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Effects of Hydroxylamine and N-Methyl-N'-Nitro-N-Nitrosoguanidine in *Mimulus cardinalis* (Scrophulariaceae): Survival Curves and Dominant Mutants

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Summary. The lethal and mutagenic effects of hydroxylamine (HA) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were investigated in the higher plant *Mimulus cardinalis*. MNNG was found to be more toxic than HA. The shapes of the survival curves obtained at different concentrations of HA and MNNG are interpreted on the basis of decreased biological activity of the solution to increased age of solution. Based on the appearance of chlorophyll-deficient mutants, MNNG is mutagenic in *Mimulus*. No albinos were detected in HA treated plants. A total of 67 putative mutants were isolated in the mutation spectra of HA and MNNG treated plants. The frequency of mutants induced by HA and MNNG are different. MNNG is mutagenic at 1/10 the concentration of HA in inducing putative mutations in M_1 plants.

Introduction

Hydroxylamine (HA) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) show strong mutagenic effects in microorganisms (Drake 1969). HA induces transitions in bacteriophages (Freese et al. 1961) and *Neurospora crassa* (Malling 1967). The strong pH dependency of its mutagenicity suggests that HA causes transitions primarily from the pair guanine-cytosine to adenine-thymine (Champe and Benzer 1962, Schuster 1961). MNNG is an extremely potent mutagen and carcinogen (Gichner and Velemínský 1967, Malling and de Serres 1969, Müller and Gichner 1964, Sugimura et al. 1966) which may act by methylating replicating DNA (Baker and Tessman 1968, Malling 1967). MNNG produces both transitions and transversions (Baker and Tessman 1968, Eisenstark et al. 1965, Freese et al. 1961, Whitefield et al. 1966, Zampieri et al. 1968).

Comparatively little information is available about the effects of HA and MNNG in higher plants (Blixt 1967, Gichner and Velemínský 1967, Kaul 1969). In this paper some results are reported on the lethal and mutagenic effects of HA and MNNG on *Mimulus cardinalis*, a plant belonging to Scrophulariaceae, the snapdragon family.

Materials and Methods

A strain of *Mimulus cardinalis* collected from Beaver Creek, Siskiyou Co., Calif., was selected as our standard (Pollock et al. 1967). Plants grown under normal greenhouse conditions were self-pollinated. The seeds collected were used as the parental generation (P_1) and these seeds when treated with mutagens are referred to as M_1 generation seeds.

The concentrations of the mutagens used were selected on the basis of solubility of the compounds, previously documented success in other test populations, and several preliminary experiments. Survival curves were established by treating seeds with concentrations of 0.02 M, 0.1 M, and 1.0 M HA and 0.001 M, 0.01 M and 0.05 M MNNG. Subsequently, solutions of 0.1 M HA and 0.01 M MNNG were used in an attempt to induce mutations. These concentrations were selected on the assumption that the most effective mutagenic treatment would be one which approaches the LD_{95} dose.

Hydroxylamine (obtained from Sigma Chemical Co., St. Louis, Mo.) and N-methyl-N'-nitro-N-nitrosoguanidine (obtained from Aldrich Chemical Co., Milwaukee, Wis.) were dissolved, immediately before use, in 0.05 M KH_2PO_4 buffer, pH 7.0. Distilled water and/or buffer solutions were used as controls. Air dried seeds of the P_1 line were immersed in freshly made treatment solutions, about 4 ml per 200 seeds, and incubated, unshaken, at room temperature (Henke and Wilson 1971, Wilson 1971). Immediately after treatment the seeds were rinsed with sterile distilled water and sterilized for one minute in 10% Clorox solution. After Clorox treatment the seeds were rinsed twice in sterile distilled water and plated on Hoagland's (Arditti and Dunn 1969) medium enriched by addition of 1 ml of micro-nutrients (Arditti and Dunn 1969) per liter of solution. The plating medium was solidified by addition of DIFCO-Bacto Agar in concentration of 2 percent (*w/v*). Petri dishes containing about 30 ml of solidified sterile medium were overlayed with the treated seeds. The standard growth conditions were the incubation of seeds at 25 °C under about 900 ft-c of continuous cool-white fluorescent light.

The number of survivors at the end of a 24 day germination period were used to determine survival curves. Plants with foliar leaves and root systems were scored as survivors. The surviving seedlings were planted in sterilized soil in $3^1/_2$ inch pots and grown under greenhouse conditions. Most mutations in the M_1 line were observed and scored while self-crossing the mature M_1 plants. When survivors were scored, a record was kept of the number of aberrant plants. The aberrations were recorded in categories as described in the next section. A few aberrant plants were recorded while scoring for germination.

[1] A portion of this work will be submitted by the senior author to the Faculty of Miami University in partial fulfillment of the Doctor of Philosophy degree.

Results

Effect of Mutagen Concentration on Survival and Growth

Survival curves were determined using three different concentrations of HA and MNNG (Fig. 1 and 2). At the higher doses the seedlings do not form foliar leaves, the cotyledons are often discolored, and the roots are poorly developed. Root length inhibition has been used as a criterion to determine mutagenic activity in M_1 plants (Gichner and Velemínský 1967, Velemínský et al. 1967). Gichner and Velemínský found in *Arabidopsis* that increased concentrations of mutagen decreases the relative root length of M_1 plants (Gichner and Velemínský 1967) . This same observation was made by us as an effect of HA and MNNG in *Mimulus*. In young plants strong growth inhibition is observed after mutagenic treatment, especially at high doses. The plants are at the outset considerably smaller than untreated plants of the same age.

A total number of 123,000 seeds were used in establishing the survival curves. The effect of lethal activity was evaluated by regressing the percent survival on dose and the natural logarithm of survival on dose. The best fit was obtained for both HA and MNNG regressing the natural logarithm of survivors on dose (Fig. 1 and 2). From the regression lines, for each concentration of mutagen used, the LD_{50} and LD_{95} was estimated. The observed lethal doses used in assaying for M_1 mutants (Table 1) were calculated and compared to the above estimates.

Survival curves may be valuable in relating the effectiveness of killing to mutagenic efficiency. The observation that the number of mutations is a function of the extent of killing has been reported to occur in many plants such as ferns (Howard and Haigh 1968), Triticale, *Vicia faba* (Gichner et al. 1963, Kaul 1969) and *Potentilla* (Asker 1966).

Mutagen Dose and Mutation Rate

The doses used, observed and estimated, in isolating mutants are shown in Table 1. The lethal doses used approach the LD_{95} level; this is in contradiction to the suggested use of an LD_{50} by many researchers (Baker and Tessman 1968, Ehrenberg und Gustafsson 1957, Ehrenberg et al. 1958). A relationship does exist between effective killing dose and the frequency of aberrant survivors in many organisms. Studies are underway to determine what lethal dose, LD_{50} or LD_{95}, is most efficient in inducing mutations in *Mimulus*. At the lethal doses used in this study HA induced 22 percent and MNNG induced 78 percent of all mutants isolated (Table 2).

Classification and Description of Putative Mutants

The mutant plants were categorized and the mutagenic efficiency of HA and MNNG was determined (Table 2).

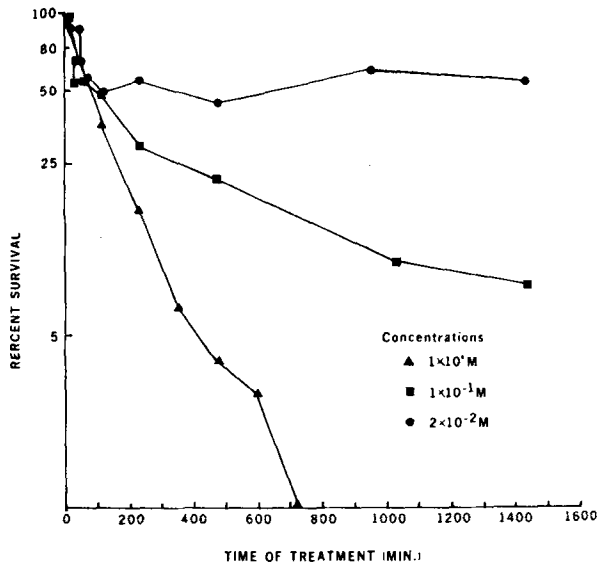

Fig. 1. Lethal effect of hydroxylamine on *Mimulus cardinalis*

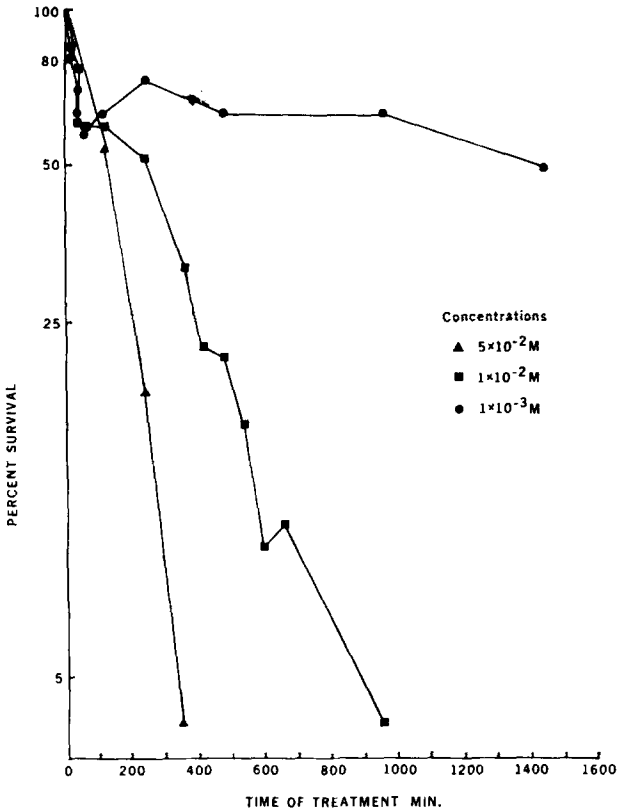

Fig. 2. Lethal effect of N-methyl-N'-nitro-N-nitrosoguanidine on *Mimulus cardinalis*

Chlorophyll-Deficient Mutants. Initial readings of chlorophyll-deficiency were made at the seedling stage to permit detection of lethals. Analogies are made here to the classification system for chlorophyll mutants in barley (Gaul 1964). Three basic patterns of chlorophyll-deficiency were observed in *Mimulus*:

Table 1. *Lethal activity of hydroxylamine (HA) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in Mimulus cardinalis*

Mutagen	Concentration	LD_{50}[a] t (min)	LD_{95}[a] t (min)	Estimated[a] t (min)	Estimated[a] LD	Observed t (min)	Observed LD	Observed n
HA	0.02 M	1,503	13,746	1,440	47	1,440	53	1,200
	0.1 M	83	1,039		97[b]		92.4[b]	18,200
	1.0 M	47	455		99+		100	1,200
MNNG	0.001 M	1,778	14,141	540	35	540	38	1,200
	0.01 M	166	886		85[b]		90.3[b]	48,800
	0.05 M	133	346		99+		100	1,200

[a] The lethal dose (LD) was estimated from a regression of the natural logarithm of the percent survival on dose.
[b] Dose used to induce M_1 mutants.

Table 2. *Mutagenic activity of hydroxylamine and N-methyl-N'-nitro-N-nitrosoguanidine in Mimulus cardinalis*

	Mutagen HA	Mutagen MNNG
Number of survivors scored[a]	18,200	48,800
Number of survivors grown to maturity[b]	267	231
Putative Mutant Classes		
Chlorophyll-deficient mutants (viable and non-viable)	0	11[c]
Anthocyanin leaf mutants	1	2
Sterility	0	3
Split and/or alternate leaf mutants	2	5
Miscellaneous morphological mutants	12	31
Total mutants	15	52

[a] All survivors were assayed for lethal albinos.
[b] Only mature survivors were assayed for viable mutants.
[c] Nine of these mutants exhibited cytoplasmic inheritance; two were dominant. The genetics and ultrastructure of these mutants have been studied (Travis et al., in prep.).

(1) "Striata type" plants with white-green variegated leaves. All leaves of this type in *Mimulus* died with a mean survival time of 2 months. The plants persisted and developed more variegated leaves. (2) "Viridis type" plants with pale yellow-green variegated leaves. The plant and leaves persisted; they did not die. (3) "Albina type" plants with stems and leaves mostly albino with some white-green and some palegreen variegated leaves. These plants did not live past the seedling stage.

Anthocyanin Leaf Mutants. Chlorophyll Absent and Anthocyanin Present in Normally Anthocyaninless Leaves. At least one of the anthocyanin leaf mutants produced flowers which appeared light-orange rather than the normal scarlet. The throat of the mutant, however, was the normal scarlet. The quantity of anthocyanin is less in the mutant flowers. Mutations affecting anthocyanin synthesis are needed for biochemical studies (Knowles-v. Wettstein 1969). The possibility that genes may affect the quantity and distribution of anthocyanins in *Mimulus* is being investigated.

Dominant Lethals. Mature Plant Produces all Sterile Flowers. Flowers of the standard possess four stamens. The mutant flowers have the normal floral parts but lack the production of mature pollen. Sterility does not show a marked increase in treated seeds as compared to the control group. Sterility is commonly a side effect of mutagenic treatments (Gichner and Velemínský 1967).

Split and/or Alternate Leaf Mutants. All split leaf aberrants also exhibit alternate leaves. All alternate leaf plants do not show split leaves. In at least two cases, involving split leaf aberrants, the flowers produced were abnormal. In one case double flowers, fused corollas, were borne at the axis of the split leaves. The double corolla possessed twice the normal reproductive floral parts, 8 stamens and two stigmas. The flowers are fertile; mature pollen is produced and upon selfing seed is harvested. In the other case of split leaf plants bearing abnormal flowers, anthocyanin production is absent in lined sectors of the corolla. The white lines originate from the connate margins of the corolla tube and extend about $^1/_2$ inch inward toward the scarlet throat of the corolla.

Miscellaneous Morphological Mutants. This category includes all obviously altered plants that could not be classified into one of the other groups and consisted primarily of alternations in leaf and flower morphology. Some of the miscellaneous aberrations scored are: corollas split and/or flowers with aberrant sepals, leaves spotted, leaves curled, growth at base of shoot, corollas forked rather than smoothly lobed and three lobed corollas rather than the normal five lobed corolla tubes.

Discussion

Mutation research in higher plants is initiated to find mutations affecting nutrition or resistance to chemicals and/or morphological characters (Hagberg and Persson 1968). An analysis of mutagens involves their mutagenic efficiencies in inducing aberrant characters. As expected many characters are reces-

sive in the M_1 plants appearing only in the M_2 generation. Yet, chlorophyll-deficient phenotypes are frequently detected in M_1 plants (Ehrenberg and Gichner 1967, Gaul 1964, Gichner and Velemínský 1965, Gustafsson and Garr 1966, Monti 1968). As already noted, chlorophyll-deficient plants have been detected in MNNG treated *Mimulus*. These chlorophyll mutants did not respond to the exogenous application of various metabolites as did some of the auxotrophic chlorophyll mutants in *Arabidopsis* (Rédei 1967) and tomato (Boynton 1966). However, the isolation of chlorophyll-deficient phenotypes (Boynton 1966, Rédei 1967), and other morphological characters such as leaf-spotting (Blixt and Mossberg 1967, Blixt and Gelin 1965), may give a more reliable indication of mutagenic effect than survival rates (Ehrenberg and Gichner 1967, Gaul et al. 1966, Kaul 1969, Monti 1968, Velemínský et al. 1967).

The results of this study indicate that MNNG is more toxic and more mutagenic than HA at similar concentrations. At the lowest concentrations of HA and MNNG, 0.02 M and 0.001 M respectively, prolonged treatment does not lead to an increased toxic effect. At these low concentrations the relationship between kill and treatment time is not linear; it appears asymptotic. At these lowest concentrations, HA solutions lose activity after approximately 14 hours, and MNNG solutions lose activity after about 12 hours. This observation of decrease of biological toxicity in relation to increased age of solution is important if mutagenic effects are to be correlated with survival (Velemínský et al. 1967). Thus the duration of the mutagenic treatment with HA and MNNG at the lowest concentrations is a critical factor after 12—14 hours, i.e. killing will cease and these treatments will not yield high kills such as required to obtain an LD_{95}.

Both HA and MNNG cause a decrease in seedling growth and in percent survival. Seedling growth was inhibited with the treatment time approaching the LD_{50}. At treatment times above the LD_{50} the inhibition of growth was nearly constant. Plants treated with high doses of HA and MNNG exhibited only a slight reduction of seed set (Table 2) as opposed to the results of others in rice (Gustafsson and Garr 1966), barley (Gaul et al. 1966), *Arabidopsis* (Gichner and Velemínský 1967), *Potentilla* (Asker 1966), peas (Blixt and Mossberg 1967), etc. MNNG induced a higher frequency of chlorophyll-deficient mutations than HA. In fact, no albinos were detected in HA treated plants. Thus, based on the appearance of chlorophyll-deficient plants, MNNG can be considered mutagenic in *Mimulus*. However, the mutagenicity of HA cannot be ruled out since some identical mutant categories have shown up in both HA and MNNG treated plants. MNNG is mutagenic at 1/10 the concentration of HA in inducing putative mutations in M_1 plants.

The results of genetic and ultrastructural analysis in preparation will aid in understanding the response of the seeds to HA and MNNG (Travis et al., in prep.).

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