفواصل تاءثیرموتاژن ها ،قرارگرفتند.اثرمتقابل موتاژنی هم‌درترکیب موتاژنها وهم‌درتکرار یک موتاژن مشاهده‌گردید.درترکیب موتاژنها ،ترتیب استفاده‌ازموتاژن وخشک شدن ویاخیساندن بذوربین دوموتاژن متوالی هردوبراثرمتقابل حاصله‌به‌نحو معنی داری اثرگذاشتند. دربرخی ازترکیبات موتاژنی ،بسته‌به‌معیارهای بیولوژیکی مورداستفاده ،اثرات تشدیدکننده ونیزاثرمتقابل کمترازاثرجمع‌شونده مشاهده‌گردید.

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INTRODUCTION

Biological effects of combined mutagenic treatments in which two physical mutagens or physical plus chemical mutagens are used have been studied by a number of investigators (1, 4, 5, 12, 17), and results ranging from antagonistic (8, 15) to synergistic (4, 10, 15, 17, 18) are reported for different biological criteria measured. However, reports dealing with the effects of combined chemical mutagen treatments are limited. Konak *et al.* (13) studied the mutagenic interaction of sodium azide (AZ) with diethyl sulfate (dES) and N. methyl N-nitrosourea (MNH) on barely. They showed that when AZ was used after MNH, a synergistic action of the two chemicals was obtained, but when azide treatment followed dES treatment, mutation yields were notably less than additive. Khalatkar *et al.* (11) investigated the effects of ethidium bromide (EB), iodoacetamide (IA) and sodium fluoride (NaF) in pre- and post- ethylmethane sulphonate (EMS) treatments on mutagenic efficiency of EMS on barley. None of the three modifiers by themselves induced chlorophyll mutations, however, the presence of EB, IA and NaF during EMS treatment significantly enhanced the mutagenic effect of the latter.

The present study reports on the single and combined effects of EMS, dES and AZ, used in different sequences, on a hull-less barely cultivar.

MATERIALS AND METHODS

Hand-threshed seeds (caryopses) of a hull-less barley (*Hordeum vulgare* L.) cultivar Himalaya, were thoroughly washed and then soaked in cold (2±1°C) distilled water for 20 hr before mutagenic treatments. After the presoak, the seeds were treated for 2 hr at 20°C with 0.024, 0.0114 and 0.0005 M of EMS, dES and AZ, respectively. EMS and dES were prepared in 0.1 M phosphate buffer at pH 7. AZ was buffered at pH^3. Another group of seeds was treated with distilled water as control. After the first mutagenic treatments (pre-treatment), seeds were separately washed for 6 min. in a strong flow of tap water and then repeatedly rinsed in large volumes of distilled water. Later, seeds were separately post-washed in large volumes of cold (2±1°C) distilled water for 20 hr. At the end of post washing half of the seeds from each treatment was air dried (dry inter treatment) for 24 hr, which decreased the moisture content of the seeds to 8-9%. The other half of the seeds was kept soaked in cold (2±1°C) distilled water (wet inter-treatment). Redried seeds of the first half were again soaked in cold (2±1 C) distilled water before receiving the second round of mutagenic treatments (post-treatment) whereas the second half received post-treatment directly from the wet state. Post treatment was carried out under exactly the same conditions as described for pre-treatment. Following final post-wash, all seeds were air dried and then stored in a refrigerator for 24 hr before being planted in the field, greenhouse and laboratory.

The design of the experiment was a 4x2x4 factorial with five, four and two replications planted in the field, greenhouse and laboratory, respectively. The number of seeds treated per replication for each treatment was 200, the plots were arranged in a completely randomized form. Seedling height was determined as the mean height of one-week old seedlings and M_1 fertility was calculated as the ratio of the number of seeds to the number of spikelets x 100 in 25 randomly selected spikes per plot. For chlorophyll mutation studies, seeds harvested from M_1 plots were planted in rows in a steam sterilized sand bed in the greenhouse. At least 10000 M_2 seedlings were analyzed from each M_1 plot whenever enough seed was available. All M_2 seedlings were counted and their chlorophyll mutations were classified according to the system originally proposed by Gustafsson (9). Data were statistically analyzed and means were compared using Duncan's multiple range test (7). The interaction effect of combined mutagenic treatments, in a given order, was determined by calculating the deviation of the observed value from the sum of corresponding single effects plus the respective control. Sequence effects were calculated as the difference between the effects of a given combination in reverse orders.

Throughout the text whenever a (+) sign is between mutagen abbreviations (e.g., AZ+EMS) it signifies the combined treatment irrespective of the order whereas a (-) sign signifies both combination and order (from left to right) of the mutagens used.

RESULTS

M_1 Seed Germination

Pre- and post- mutagenic treatments , inter-treatments and all of their interactions, except pre-treatment X inter-treatment, resulted in highly significant effects on seed germination ($p < 0.01$). Pre-treatment with EMS did not decrease seed germination compared to the control, whereas dES and AZ treatments both caused a 10% reduction in seed germination. Dry-back inter-treatment also significantly ($p < 0.01$) reduced seed germination in all cases (Table 1).

Post-treatment with EMS did not reduce seed germination whereas dES and AZ caused 9 and 42% reductions, respectively. Seed redrying followed by AZ post-treatment resulted in a drastic (51%) reduction in seed germination whereas the same inter-treatment caused only 11 and 2% reductions in treatments receiving dES and EMS post-treatments, respectively.

Interactions between pre- and post-treatments, ignoring inter-treatments, resulted in different effects on seed germination. The lowest seed germination (30%) was obtained with the double AZ treatment followed by the dES-AZ, 0-AZ, EMS-AZ and dES-dES treatments with 53, 66, 70 and 73% germinations, respectively. All other combinations were not

Table 1. Biological effects of various combinations and sequences of EMS, dES and AZ on Himalaya barley.

Treatment	Reduction in M_1 germination (%)	Seedling injury (%)	M_1 sterility (%)	Chlorophyll M_2 mutation frequency x (10^{-4})
0-W-0†	0a*	0ab	31a	40.2mn
0-W-AZ	17b	12b	77j-m	609.8b-g
0-W-EMS	1a	4a-c	53d-f	342.2f-l
0-W-dES	2a	16b-e	55e-g	321.2g-l
0-D-0	0a	1ab	31a	16.8n
0-D-AZ	50cd	23d-g	77j-m	862.6ab
0-D-EMS	1a	5a-c	46b-d	268i-m
0-D-dES	1a	19c-f	50c-e	265.8j-n
AZ-W-0	1a	10a-d	68h-j	603.4b-g
AZ-W-AZ	55d	23d-g	61f-h	469.8c-j
AZ-W-EMS	3a	22d-g	79l-n	700.0b-d
AZ-W-dES	5ab	27e-h	55e-g	340.6g-l
AZ-D-0	3a	18c-e	76j-m	756.8b-c
AZ-D-AZ	70e	34g-i	87n	1047.8a
AZ-D-EMS	4ab	20d-g	83l-n	659.4b-e
AZ-D-dES	14b	34h-i	71i-k	470.2c-j
EMS-W-0	2a	-1.0a‡	30a	56.0mn
EMS-W-AZ	18b	18c-e	76j-l	647.8b-e
EMS-W-EMS	0a	13a-e	50c-e	300.0h-m
EMS-W-dES	0a	23d-g	64j-i	369.6e-l
EMS-D-0	0a	2ab	41bc	201.4j-n
EMS-D-AZ	42c	39hi	86mn	695.4b-d
EMS-D-EMS	1a	8a-c	61f-h	385.8e-l
EMS-D-dES	6ab	40hi	72i-k	460.8c-j
dES-W-0	1a	14a-e	39ab	149.6l-n
dES-W-AZ	48cd	42hi	45b-d	159.6k-n
dES-W-EMS	1a	40hi	70h-k	567.0c-h
dES-W-dES	11a	57j	79k-n	574.4b-h
dES-D-0	1a	12a-e	38ab	156.2l-n
dES-D-AZ	45cd	46ij	45b-d	237.2j-n
dES-D-EMS	4ab	33fi	68h-g	437.2d-k
dES-D-dES	42c	61k	73i-k	523.2c-i

†From left to right, pre-treatment, inter-treatment and post-treatment. 0=No mutagen W=Wet D=Dry dES=Diethyl sulphate EMS=Ethylmethane sulphonate AZ=Sodium azide

*Means within a column followed by the same letter are not significantly different at the 5% level of probability according to Duncan's Multiple Range Test.

‡Negetive seedling injury.

different from the control.

Double AZ treatment with redrying inter-treatment was the most injurious treatment to seed germination followed by the AZ-W-AZ combination (Table 1). In general, wherever AZ treatment followed by drying inter-treatment, enhanced seedling damage.

M_1 Seedling Height

In both pre- and post-treatment, dES caused the highest reduction in M_1 seedling height followed by AZ and EMS.

As with seed germination, the interaction between pre-treatment and inter-treatment was not significant. However, the interaction between inter-treatment and post-treatment was significant ($p < 0.01$). Dry inter-treatment followed by either dES or AZ treatment significantly reduced seedling height, whereas no significant was observed when EMS compared to wet inter-treatment.

Combined mutagenic treatments affected seedling height differently. The most damaging combination was dES-dES followed by dES AZ, dES-EMS and AZ-dES. Among all treatment and inter-treatment combinations, however, the highest reduction in seedling height was obtained in dES-dry-dES treated seeds.

M_1 Sterility

M_1 sterility was significantly affected by mutagens and their various combinations and sequences. However, inter-treatment X post-treatment interactions proved to be non-significant.

In pre-treatments, AZ caused the highest sterility followed by EMS and dES (Table 1). Seed drying as inter-treatment caused the highest M_1 sterility in AZ treated plants, while dES treated plants did not suffer from drying and EMS treated plants suffered significantly but less than azide treated plants.

In post-treatments, AZ again caused the highest sterility (70%) followed by dES and EMS, which had similar effects. Drying inter-treatment interacted only with AZ post-treated seeds and caused a 10% increase in seed sterility.

In combined mutagenic treatments, the lowest seed set was obtained from AZ+EMS combinations in both sequences with dES+dES and AZ+AZ combinations next in order. The highest percentage of seed sterility was observed for AZ-D-AZ combination.

Frequency of Chlorophyll Mutations

In pre-treatments, AZ produced the highest frequency of chlorophyll mutations followed by EMS and dES. Drying, in general, increased mutation rate compared to that of the wet treatment; however, this increase was mostly due to the increased mutation rate for the AZ treated seeds with no significant effect on EMS or dES treated seeds (Table 1).

In post-treatment, as pre-treatment, AZ again produced the highest mutation rate followed by EMS and dES.

Drying inter-treatment preceding AZ treatment significantly increased mutation rate over that of the wet condition; however, with other post-treatments the rates were statistically nonsignificant.

Combined mutagen treatments caused significantly different effects on the M_2 mutation rate. The highest rate was obtained from double AZ treatment followed by single AZ treatments and AZ+EMS combinations. The highest mutation frequency (1047×10^{-4}), however, was obtained from double AZ treatment with dry inter-treatment (Table 1). The same combination when intermediated by wet condition resulted in 2.2 times less mutation frequency.

DISCUSSION

The main purpose of this study was to investigate the interaction and sequence effects of three known chemical mutagens on various biological criteria of Himalaya barely, a widely used experimental material in radiation and chemical mutagenesis. Low mutagen concentrations were deliberately used in order to have: first, a low physiological damage even with repeated injurious treatments, and second, enough unreacted loci available for post-treatments so that additivity, if present, could be manifested.

As shown in Table 2, various combinations and sequences of the mutagens exerted different interaction effects on biological criteria measured. When AZ was used as pre-treatment the interaction effect on germination reduction varied from highly synergistic for double AZ treatment to merely additive for AZ-EMS combinations. When EMS was used as pre-treatment, interaction effects on germination reduction were generally but slightly antagonistic. With dES pre-treated seeds the interaction effects on germination were moderately antagonistic to highly synergistic. Wet and dry inter-treatments, respectively, reversed the interaction effect of dES-AZ from synergistic to antagonistic as regards seed germination. It is interesting to note that the two highest synergistic effects were obtained with double AZ as well as double dES treatments; this indicates that repeated applications of the same mutagen also may show interactions.

As regards M_1 seedling injury, the interaction effects were mostly synergistic as shown in the third column of Table 2. The highest synergistic interactions, however, were noticed when dES was used as pre-treatment with EMS next in order. The over-all interaction effect of combined treatments, in which AZ was used as pre-treatment was close to additivity. Repeated treatments with dES, again, resulted in the highest synergistic interaction on seedling injury. The above observations may suggest that, in the mutagenic combinations studied, pre-treatment has a decisive effect on seedling height.

The strongest antagonistic effect on seed sterility was noticed for repeated AZ treatments followed by dES+AZ

Table 2. Interaction effects of various mutagenic combinations and sequences on seed germination, seedling height, M_1 spike sterility and M_2 chlorophyll mutation frequency.

Treatment	Reduction in M_1 germination (o-c)† %	Seedling injury (o-c) %	M_1 sterility (o-c) %	Chlorophyll M_2 mutation frequency x (10^{-4}) (o-c)
AZ-W-AZ‡	+37§	+ 1	-53	-703
AZ-D-AZ	+17	- 6	-35	-555
AZ-W-EMS	+ 1	+ 8	-11	-205
AZ-D-EMS	+ 0	- 2	- 8	-349
AZ-W-dES	+ 2	+ 2	-37	-544
AZ-D-dES	+10	- 2	-24	-536
EMS-W-AZ	- 1	+ 8	0	+ 22
EMS-D-AZ	- 8	+15	- 1	-352
EMS-W-EMS	- 3	+11	- 2	- 58
EMS-D-EMS	0	+ 2	+ 5	- 67
EMS-W-dES	- 4	+ 8	+10	+ 33
EMS-D-dES	+ 5	+20	+12	+ 10
dES-W-AZ	+30	+17	-40	-560
dES-D-AZ	- 6	+12	-39	-765
dES-W-EMS	- 1	+22	+ 6	+118
dES-D-EMS	+ 2	+17	+15	+ 27
dES-W-dES	+ 8	+28	+16	+144
dES-D-dES	+40	+31	+16	+118

†Observed minus calculated.

‡From left to right; pre-treatment, inter-treatment and post-treatment.

W=Wet

D=Dry

dES=Diethyl sulphate

EMS=Ethylmethane sulphonate

AZ=Sodium azide

§Each figure represents deviation of the observed value from the sum of corresponding single treatments and respective control.

combinations in both orders. The highest synergistic effects, on the other hand, were obtained with repeated dES treatments.

Chlorophyll mutation rates were also affected differently by combined treatments as shown in the last column of Table 2. In all combinations, where AZ was used as a pre-or post-treatment, strong antagonistic interactions were observed with the exception of the EMS-W-AZ combination which showed a moderate synergistic effect. This is in agreement with the results reported by Konzak *et al.* (13) who showed that when AZ treatment followed dES treatment mutation yields were notably less than additive.

EMS used before or after dES, with wet or dry inter-treatment, resulted in synergistic interactions on mutation frequency. The same was also true for double dES application whereas antagoistic effects were observed with repeated EMS applications. In general, the observed frequency of mutants (Table 2) was substantially lower than expected in nine, close to additive in six and more than additive in three out of 18 combinations studied. This is in agreement with results obtained in combined physical and chemical mutagenic treatments by Doll and Sandfer (6) and Favret (8) who reported that the mutation frequency induced by combined treatments was not generally higher than that expected from a simple additive effect of the mutagens. The over-all combined mutagenic effect of EMS+dES, however, could be considered more than additive which also agrees with results obtained by Arnason *et al.* (2) who showed that the combined effect of EMS and ethylenimine was more than additive as far as chlorophyll mutations in barley are concerned. As it is evident from Table 1, 2 and 3, among the biological criteria measured in this study, M_1 sterility and M_2 mutation rates are, in most cases, similarly affected by a given combination sequence. Seed germination and seedling height, however, may differ from mutation rate with respect to the effect of various treatments. This may suggest that M_1 sterility and M_2 chlorophyll mutation are caused by similar phenomena (e.g. gene mutation) whereas seed germination and seedling height are affected through a different mechanism.

Various biological criteria measured in this study were also differentially affected by treatment sequence (Table 3). In the sequence, each figure represents the subtracted value of the alternative order from the one listed in the table. The biological damage induced by AZ, when used singly as a post-treatment, was more than its single effect as pre-treatment. This is true under both wet and dry inter-treatment although the effects are more pronounced for mutation yield and seed germination with dry condition. The same also applies to EMS and dES especially in regard to mutation frequencies. In general, the differential effect of sequence in single treatments could be attributed to post-wash duration. With increased post-washing, all criteria of biological damage decreased irrespective of the inter-treatments they received. This is in general agreement with results obtained by Bender and Gaul (3) who found that in EMS treated seeds increased post-washing time increases seedling height, survival rate and fertility. This seems to be equally true for all the three mutagens used in the present study. However, their results showed that, the period of post-washing did not have any influences on the frequency of chlorophyll mutations, varies from those obtained in this study. The discrepancy observed in the results, could be attributed to the effect of post-washing temperatures, namely 25°C in Bender and Gaul's experiment, and 2±1°C in the present study. Higher post-washing temperatures could also be regarded as an incubation period which enhances the metabolic activity of the seeds and the rate of reaction between the chemical and the genetic material. Post-washing at low temperatures, on the order hand, does not permit extensive reaction between mutagens and the target. Bender and Gaul (3) also found that post-washing for 24 hr at 10°C

Table 3. Sequence effect of single and combined mutagenic treatments on seed germination, M_1 seedling height, M_1 sterility and M_2-chlorophyll mutation frequency.

Treatment	Reduction in M_1 germination (%)	Seedling injury (%)	M_1 sterility (%)	M_2 chlorophyll mutation frequency $x(10^{-4})$
0-W-AZ†	+16‡	+11	+ 9	+ 6
0-D-AZ	+47	+ 5	+ 1	+106
0-W-dES	+ 1	+ 2	+16	+172
0-D-des	0	+ 7	+12	+109
0-W-EMS	- 1	+ 4	+23	+286
0-D-EMS	+ 1	+ 3	+ 5	+ 67
AZ-W-EMS	-15	+ 4	+ 3	+ 53
AZ-D-EMS	-38	-19	- 3	- 36
AZ-W-dES	-43	-15	+10	+181
AZ-D-dES	-28	-12	+26	+233
EMS-W-dES	- 1	-17	- 6	-198
EMS-D-dES	+ 2	+ 7	+ 4	+ 33

†From left to right; pre-treatment and post-treatment.

0= No mutagen

W= Wet

D= Dry

dES= Diethyl sulphate

EMS= Ethylmethane sulphonate

AZ= Sodium azide

‡Each figure represents the subtracted value of the alternative sequence from the one listed under treatment.

(their lowest post-washing temperature) unlike post-washing at 25°C gave lower mutation rates. It should be noted that pre- and post-single treatments are also accompanied by shorter and longer pre-soaking, respectively. It has been reported that pre-soaking enhances sensitivity of barley seeds to EMS, dES (14) and AZ (16). Thus, the sequence effect in single treatment is a compound effect of pre-soaking and post-washing. Their combinations lead to decreased and increased biological damage in pre- and post-treated seeds, respectively.

AZ applied before dES caused an increase in mutations, however, the effects on mutation yield were reversed under wet and dry inter-treatments. In EMS+dES combinations, the sequence effects on all biological criteria measured were also reversed in wet and dry inter-treatments.

The following concluding remarks could be derived from the foregoing discussion.

1- Synergistic and antagonistic phenomena observed in combined mutagenic treatments are not limited to combinations of different mutagens. The same phenomena could be encountered in repeated applications of the same mutagen. Thus, some of the observed mutagenic interactions may simply be due to repeated treatments rather than combined treatments.

2- A given combined treatment may show synergistic as well as antagonistic effects when different biological criteria are used.

3- Wet and dry inter-treatments may, in some cases, reverse the interaction effect from synergistic to antagonistic and *vice versa*.

4- Reverse orders of a given mutagenic combination may result in opposite biological consequences.

5- Biological effects of a given mutagenic sequence may be reversed under the influence of wet and dry inter-treatment.

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