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Peroxidase activities in the floral tissues of *Tradescantia* clone BNL 4430 treated with maleic hydrazide alone, X rays alone, or in combinations

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Changes in peroxidase activity in young inflorescences of *Tradescantia* clone BNL 4430 were investigated after treating with maleic hydrazide (MH) alone, X rays alone, or MH and X rays in combinations. MH is a promutagen known to be activated into a mutagen in plant cells. The treatments with MH were conducted for 4 h at a fixed concentration of 1 mM, and the X-ray doses applied were 768 to 882 mGy. For the combined treatments, X rays were delivered either 20 h before starting, or 2 or 20 h after completing the 4-h MH treatments. The treatments with MH alone and X rays alone both increased peroxidase activity, as compared with the controls. The combined treatments of delivering X rays 20 h before starting the MH treatments resulted in marked increases in the activity of this enzyme. However, the increases in peroxidase activity after the combined treatments of X-raying 2 h after completing the MH treatments were small, and the activity rather decreased by X-raying 20 h after completing the MH treatments. These results are consistent with our earlier findings that clear synergistic effects of MH and X rays in inducing somatic pink mutations in the stamen hairs of clone BNL 4430 were detected by treating with X rays before MH treatments, whereas antagonistic effects of MH and X rays were often observed by delivering X rays during or after MH treatments (Xiao and Ichikawa, 1995). It is therefore highly likely that peroxidase is certainly involved in the activation of MH into a mutagen, and that the increases and decreases in peroxidase activity after different combined treatments are related to the occurrences of synergism and antagonism, respectively, between MH and X rays.

INTRODUCTION

Many different types of mutagens and/or carcinogens (mostly produced by human activities) exist together in our environment, but most of mutagenicity or carcinogenicity testings have dealt with the effects of individual agents. Since combinations of two or more different agents may results in unknown effects significantly modified from those expected from their additive effects, intensive studies on interactions between different agents are urgently needed. Surveys of synergistic effects between different agents are particularly important, because the occurrence of synergism has implications for current risk evaluation of individual agents (Ichikawa, 1992; Ichikawa et al., 1993; Shima and Ichikawa, 1994, 1995; Xiao and Ichikawa, 1995). In fact, mutagenic synergisms among several monofunctional alkylating agents and X rays have clearly been demonstrated using *Tradescantia* stamen-hair system (Cebulska-Wasilewska et al., 1981; Ichikawa, 1992; Ichikawa et al., 1993; Shima and Ichikawa, 1994, 1995).

Mutagenic interactions between maleic hydrazide (MH) and X rays have also been studied in the stamen hairs of *Tradescantia* clone BNL 4430, and clear synergistic effects of MH and X rays in inducing somatic mutations have been detected by treating with X rays before MH treatments, whereas antagonistic effects of MH and X rays have often been observed by delivering X rays during or after MH treatments (Xiao and Ichikawa, 1995). MH has been extensively applied as a herbicide, fungicide, growth inhibitor and growth regulator in agriculture (Fishbein, 1972), and is a promutagen known to be activated into a mutagen in plant cells (Gichner et al., 1982; Plewa and Gentile, 1982; Heindorff et al., 1984; Veleminsky and Gichner, 1988). The mechanism of the activation of MH in plant cells has not yet been fully understood, but the

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involvement of peroxidase has been thought very likely, as discussed earlier (Xiao and Ichikawa, 1995). If MH is activated by peroxidase, it becomes easier to understand the antagonistic effects of MH and X rays in cases of X-raying during or after MH treatments as the result of X-ray-caused suppression of the activation of MH (Xiao and Ichikawa, 1995).

This paper describes the results of a series of experiments which have been conducted in order to clarify the roles of peroxidase in activating MH into a mutagen and also in the mechanisms of the occurrences of both synergism and antagonism between MH and X rays.

MATERIALS AND METHODS

Clone used. Young inflorescence-bearing shoots with roots of *Tradescantia* clone BNL 4430 as described earlier (Shima and Ichikawa, 1994; Ichikawa et al., 1995) were used as the tester plants in the present study. This clone is a diploid hybrid ($2n$=12) between a blue-flowered *T. hirsutiflora* Bush and a pink-flowered *T. subacaulis* Bush (Emmerling-Thompson and Nawrocky, 1980), thus is a blue/pink heterozygote. This clone has been frequently used in studies of induced somatic mutations in the stamen hairs as reviewed earlier (Schairer and Sautkulis, 1982; Schairer et al., 1983; Ichikawa, 1992), and has been shown to be more sensitive to alkylating agents than other clones (Sparrow et al., 1974; Nauman et al., 1976; Ichikawa et al., 1993).

Growing conditions. The young inflorescence-bearing shoots with roots were cultivated in a nutrient solution circulating (NSC) growth chamber (Kyosin Riko Co.) designed for our requirements (Shima and Ichikawa, 1994). The environmental conditions in the NSC growth chamber were 22.0±0.5°C during the 16-h day with a light intensity of 7,500 lux from white fluorescent tubes, and 20.0±0.5°C at night. The nutrient solution used was 1/2,000 Hyponex solution.

Treatments with MH. Young inflorescences just before initiating flowering were treated with 1 mM MH (CAS registry no. 123-33-1; Wako Pure Chemical Industries, Ltd) dissolved in phosphate buffer (pH 7.0), for 4 h at 23.0± 0.5°C. The methods used for the treatments were identical to those in our earlier study (Xiao and Ichikawa, 1995). The methods were originally developed by Ichikawa and Takahashi (1978) for treating *Tradescantia* inflorescences with aqueous chemical mutagens, and have been used for treating with alkylating agents (Ichikawa and Takahashi, 1978; Ichikawa et al., 1990, 1993, 1995; Sanda-Kamigawara et al., 1991; Ichikawa, 1992; Shima and Ichikawa, 1994, 1995). Control inflorescences were treated with the phosphate buffer.

X-ray treatments. A Hitachi MBR-1505R X-ray generator was used for X-ray treatments of young inflorescences. The treatments were conducted acutely at 150 kVp and 4 mA with a 0.5 mm Al+0.1 mm Cu filter at 23.0±0.5°C. The exposures were measured simultaneously with thermoluminescence dosimeter (TLD) elements (National UD-170L) set at the same target distance as the inflorescences treated, and with a thermoluminescence reader (National UD-502B). The exposure data obtained in R were converted into absorbed doses in Gy with a converting factor of 9.57×10^{-3} (i.e., 1 R=9.57 mGy). The doses applied were relatively uniform being 768 to 882 mGy.

Combined treatments with MH and X rays. Young inflorescences were treated with 1 mM MH for 4 h and with 768 to 882 mGy X rays in three different ways. Namely, the X-ray treatments were conducted either 20 h before starting, or 2 or 20 h after completing the 4-h MH treatments.

Peroxidase assay. Peroxidase activities were measured following the methods described by Gichner et al. (1994), except for minor modifications. The inflorescences treated with MH alone, X rays alone, MH and X rays in combinations or with the phosphate buffer (control) were harvested either immediately, 12 h, or 24 h after completing these single or combined treatments, and were quickly homogenized in liquid nitrogen. The resultant powder was solubilized in cold homogenation buffer (2.5% polyvinylpyrrolidone, 50 mM citrate phosphate buffer, pH 6.5) at a concentration of 1 g powder to ca. 7 ml buffer, and was kept at 4°C for 1 h. The suspension was centrifuged at 15,000 x g for 10 min, and the supernatant fluid recovered was kept at 4°C. For each sample, 450 µl of the supernatant was retained and heated in a boiling water bath for 10 min for use in the preparation of the spectrophotometric blank. The reaction mixture consisted of 1 ml of 0.3% hydrogen peroxide, 200 µl of the supernatant, 800 µl of 50 mM citrate phosphate buffer (pH 6.5), and 1 ml of 1% guaiacol, the final volume being 3 ml. Adding the guaiacol solution last, the cuvette was quickly turned upside down twice, and the absorbance at 470 nm was measured every 30 sec for 5 min using a Beckman DU-50 series spectrophotometer. The measurements were made on five samples (each taken from one inflorescence) per treatment, and the intermediate three out of the five values obtained (i.e., excluding the highest and lowest) were averaged to determine the peroxidase activity.

Protein analysis. The protein content of the supernatant fluid of each sample was determined by Bio-Rad protein assay, and a protein concentration standard curve was prepared for each treatment.

RESULTS

X-raying 20 h before starting 4-h MH treatment. The peroxidase activity measured immediately after completing the combined treatment of exposing to 793 mGy of X rays 20 h before starting 4-h 1 mM MH treatment was almost comparable to that determined immediately after the MH treatment alone (Fig. 1A). These activities were slightly lower than those 24 h after the X-ray treatment alone and in the control as seen in the same figure.

A greatly increased peroxidase activity was measured 12 h after completing the combined treatment of 822 mGy X rays 20 h before starting the MH treatment (Fig. 1B). The activities measured 36 h after the X-ray treatment alone and 12 h after the MH treatment alone also increased as seen in the same figure.

The peroxidase activity determined 24 h after completing the combined treatment of 793 mGy X rays 20 h before starting the MH treatment was still considerably higher than that in the control, although the activity decreased to levels closer to the control 24 h after the MH treatment alone and 48 h after the X-ray treatment alone (Fig. 1C).

These results showed that X-raying 20 h before starting MH treatment resulted in increased peroxidase activities 12 to 24 h after completing the combined treatment.

X-raying 20 h after completing 4-h MH treatment. The peroxidase activity determined immediately after completing the combined treatment with 1 mM MH for 4 h and 857 mGy X rays 20 h after the MH treatment was nearly comparable to those 20 h after the MH treatment alone and immediately after the X-ray treatment alone (Fig. 2A). These activities were higher than that in the control as seen in the same figure.

The peroxidase activity increased 32 h after the MH treatment alone (Fig. 2B). On the other hand, the activity decreased 12 h after completing the combined treatment with MH plus 768 mGy X rays and 12 h after the X-ray treatment alone, becoming comparable to that in the control.

A decrease in the peroxidase activity was observed 44 h after the MH treatment alone, but the activity was still higher than in the control. The activity 24 h after 857 mGy X-ray treatment alone showed a slight increase and was comparable to that 44 h after the MH treatment alone (Fig. 2C). The activity 24 h after the combined treatment was lower than that in the control.

These results showed that X-raying 20 h after the MH treatment resulted in lower peroxidase activities 12 to 24 h after completing the combined treatment, as compared with the MH treatment alone.

X-raying 2 h after completing 4-h MH treatment. The peroxidase activity measured immediately after completing the combined treatment with 1 mM MH for 4 h and 882 mGy X rays 2 h after the MH treatment was slightly lower than those immediately after the X-ray treatment alone and in the control, but was slightly higher than that 2 h after the MH treatment alone (Fig. 3A).

The peroxidase activity 12 h after completing the combined treatment with MH plus 790 mGy X rays was somewhat higher than those 12 h after the X-ray treatment alone, 14 h after the MH treatment alone and in the control (Fig. 3B).

The activity 24 h after completing the combined treatment with MH plus 882 mGy X rays was higher than that in the control, but was obviously lower than that 26 h after the MH treatment alone and also lower than that 24 h af-

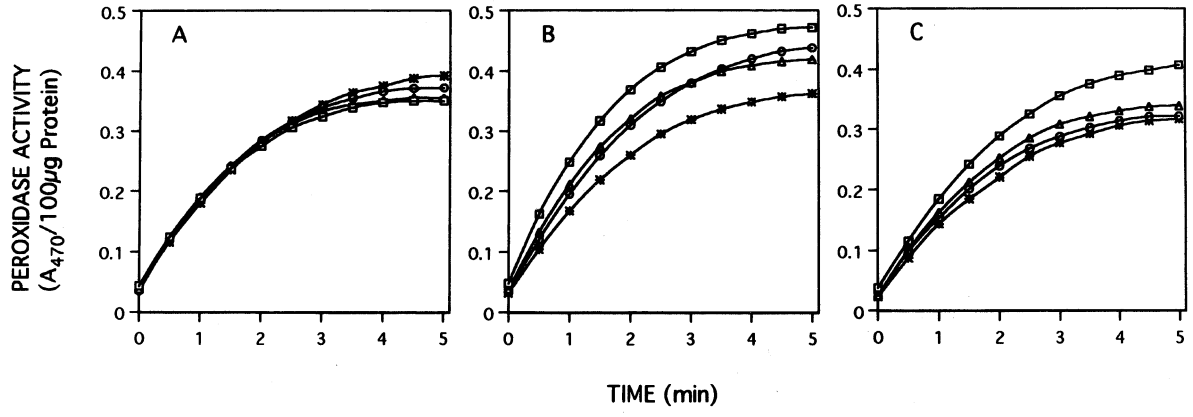

Fig. 1. Peroxidase activities in the young inflorescences of clone BNL 4430 exposed to X rays 20 h before starting 4-h MH treatment at 1 mM (□), treated with MH alone (△), exposed to X rays alone (○), and treated with phosphate buffer as controls (*). The data obtained 0 (immediately), 12 and 24 h after the combined treatments and treatments with MH alone or the buffer (24, 36 and 48 h after X-ray treatments alone) are shown in A, B and C, respectively. The data were taken every 30 sec for 5 min. The X-ray doses applied were 793 mGy in A and C and 822 mGy in B.

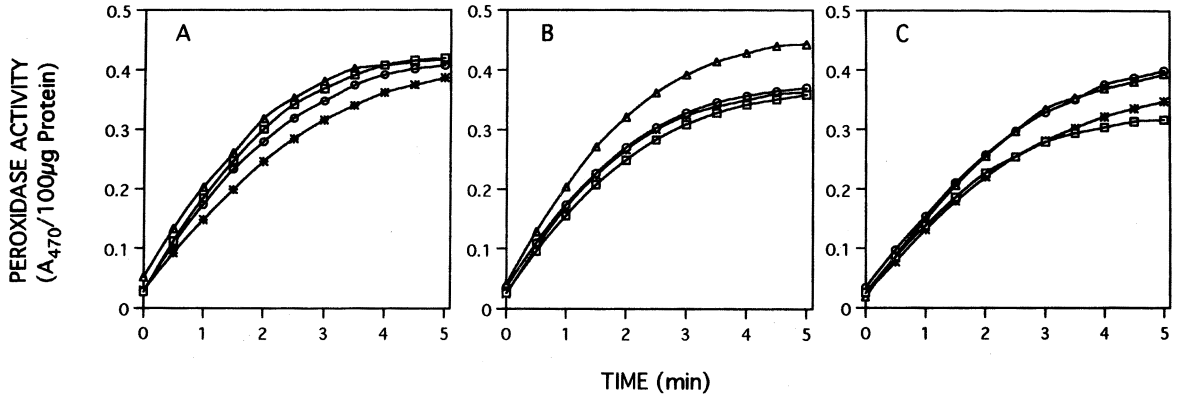

Fig. 2. Peroxidase activities in the young inflorescences of clone BNL 4430 exposed to X rays 20 h after completing 4-h MH treatment at 1 mM (□), treated with MH alone (△), exposed to X rays alone (○), and treated with phosphate buffer as controls (*). The data obtained 0 (immediately), 12 and 24 h after the combined treatments and X-ray treatments alone (20, 32 and 44 h after treatments with MH alone or the buffer) are shown in A, B and C, respectively. The data were taken every 30 sec for 5 min. The X-ray doses applied were 857 mGy in A and C and 768 mGy in B.

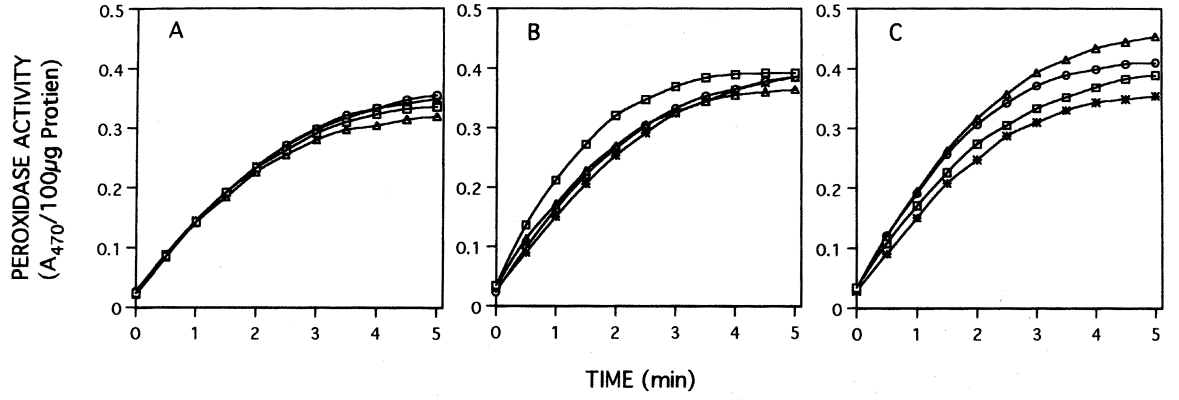

Fig. 3. Peroxidase activities in the young inflorescences of clone BNL 4430 exposed to X rays 2 h after completing 4-h MH treatment at 1 mM (□), treated with MH alone (△), exposed to X rays alone (○), and treated with phosphate buffer as controls (*). The data obtained 0 (immediately), 12 and 24 h after the combined treatments and X-ray treatments alone (2, 14 and 26 h after treatments with MH alone or the buffer) are shown in A, B and C, respectively. The data were taken every 30 sec for 5 min. The X-ray doses applied were 882 mGy in A and C and 790 mGy in B.

ter the X-ray treatment alone (Fig. 3C).

These results showed that X-raying 2 h after the MH treatment resulted in a higher peroxidase activity 12 h after completing the combined treatment, as compared with the MH treatment alone, but the activity 24 h after completing the combined treatment was lower than that after the MH treatment alone.

DISCUSSION

Fluctuations in peroxidase activity in controls. For comparing and evaluating the results of peroxidase assays after different treatments or at different times after each treatment, it is necessary to examine the extent of fluctuations in the peroxidase activity in control inflorescences. The measurements of the peroxidase activity were conducted three times on the inflorescences of the shoots with roots cultivated in the NSC growth chamber. The peroxidase activities in these inflorescences were 0.277 to 0.359 A_{470}/100 μg protein at 5 min (after starting the measurements). On the other hand, the peroxidase activities measured in the nine controls (3 treatments x 3 assaying times) ranged between 0.310 to 0.393 A_{470}/100 μg protein at 5 min. Considering these fluctuations in the peroxidase activity in control inflorescences, it was judged to be necessary to compare and evaluate the peroxidase activities after various treatments as relative values against each control.

Peroxidase activities after MH treatment alone. The peroxidase activities 0 to 44 h after the 1 mM MH treatments for 4 h are seen in Figs. 1 to 3. Namely, the activities measured 0 (Fig. 1A), 2 (Fig. 3A), 12 (Fig. 1B), 14 (Fig. 3B), 20 (Fig. 2A), 24 (Fig. 1C), 26 (Fig. 3C), 32 (Fig.

2B) and 44 h (Fig. 2C) after the MH treatments are shown in these figures. When these activities are converted into relative values against each control, it becomes clear that the relative activity increased 20 to 44 h after the MH treatments, the values of 1.281±0.209 and 1.224±0.049 for 26 and 32 h after the MH treatments, respectively, being especially high (Table 1).

These results agree with earlier reports that the activity of peroxidase increased very much when MH existed, thus MH was considered to become the substrate of this enzyme (Andreae and Andreae, 1953: Kenten, 1955). More recently, some indirect evidences on the activation of MH by peroxidase have been presented (Heindorff and Rieger, 1984; Veleminsky and Gichner, 1988; Gichner et al., 1988). For example, it has been reported that the MH-induced somatic mutation frequency in the stamen hairs of clone BNL 4430 decreased remarkably by the pre-treatment with diethyldithiocarbamate (DEDTC), an agent suppressing peroxidase activity (Gichner et al., 1988). It has also been shown that peroxidase is important for activating some other promutagens into mutagens in plant cells (Stiborova and Anzenbacher, 1991; Plewa et al., 1991; Plewa and Wagner, 1993; Seo et al., 1993; Gichner et al., 1994). The present results show that peroxidase is certainly involved in the activation of MH in *Tradescantia* cells, and also that the activation of MH starts about 20 h after the MH treatment and is still in progress even 44 h after the MH treatment.

Table 1. Relative peroxidase activities against each control 0 to 44 h after completing 4-h MH treatments at 1 mM

Hours after MH treatment	Relative activity of peroxidase (±SD)	Corresponding figure
0	0.901±0.091	Fig. 1A
2	0.914±0.131	Fig. 3A
12	1.159±0.075	Fig. 1B
14	0.946±0.027	Fig. 3B
20	1.081±0.029	Fig. 2A
24	1.068±0.073	Fig. 1C
26	1.281±0.209	Fig. 3C
32	1.224±0.049	Fig. 2B
44	1.132±0.088	Fig. 2C

Peroxidase activities after X-ray treatment alone. The peroxidase activities 0 to 48 h after acute 768 to 882 mG X-ray treatments are also seen in Figs. 1 to 3. Namely, the activities measured 0 (Figs. 2A and 3A), 12 (Figs. 2B and 3B), 24 (Figs. 1A, 2C and 3C), 36 (Fig. 1B) and 48 h (Fig. 1C) after the X-ray treatments are presented in these figures. When these activities are converted into relative values against each control as shown in Table 2, it is seen that the highest relative activity was observed 36 h after the X-ray treatment. Since the X-ray doses applied were different between treatments, the relative activities per average X-ray dose (818.7 mGy) are also calculated as shown in Table 2. It is more clearly seen in this table that the relative activity started to increase 12 h after, and became highest (1.206±0.109) 36 h after the X-ray treatment.

These increases in the peroxidase activity are considered to be related to the production of free radicals from water by X rays and the resultant production of hydrogen peroxide which is required for peroxidative reaction catalyzed by peroxidase.

Peroxidase activities after combined treatments with MH and X rays. Combined treatments with acute 793 or 822 mGy X rays 20 h before the 1 mM MH for 4 h resulted in marked increases in peroxidase activity 12 and 24 h after completing the treatments (Figs. 1B and 1C). It is easy to understand the increased peroxidase activities occurred 12 and 24 h after completing the combined treatments (36 and 48 h after the X-ray treatments),

Table 2. Relative peroxidase activities against each control and those per average X-ray dose (818.7 mGy) 0 to 48 h after acute X-ray treatments with 768 to 882 mGy

Hours after X-ray treatment	Relative activities of peroxidase (±SD)	Per average X-ray dose (±SD)	Corresponding figure
0	1.055±0.017	1.008±0.016	Fig. 2A
	1.018±0.049	0.945±0.045	Fig. 3A
		(av. 0.977)	
12	1.021±0.165	1.088±0.176	Fig. 2B
	1.002±0.060	1.038±0.062	Fig. 3B
		(av. 1.063)	
24	0.949±0.062	0.980±0.064	Fig. 1A
	1.151±0.087	1.100±0.083	Fig. 2C
	1.157±0.121	1.074±0.112	Fig. 3C
		(av. 1.051)	
36	1.211±0.109	1.206±0.109	Fig. 1B
48	1.014±0.089	1.047±0.092	Fig. 1C

since the MH treatment alone increased the peroxidase activity 20 to 44 h later with a peak at 26 to 32 h (Table 1), and X-ray treatments alone also increased the activity 12 to 48 h later with a peak at 36 h (Table 2). The enhanced peroxidase activities are consistent with our earlier detection of clear synergistic effects of MH and X rays in inducing somatic pink mutations in the stamen hairs of clone BNL 4430 by treating with X rays before MH treatment (Xiao and Ichikawa, 1995), and this clear relationship between the enhanced peroxidase activities and the occurrences of mutagenic synergism supports strongly the above conclusion that peroxidase is certainly involved in the activation of MH in *Tradescantia* cells.

On the contrary, by exposing acutely to 857 or 768 mGy X rays 20 h after the 1 mM MH treatment for 4 h, peroxidase activities measured 12 and 24 h after the combined treatments were lower than those 32 and 44 h after the MH treatment alone or 12 and 24 h after the X-ray treatments alone and in the controls (Figs. 2B and 2C). Since it is certain that peroxidase is involved in the activation of MH, the lower peroxidase activities confirmed are considered to be the results of suppressions of the activation of MH by X rays which were delivered 20 h after the MH treatment, i.e., at the time when MH was going to be activated (see Table 1). Peroxidase catalyzes not only peroxidative reaction but also oxidative reaction which requires oxygen. Early report of highly significant decrease of MH-induced chromosomal aberrations in *Vicia faba* under anoxic conditions (Kihlman, 1956) indicates that oxygen is required for the activation of MH. On the other hand, an obvious oxygen enhancement effect on X-ray-induced mutations in the stamen hairs of *Tradescantia* has been demonstrated (Underbrink et al., 1975). Namely, oxygen is required for the appearance of X-ray-induced genetic damages and also for the activation of MH. The suppressed peroxidase activities measured after the combined treatments of X-raying 20 h after the MH treatment are therefore considered to be the results of competitive requirements of oxygen between the genetic effects of X rays and the activation of MH, as discussed earlier (Xiao and Ichikawa, 1995). The suppressed peroxidase activities are also consistent with our earlier findings that antagonistic effects were often detected between MH and X rays in inducing pink mutations in the stamen hairs of clone BNL 4430 by treating with X rays after MH treatment (Xiao and Ichikawa, 1995).

Combined treatment with acute 790 mGy X rays 2 h after the 1 mM MH for 4 h produced a higher peroxidase activity 12 h after the combined treatment than after the MH treatment alone or X-ray treatment alone (Fig. 3B), but the activity measured 24 h after the combined treatment with acute 882 mGy X rays 2 h after the MH treatment was lower than those after the MH or X-ray treatment alone, although it was higher than that in the control (Fig. 3C). Also, the increases in peroxidase activity observed were much smaller than in the case of X-raying 20 h before the MH treatment (see Figs. 1B and 1C). The results obtained seem to agree with our earlier detection of the antagonistic effect of MH and X rays in one of the three experiments in which X rays were delivered during the MH treatment (Xiao and Ichikawa, 1995).

In the present study, however, the peroxidase activities measured were those for all species of peroxidases which existed in *Tradescantia* cells. Since it is well known that many different peroxidase isozymes are found in plant tissues, it seems necessary to identify the peroxidase species which activate(s) MH into a mutagen, for elucidating fully the mechanisms of activation of MH in plant cells.

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