GENETIC EFFECTS OF ETHYL METHANESULFONATE IN COMBINATION WITH COPPER AND ZINC IONS ON *ARABIDOPSIS THALIANA*[1]

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Received April 13, 1965

VARIATION in experimental conditions is known to modify the observed effect of ethyl methanesulfonate (EMS) and other chemical mutagens (NILAN *et al.* 1964; KONZAK *et al.* 1964). MOUTSCHEN-DAHMEN (1963) observed marked differences with regard to chromosome breakage following EMS treatment in distilled water from different sources, increased chromosome breaks being associated with contamination of zinc, copper or both. In further experiments with *Vicia faba* and barley seeds, addition of Cu and Zn ions to EMS solution in double distilled water enhanced the number of chromosomal aberrations in the first mitosis after seed germination. The synergistic effect of metal ions varied with the pH. A similar effect of Cu ions was observed on wheat chromosomes (BARI 1963). MOUTSCHEN, MOES and GILOT (1964) did not observe any increased meiotic damage due to Cu and Zn ions in M_1 spikes of barley but the proportions of various types of aberrations were different. Metal ions themselves are known to have radiomimetic (GLASS 1956) and mutagenic effects and are also reported to modify X-ray sensitivity of seeds (VON ROSEN 1957, 1964). In contrast, protective action of metal ions on radiation sensitivity of seeds (HAZAMA, HAZAMA and EHRENBERG 1963), has been reported.

This study, using *Arabidopsis thaliana* as the test plant, was undertaken to investigate the genetic effects of Cu and Zn ions alone and in combination with EMS at three levels of hydrogen ion concentration. In addition, it was desirable to determine if the metal ions and different hydrogen ion concentrations can be used for increasing the efficiency of chemical mutagens. The M_2 analysis of chlorophyll deficient mutants used in this study permits a rapid evaluation of genetic effects following treatment with mutgaens under highly controlled environmental conditions.

MATERIALS AND METHODS

Seeds from a single plant of *Arabidopsis thaliana* race Landsberg type "erecta" were increased and used for this experiment. Dormancy was eliminated by storing the seeds at 3°C on wet filter paper for five days. The seeds were then dried and equilibrated to 32% relative

[1] Part of the research carried out at Brookhaven National Laboratory under the auspices of the U.S. Atomic Energy Commission.

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humidity over Ca Cl_2 for eight days. For treatment, seeds were soaked for 24 hours at 24 ± 0.5°C in Sorensen's buffers (0.1 M mono and disodium phosphate) made in double distilled water at pH 5, 7, and 9, buffers + 10^{-3} mM $ZnSO_4$, buffers + 10^{-3} mM $CuSO_4$ and all the nine combinations with 10 mM EMS. In total there were 19 treatments including one lot of seeds soaked in double distilled water to serve as control. The concentration of EMS and duration of treatment were based on previous experience with this compound on the same genotype. The concentration used was known to give high survival and fertility in the M_1 generation with approximately half of the families segregating for chlorophyll mutations in M_2.

Following the treatment, seeds were washed in double distilled water and planted on neutral agar in petri dishes, germinated in light for 36 hours at 24 ± 0.5°C. This was followed by a dark period of 36 hours to etiolate the seedlings for easy handling during transplantation. Five-day old seedlings were transplanted into pots and raised to maturity in an air conditioned greenhouse at 24 ± 2°C, under continuous illumination. Ten pods were harvested from each M_1 plant and the M_2 population was grown on nutrient agar in petri dishes under sterile conditions. Petri dishes were kept at 3°C for five days to eliminate dormancy effects and to get uniform germination. The M_2 population was raised in growth chambers at 24 ± 0.5°C. Chlorophyll mutations were scored at the four-leaf stage. Each experiment was replicated twice and in every treatment at least 120 M_1 families were analyzed.

RESULTS

Germination of the treated seeds was over 95% and M_1 survival to maturity was in the range of 60 to 70%. No appreciable effect of metal ions or pH on germination and survival was observed. Zinc, especially at pH 9, showed a stimulatory effect on the growth of seedlings.

In the M_2 generation a variety of chlorophyll deficient mutants were observed in segregating families. The most frequent were *albina*, *xantha* and *viridis* types. However, as it was not possible to analyze all mutants genetically, we preferred to group them in one class as chlorophyll mutants. There were other morphological mutants which could be detected with certainty at the four-leaf stage but these are not included in the data reported. The results in the M_2 generation are presented in Table 1. Mutation rate is expressed both as the percentage of M_1 families segregating and as the percentage of mutant plants in the M_2 population. Neither the buffers alone nor the concentration of Cu and Zn ions used were found to induce any genetic changes. EMS enhanced the mutation rate in all treatments.

An analysis of variance and a chi-square test applied to the data show a significant effect of pH at 1% level both on the basis of the percent of M_1 families segregating and the number of mutants per 100 M_2 plants. The addition of metal ions caused an increase, significant at 1% level, in the percentage of M_2 mutant plants but not in the percentage of M_1 families segregating. From the mutagenic effect of EMS + metal ions relative to the EMS alone (Figure 1), it appears that the effect of Zn is more pronounced at pH 7, whereas for copper a pH of 9 is optimum.

DISCUSSION

The results show that, at the concentrations used, Zn and Cu ions alone are not mutagenic, but they enhance the mutagenic action of EMS. Differences in pH significantly alter the frequency of chlorophyll mutations following EMS treat-

TABLE 1

Effect of EMS alone and in combination with Cu and Zn ions on chlorophyll mutation rate in Arabidopsis

Treatment	No. of M_1 families		No. of plants		Mutation rate	
	Scored	Segregating	Scored	Mutants	Percent M_1 families	Per 100 M_2 plants
Double distilled water	134	0	22140	0	....	...
Buffer pH 5	128	0	21502	0	....	...
Buffer pH 7	132	0	19003	0	....	...
Buffer pH 9	140	1	18369	3	0.76	0.01
Buffer pH 5 + Zn	132	0	19637	0	....	...
Buffer pH 7 + Zn	143	0	21079	0	....	...
Buffer pH 9 + Zn	142	0	20219	0	....	...
Buffer pH 5 + Cu	147	0	23213	0	....	...
Buffer pH 7 + Cu	147	0	19932	0	....	...
Buffer pH 9 + Cu	132	0	19067	0	....	...
Buffer pH 5 + EMS	124	71	19755	1411	57.26	7.14
Buffer pH 7 + EMS	133	62	22020	1224	46.62	5.56
Buffer pH 9 + EMS	148	56	21029	979	37.84	4.55
Buffer pH 5 + Zn + EMS	139	88	18840	1640	63.31	8.70
Buffer pH 7 + Zn + EMS	129	71	17752	1484	55.04	8.36
Buffer pH 9 + Zn + EMS	162	70	22994	1684	43.21	7.32
Buffer pH 5 + Cu + EMS	139	77	17936	1360	55.40	7.58
Buffer pH 7 + Cu + EMS	136	72	17554	1348	52.94	7.68
Buffer pH 9 + Cu + EMS	155	82	20855	1799	52.00	8.63

ment. Both metal ions and the hydrogen ion concentration may directly amplify the initial damage or may act by slowing down the recovery processes. The modifying action of the metal ions appears to be a very complex one involving several mechanisms. Metals are known to function as active groups of several

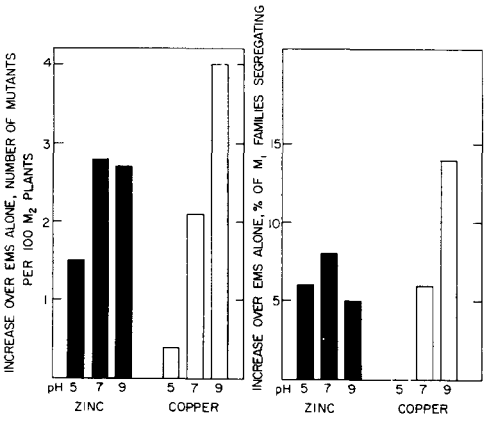

FIGURE 1.—Increase in mutation rate in presence of zinc and copper ions over EMS alone, both as percentage of M_1 families segregating and number of mutants per 100 M_2 plants. The optimal effects are significant at 5% and 1% level for Zn and Cu respectively.

enzymes. von Rosen (1957) attributes mutagenic action of metal ions to enzymatic imbalance caused both by deficiency and excess of metal ions. Moutschen-Dahmen (1963) also suggested the possibility of an enzyme inhibition for increase in EMS induced chromosomal aberrations in presence of metal ions. von Rosen (1964) has suggested the possibility for formation of strong metal chelates in presence of Cu and Zn. Metallic ions like calcium and magnesium are essential for the integrity of the chromosomes (Steffensen and Bergeron 1959) and alkylating agents used in combination with metal chelators enhanced the frequency of chromosomal breaks in *Vicia faba* (Cohn 1961).

The influence of pH on the effectiveness of chemical mutagen treatment has been referred to by Nilan (1964) and by Froese-Gertzen *et al.* (1964). *In vitro* reactions of alkylating agents are discussed by Ross (1957), who suggests that the fraction of sites available for alkylation varies with the pH, depending upon the dissociation constant of the particular group. In addition, it is known that the reaction rate of a mutagen like EMS can be altered considerably by slight modifications in pH (Froese-Gertzen *et al.* 1964). EMS hydrolyzes much faster in basic solutions. The pH of the cell is known to be fairly constant and we do not know the extent to which buffer solution entering the cells of dry seeds can alter the pH. Apparently, the end results do show significant differences. The variation in synergistic effect of metal ions at different pH levels may be due to the availability of ions. In plant nutrition experiments Zn is maximally available in the pH range 5.5 to 7. Outside this range Zn deficiency symptoms can be observed in spite of the presence of Zn in the nutrient.

A large number of loci are known to be associated with chlorophyll development in Arabidopsis and alteration or inactivation of one or more may give a similar phenotypic effect. The present data do not show if the enhanced mutation rate is due to increased chromosomal aberrations or whether the metal ions also increase true gene mutations. Effects of metal ions on mutability of specific loci and the mechanism of their action needs further investigations.

We are grateful to Drs. H. H. Smith and R. E. Heiner for going through the manuscript and to Professor R. Prakken for his interest. The assistance of Mr. Keith Thompson in statistical analysis is also gratefully acknowledged.

SUMMARY

Effects of Cu and Zn ions on the frequency of chlorophyll deficient mutants were studied alone and in combination with 10 mM ethyl methanesulfonate (EMS) at pH 5, 7 and 9. EMS is more mutagenic in the presence of metal ions. Zinc shows a maximum effect at pH 7, Cu at pH 9. The mutagenic efficiency can thus be enhanced in treatments applied at experimentally determined optimal pH and metal ion concentration.

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