# EFFECT OF MH APPLICATION TIME ON METHANOL-SOLUBLE AND INSOLUBLE RESIDUES IN FLUE-CURED TOBACCO[1]

TOBACCO SCIENCE

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Field experiments were conducted in 1983 and 1986 to determine the relationships among sucker control, level of methanol-soluble and methanol-insoluble MH, and the time of day that MH was applied. Residues for both fractions were highest from applications made at 1000 and 1400 h and lowest from applications made at 0200 and 0600 h. Residue concentrations from applications made at 1800 and 2200 h tended to be intermediate. Sucker control varied with time of day of MH application. The best control was obtained with application times that gave the highest MH residues.

**Additional key words: *Nicotiana tabacum*, pesticide residues.**

## INTRODUCTION

The plant growth regulator MH (1,2-dihydro-3,6-pyridazinedione), which is also known by the trivial name maleic hydrazide, has been used for about 40 years as a systemic inhibitor of axillary buds, or suckers, on decapitated tobacco plants (12). After application, MH is readily taken up by the leaves. Absorption is highest under high humidity conditions when leaf cells are turgid (25). Sucker control has been shown to be better with applications made between 0700 and 0800 h or 1300 to 1400 h as opposed to applications made between 1900 and 2000 h (20). These results suggest that time of day may affect the amount of MH absorbed, or if MH is equally absorbed throughout the day, some detoxification of MH may occur or the mode of action may be altered by the time of application. Once inside the plant, MH is translocated through the xylem and phloem and concentrated in the meristematic regions of the plant (8, 9, 10, 11) where inhibition of growth occurs.

The growth regulating activity of MH appears to be due to inhibition of cell division (6, 13). The binding site is still unknown, but several hypotheses have been proposed, including the incorporation or binding of MH with nucleic acids. The close structural similarity between MH and the pyrimidine base, uracil, has led some investigators to hypothesize that MH replaces uracil in the ribonucleic acid (RNA) molecule (3, 4, 6, 7). Baker (2) reported a high concentration of radioactivity associated with or incorporated into nuclei and cytoplasm of the meristematic region of tobacco roots treated with $^{14}C$-labeled MH. Callaghan and Grun (4) showed that the $^{14}C$ from labeled MH was incorporated into the chromosomal material of the nuclei of root tips of three different plant species. Later, Callaghan et al. (3) showed that MH was incorporated into the RNA of yeast cells. Coupland and Peel (6) also found that labeled MH was incorporated into RNA of willow tree root tips. Appleton et al. (1) recently found that MH replaced cytosine in the RNA of yeast cells. In addition, MH has been found bound to protein (2, 6, 14, 16, 18).

The effectiveness of MH for suppressing axillary bud growth appears to be influenced by the amount of MH absorbed into the plant and ultimately bound, and by the time of day it is applied. The present study was undertaken to investigate further the effect of the time of day when MH is applied on retardation of sucker growth and on the levels of methanol-soluble and methanol-insoluble MH.

## MATERIALS AND METHODS

### Field Procedures

Twenty-two flue-cured tobacco (*Nicotiana tabacum* L.) plants were transplanted in rows 1.1 m apart and 0.56 m in the row on May 17, 1983, and May 6, 1986, on the Central Crops Research Station near Clayton, N.C. In 1983, 'McNair 944' was grown on a Marlboro loamy sand, and in 1986 'McNair 373' was grown on a Dothan loamy sand. The one-row experimental plots alternated with untreated guard rows. The experimental design was a randomized block with four replications. Cultural practices were those recommended for the location.

The plants were topped in the early flower stage, and a contact sucker control agent containing long-chain fatty alcohols ($C_6$-0.5%, $C_8$-42%, $C_{10}$-56%, and $C_{12}$-1.5%) was applied to all plots that were to receive MH. As more plants flowered, they were topped and additional contact agent applications were made at weekly intervals, two in 1983 and one in 1986. MH was applied at a rate of 2.5 kg a.i./ha about one week after the last application of the contact agent. The experimental variable was the time of day for application: 0200, 0600, 1000, 1400, 1800, and 2200 h. Two nonchemical controls were hand suckered (HS), where the lateral buds or suckers were removed by hand at least weekly, and topped but not suckered (TNS), where the lateral buds or suckers were allowed to grow. Rainfall was recorded for the experimental period both years.

The potassium salt formulation of MH was diluted with water and applied with a knapsack sprayer and a one-row boom equipped with three solid cone nozzles with pressure and speed to deliver 470 L/ha.

Green leaves were sampled 24 h after each treatment time. The two uppermost leaves were removed from 10 alternate plants in each plot. The green samples were placed in polyethylene bags in an insulated box with dry ice and transported to the laboratory for storage at -18°C.

All other leaves were harvested in the usual manner as ripening occurred and were cured in bulk curing barns. Yields were recorded and a sample for each plot, composited over stalk positions, was saved for alkaloid and reducing sugar analyses by the Tobacco Chemistry Laboratory at N.C. State University. Samples of cured leaf from the last harvest were saved for MH analysis.

Sucker weights were taken after the last harvest. Suckers approximately 5 cm or longer were pulled, and the fresh weight was recorded for suckers from all 20 competitive plants within each plot. The percent control was calculated using the green weight of suckers from the TNS plots as the base value.

### Sample Preparation

Green leaf samples were removed from the freezer and chopped while still frozen with a food chopper. Approximately 400 g of each thoroughly mixed sample were placed in a 950-mL glass jar and stored at -18°C until they

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were analyzed for MH residues.

Cured subsamples from each harvest were dried at 65°C in a forced draft oven and ground in a Wiley mill to pass a 1-mm mesh screen. Samples were stored in glass jars in the freezer until analyzed.

Moisture content of the samples was determined at the time of analysis.

### Analytical Procedure

MH residues were determined by the spectrophotometric method of Lane (17). An additional step, however, was included in which the hydrazine distillate collected was shaken with activated charcoal prior to color development as prescribed in the CORESTA method No. 4 (5).

The methanol-soluble and methanol-insoluble forms of MH were separated according to procedures briefly described by Seltmann and Powell (21). A 5-g sample of green tissue was placed in a 250-mL Erlenmeyer flask with 50 mL of 70% methanol in water and shaken with a mechanical shaker for 30 min. The mixture was filtered through two Whatman Glass Microfibre filter pads with suction into a 125-mL filtering flask. The filtercake containing the methanol-insoluble fraction was rinsed three times with approximately 20 mL of 70% methanol. The filtercake was placed in a 300-mL double thickness distilling flask and heated with concentrated sodium hydroxide. Zinc and ferrous chloride were then added. With additional heating, the zinc and ferrous chloride reacted with the sodium hydroxide to release hydrogen. The hydrogen reduced MH to succinic hydrazide which was then hydrolyzed to liberate hydrazine. The hydrazine was distilled into a receiving flask of sulfuric acid and reacted with p-dimethylaminobenzaldehyde to form a yellow azine which was measured spectrophotometrically.

The methanol washes were combined with the filtrate containing the methanol-soluble MH in a 300-mL double thickness distilling flask. The solution was concentrated to a volume of 15 mL by use of a hot water bath (65°C) and air stream, and MH was determined as previously described.

Two-gram samples of cured tobacco from the last harvest were extracted with 70% methanol and subjected to the same procedure as the green samples. The sums of the MH in the filtercake and filtrate fractions were compared to total MH determinations on paired samples of tissue carried through the same procedure as the fractions for methanol-insoluble MH residues.

A standard curve for each fraction was obtained by fortifying samples with known amounts of MH in the concentration range for which the unknown samples were expected to fall. The curves were fitted by regression analysis. Correlation coefficients of 0.96 to 0.99 were obtained over the range of 0 to 1000 ppm. The lowest detectable limits for MH in the filtercake (methanol-insoluble), filtrate (methanol-soluble), and the total in green tobacco were found in 1983 to be 16, 14, and 32 ppm, respectively, with the residue values converted to the concentration in tobacco containing 13% moisture, the approximate water content of tobacco moving in commerce. The lowest detectable limits for methanol-soluble, methanol-insoluble, and total MH in cured tobacco at 13% moisture were 5, 6, and 11 ppm, respectively, in 1983. In 1986, the detectable limit for all samples was 10 ppm.

### Statistical Analyses

MH residues for green and cured leaf were based on a moisture content of 13%. Standard deviations of MH residue values varied directly with the means; therefore, values were transformed to natural logarithms for the analysis of variance. Sucker control and yield data did not require transformation and, thus, were analyzed directly. Duncan's Multiple Range Test was used to evaluate the results.

## RESULTS AND DISCUSSION

Levels of MH in green leaves sampled 24 h after each MH application varied with the time of day that MH was applied (**Table 1**). In 1983, residue levels were highest for the 1000 and 1400 h application times. The residue pattern in the 1986 experiment was similar to that for 1983, except that in 1986 the residues at 1800 h were not different from those found for 1000 and 1400 h (**Table 1**). The best sucker control was obtained with application times that gave the highest MH residues (**Table 2**). The lowest MH residues were found for applications at 0200 and 0600 h. The lowest values for percent control were also obtained for these application times.

**Table 1. Effect of time of day of MH application on methanol-insoluble and soluble MH in green tobacco 1 day (24 h) after application and on cured tips of flue-cured tobacco**

| Year | Sampling time[a] (days after treatment) | Treatment | Methanol-insoluble MH[b] | | Methanol-soluble MH[b] | |
|---|---|---|---|---|---|---|
| | | h | ppm | ln ppm | ppm | ln ppm |
| 1983[c] | 1 | 0200 | 40 | 3.654c | 64 | 4.142c |
| | | 0600 | 35 | 3.359c | 61 | 4.110c |
| | | 1000 | 109 | 4.694a | 242 | 5.479a |
| | | 1400 | 96 | 4.556a | 222 | 5.404a |
| | | 1800 | 65 | 4.173b | 131 | 4.851b |
| | | 2200 | 72 | 4.236b | 144 | 4.903b |
| | 45 | 0200 | 8 | 2.029bc | 24 | 3.169c |
| | | 0600 | 7 | 1.884c | 26 | 3.246c |
| | | 1000 | 11 | 2.414a | 51 | 3.895a |
| | | 1400 | 11 | 2.414a | 49 | 3.888a |
| | | 1800 | 9 | 2.237ab | 47 | 3.844a |
| | | 2200 | 10 | 2.253ab | 36 | 3.582b |
| 1986[c] | 1 | 0200 | 20 | 3.004c | 49 | 3.987c |
| | | 0600 | 18 | 2.880c | 54 | 3.885b |
| | | 1000 | 31 | 3.412a | 90 | 4.500a |
| | | 1400 | 29 | 3.350a | 90 | 4.502a |
| | | 1800 | 27 | 3.283ab | 87 | 4.462a |
| | | 2200 | 22 | 3.079bc | 66 | 4.184b |
| | 55 | 0200 | 16 | 2.793ab | 46 | 3.828b |
| | | 0600 | 14 | 2.667b | 32 | 3.448c |
| | | 1000 | 20 | 2.994a | 53 | 3.971ab |
| | | 1400 | 21 | 3.036a | 62 | 4.121a |
| | | 1800 | 18 | 2.903ab | 64 | 4.147a |
| | | 2200 | 16 | 2.726b | 46 | 3.814b |

[a] The one-day sampling time was 24 h after application. The 45- and 55-day sampling times were normal harvests for curing. The one-day samples were analyzed green.

[b] Means within a sampling time and year that are followed by the same letter are not significantly different at the 0.05 level of probability. All residue values are on a 13% moisture basis.

[c] The first application in 1983 and 1986 was made at 0600 h and 0200 h, respectively.

Dew was present when the MH application was made at 0600 h on August 5, 1983. The presence of moisture on the leaf surfaces may have afforded some dilution and drip-off of the MH spray solution. The dew had evaporated by the time of 1000 h application.

A light rain of 0.05 cm at 2330 h on August 5, 1983 the day of the MH applications (**Table 3**), may have washed off a small part of the MH residue from the 2200 h application and less from the 1800 h application (22). A second light rain of 0.07 cm occurred at 1630 h on August 6 before the last of the 24-h samples had been taken. Plots that had not been sampled at the time of this rain were those sprayed at 1800, 2200, and 0200 h. The time intervals between MH application and the rain were 21.5, 17.5, and 13.5 h, respectively. Washoff could have reduced MH residues some, but the effect would have been minimal (22).

**Table 2. Control of suckers on flue-cured tobacco after application of MH at different times of day in 1983 and 1986.**

| Treatment time | 1983[a] | 1986[a] |
|---|---|---|
| h | ----------% | ---------- |
| 0200 | 92b | 87bc |
| 0600 | 86c | 82c |
| 1000 | 99a | 95ab |
| 1400 | 99a | 98a |
| 1800 | 97ab | 96ab |
| 2200 | 97ab | 91ab |

[a] Percent sucker control was calculated by dividing the weight of suckers from MH-treated plots by the average weight of suckers from the TNS plots. Means within a column that are followed by the same letter are not significantly different at the 0.05 level of probability.

**Table 3. Rainfall and irrigation data and dates of application and harvest for the experimental periods in 1983 and 1986.**

| Day of month | 1983 Aug | 1983 Sept | 1986 July | 1986 Aug | 1986 Sept |
|---|---|---|---|---|---|
| | ----------cm---------- | | | | |
| 1 | | | 1.50 | | |
| 2 | | | 1.17 | | 0.23 |
| 3 | | | | 3.53 | |
| 4 | | | | 0.08 | |
| 5 | 0.05 MH[a] | | | I | |
| 6 | 0.07 | | | | 0.36 |
| 7 | 0.89 | | | | |
| 8 | | | | | |
| 9 | I[b] | | | | 0.51 |
| 10 | | | 0.71 | 0.86 | |
| 11 | | | | 3.68 | III |
| 12 | 2.54[c] | 1.37 | | 1.63 | |
| 13 | | 5.94, III | 0.28 | 1.19 | |
| 14 | | 0.81 | 1.90[c] | | |
| 15 | | | | | |
| 16 | | | | | |
| 17 | 2.54[c] | | | 0.76 | |
| 18 | | | | 0.15 | |
| 19 | | | | 4.14,II | |
| 20 | | 0.48, IV | | 2.11 | |
| 21 | | 1.85 | 2.74[c] | 2.69 | |
| 22 | 2.54[c] | | | 1.19 | |
| 23 | 2.13, II | | 0.41 | | IV |
| 24 | | | | | |
| 25 | | | | | |
| 26 | | | 0.18 | | |
| 27 | | | 1.07 | | |
| 28 | | | | 3.84 | |
| 29 | | | | 0.61 | |
| 30 | | | MH[a] | | |
| 31 | | | | | |

[a] Day of MH application.
[b] Denotes dates of harvests.
[c] Overhead sprinkler irrigation.

Residues found 24 h after application were greater for all application times in 1983 than in 1986 (**Table 1**). Differences of this magnitude of MH residues among locations and years have been observed in other studies in which the same application rate and method of application were used (23, 24). Such differences can not be readily explained.

Smith et al. (25) reported that rapid absorption of MH occurred when the humidity was high and the leaves turgid. Although such conditions will usually exist during early morning hours (0200 and 0600 h), our results indicated that sucker control and total recovered MH were lowest in both years at these times.

Based on statistical analysis, the sum of the methanol insoluble and soluble MH residues was not significantly different from the values found for the total MH determination at the 0.05 probability level (**Table 4**).

**Table 4. The effect of time of day of MH application on the methanol-insoluble and methanol-soluble MH, total MH by analysis, and the insoluble fraction as a percentage of the total of the insoluble and soluble forms.**

| Year | Sample time[a] | Treatment time | Methanol Insoluble + soluble MH[b] | | Total MH by analysis[b] | | Insoluble MH[b] |
|---|---|---|---|---|---|---|---|
| | Days after appl. | h | ppm | ln ppm | ppm | ln ppm | % |
| 1983 | 1 | 0200 | 104 | 4.630c | 104 | 4.630c | 38a |
| | | 0600 | 96 | 4.560c | 89 | 4.489c | 36a |
| | | 1000 | 352 | 5.856a | 323 | 5.774a | 31a |
| | | 1400 | 318 | 5.763a | 293 | 5.679a | 30a |
| | | 1800 | 196 | 5.266b | 188 | 5.221b | 34a |
| | | 2200 | 216 | 5.323b | 211 | 5.312b | 34a |
| | 45 | 0200 | 29 | 3.448c | 29 | 3.372c | 24a |
| | | 0600 | 31 | 3.478c | 28 | 3.336c | 21a |
| | | 1000 | 62 | 4.102a | 55 | 3.991a | 19a |
| | | 1400 | 60 | 4.098a | 57 | 4.046a | 19a |
| | | 1800 | 52 | 4.028a | 53 | 3.963a | 17a |
| | | 2200 | 41 | 3.819b | 43 | 3.757b | 21a |
| 1986 | 1 | 0200 | 69 | 4.232c | 76 | 4.295c | 29a |
| | | 0600 | 72 | 4.273c | 71 | 4.253c | 25ab |
| | | 1000 | 121 | 4.791a | 118 | 4.768a | 25ab |
| | | 1400 | 119 | 4.779a | 123 | 4.809a | 24b |
| | | 1800 | 114 | 4.732a | 123 | 4.809a | 24b |
| | | 2200 | 88 | 4.472b | 91 | 4.514b | 25ab |
| | 55 | 0200 | 62 | 4.136b | 58 | 4.060b | 26ab |
| | | 0600 | 46 | 3.828c | 41 | 3.713c | 32a |
| | | 1000 | 73 | 4.291ab | 70 | 4.228ab | 27ab |
| | | 1400 | 83 | 4.416a | 75 | 4.298a | 25ab |
| | | 1800 | 82 | 4.402a | 76 | 4.321a | 22b |
| | | 2200 | 62 | 4.117b | 69 | 4.226ab | 26ab |

[a] The one-day sampling time was 24 h after application. The 45- and 55-day sampling times were normal harvests for curing. The one-day samples were analyzed green.
[b] Means within a sampling time and year that are followed by the same letter are not significantly different at the 0.05 level of probability. All residue values are on a 13% moisture basis.

**Table 5. Yields of flue-cured tobacco after application of MH at different times of day in 1983 and 1986.**

| Treatment | | 1983[a,b] | 1986[b] |
|---|---|---|---|
| | | ----------kg/ha---------- | |
| MH at | 0200 | 2840a | 3233a |
| | 0600 | 2987a | 3306a |
| | 1000 | 3018a | 3168a |
| | 1400 | 2862a | 3296a |
| | 1800 | 3045a | 3216a |
| | 2200 | 3117a | 3024a |
| | HS | 2813 | 3032a |
| | TNS | 2742 | 2314b |

[a] Values for the HS and TNS treatments were not included in the analysis of variance in 1983.
[b] Means within a column followed by the same letter are not significantly different at the 0.05 probability level.

Total MH residues on cured tobacco from the last harvest for all the treatment times fell within the range of values previously determined where MH was applied at a rate of 2.5 kg/ha. Studies by Hunt et al. (15) and Sheets and Seltmann (24) showed that residue values on cured samples from the last harvest ranged from 47 to 68 ppm and 23 to 116 ppm, respectively.

Sucker control was good when MH was applied at 1000, 1400, and 1800 h in 1983 and 1986; in addition, sucker control was good for the MH application at 2200 in 1983 (**Table 2**). Control was only fair for applications at 0200 and 0600 h in both years. The concentrations of methanol-insoluble, methanol-soluble, and total MH residues (**Tables 1,**

4) were highest for those treatments resulting in the best sucker control (**Table 2**). Hence, no clue was found to the question of whether the methanol-soluble or methanol-insoluble residues are responsible for growth inhibition.

Yield was not affected by the time of day of MH applications (**Table 5**). Although there may not have been significant differences between the MH treatments and the nonchemical treatments in these studies, the fact has been well established that sucker control through the use of MH will usually result in a significantly greater yield when compared to manual control (19) .

For samples taken 24 h after MH application, statistical analyses implied that the amount of methanol insoluble MH, expressed as a percentage of the total present in and on the leaf, did not differ among the six application times, with values ranging from 30 to 38% in 1983 and 24 to 29% in 1986 (**Table 4**). Therefore, the time of day of applications did not appear to influence the percentage of methanol-insoluble MH for samples taken 24 h after application.

Because the potassium salt of MH is water soluble, it can be assumed that by harvest time most, if not all, of the surface MH had been removed from all treatments by rain and heavy dews. Furthermore, it has been shown that MH on the green leaves at harvest is not lost during flue-curing (24). Therefore, the percentage of methanol-insoluble MH found in the cured leaf depended only upon the amount absorbed. MH may bind to several cellular components such as DNA, RNA, and protein, and hypotheses have been advanced that the growth inhibitions are mediated by "bound" MH (1, 2, 3, 4, 6, 14, 16, 18). We believe that the extraction method utilized in this study separated the methanol-insoluble from the methanol-soluble MH. Sucker control values were highest for those treatments that provided the highest levels of methanol-insoluble MH 24 h after MH application. Because high levels of insoluble MH were accompanied by high levels of soluble MH, and hence total MH residues, we cannot conclude which fraction was most closely associated with growth inhibition. It is interesting to note, however, that the largest portion was methanol-soluble suggesting that some MH was mobile in the plant throughout the period of application and final leaf harvest and that some may have been metabolized and (or) exuded through the roots. There seems to be a tendency for lower MH residues in tobacco crops that have grown under good moisture conditions, that are succulent during MH application, and that have adequate moisture during the harvest season.

Once the MH is applied and absorption has occurred, one can speculate what environmental influences such as heavy dews and rain will have on the removal of surface residues as a mechanism whereby MH residues are reduced. The excess residues can be removed through sprinkler irrigation 24 h after application. Also, a delay between MH application and the next harvest will reduce residues. Such a delay is exemplified in burley tobacco where harvest usually occurs 3 to 4 weeks after MH application as evidenced by the fact that MH residues usually are not excessive.

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