Blight Development and Methyl-2-Benzimidazole Carbamate Levels in Bark Tissues of American Chestnut Trees Following Soil Injection of Benomyl

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ABSTRACT.—If a method of injecting systemic fungicides into the soil for control of chestnut blight can be perfected, there are several advantages to this approach. To evaluate the efficacy of this, America chestnut stump sprouts were soil-injected with various levels of aqueous solution of benomyl using different techniques. The potential for chemical control of bark pathogens by soil injection of fungitoxicants was demonstrated for tap-rooted trees such as American chestnut. Water extraction would appear to be the method of choice for determining available levels of fungitoxicants in plant tissues.

INTRODUCTION

Chemical control of chestnut blight of American chestnut *(Castanea dentata* [Marsh.] Borkh) caused by *Endothia parasitica* (Murr.) P.J. and H.W. And. would permit the establishment of specimen American chestnut trees in landscape and/or arboreta plantings. Early failures of chemical control of chestnut blight included injection of a great number of chemicals into stems of American chestnut trees (Rumbold, 1920). Interest in chemical control of chestnut blight was revived by a report that benomyl (methyl 1-(butylcarbomayl)-2-benzimidaz ole carbamate) inhibited *E. parasitica in vitro.* Jaynes and Anagnostakis (1971) observed reduced fungal growth following inoculation with *E. parasitica* of two-year-old American chestnut trees treated with a soil drench of an aqueous suspension of benomyl, as compared to untreated inoculated controls. Subsequent experiments by Jaynes and Van Alfen (1974, 1977) on stem injection of six-year-old American chestnut trees with soluble salt solutions of methyl-2-benzimidazole carbamate (MBC), a fungitoxic breakdown product of benomyl, showed that canker development on inoculated, treated trees was restricted. However, the concentration of MBC salts necessary for protection was high enough that foliage injury often resulted.

The general objective of the present study was to evaluate the efficacy of soil injection of benomyl in protecting American chestnut trees from chestnut blight. Soil injection of water-insoluble benomyl offers advantages over stem injection by providing long-term protection with a single treatment and without injury to the tree (Biehn, 1973; Stipes, 1975). The specific objective was to monitor MBC levels in bark tissue, the infection locus of *E. parasitica.* A preliminary report of this work has been presented (Elkins *et al.,* 1977).

Materials and Methods

Soil injection. In April, 1974, the root zones of eight small, blight-free American chestnut stump sprouts (2-3 cm diameter at the base) located in the Jefferson National Forest near Blacksburg, Virginia, were injected with an aqueous suspension of benomyl (Benlate, E.I. duPont de Nemours) to a maximum depth of 25 cm using a soil needle adapted to a hand-carried pressurized sprayer (30 psi). For this experiment (experiment I), each of two trees received 12 1 of benomyl at concentrations of 4,000, 10,000, 20,000 or 40,000 mg/1.

During May, 1975, the root zones of seven larger American chestnut trees (5-17 cm diameter at breast height) located in the Jefferson National Forest (four trees) and near Flat Top Lake in West Virginia (three trees) were excavated to a depth up to 25 cm for a distance of 2 m around the trees. In this study, experiment II, the trees were then treated with a total of 72 1 of an aqueous suspension of benomyl at a concentration of 10,000 mg/1. A soil auger was used to drill holes with a diameter of 2.5 cm and a depth up to 75 cm in the soil adjacent to the tap roots and along both sides of the lateral roots. The holes were filled with 48 1 of the aqueous suspension of benomyl and then 24 1 of the benomyl suspension were injected with the soil probe under pressure along and between the lateral roots. Following treatment, the soil was replaced.

Bioassay of MBC in bark. Terminal portions of branchlets of trees in experiment I were collected in September, 1974, five months after treatment. Bark slivers, 1 mm x 3 mm, with total dry weights of 29-112 mg, were removed from each branchlet and inserted side by side, with the narrow end down in two rows and with the cambium side facing out, into potato-dextrose agar (PDA) plates which had been

surface-seeded with a suspension of *Penicillium expansum* Link conidia using a cotton-tipped applicator stick. The plates were placed in a refrigerator overnight to allow for diffusion of bark constituents into the medium and then incubated at room temperature until the *P. expansum* mycelium developed. Net zones of inhibition, width and length, were then determined. In a similar experiment, terminal branchlets from all four quadrants (N,S,E,W) of trees in Experiment I were collected in October, 1974. Bark slivers with total dry weights of 11-79 mg from the tips and 26-152 mg from the bases of the branchlets were assayed to determine the distribution of MBC in the trees.

HPLC analysis of MBC in bark. High performance liquid chromatographic (HLPC) analyses of MBC in bark tissues were carried out with modifications of the method developed for MBC residue analysis (Kirkland *et al.,* 1973). In the first modification, 2 g of milled, air-dried bark from terminal branchlets about 50 cm long or from bark "patches," about 5 cm^2, from the lower main stem were extracted continuously with ethyl acetate in a Soxhlet extractor until all the chlorophyll was removed. The ethyl acetate extract was then concentrated with a rotary evaporator to approximately 50 ml and extracted twice with 20 ml portions of 0.1 N HC1. The HC1 solution was made basic (about pH10) with 3 ml of 6.5 N NaOH at which time the solution turned deep red. The basic solution was then extracted four times with 50 ml of ethyl acetate. The ethyl acetate was removed from the extract in two stages with a rotary evaporator. In the first stage, it was concentrated to approximately 15 ml. The concentrate was then introduced into a 25-ml flask containing 1 ml of 0.1 M H3PO4. The remainder of the ethyl acetate was removed and the aqueous solution was transferred to a 2-ml volumetric flask and made up to the mark with 0.1 M H3 PO4. Any soluble residue was removed by filtering through a Swinney filter.

Analysis by HPLC of the purified extract was carried out by injecting 100p1 of the extract into a 1 m x 2.1 mm i.d. Zipax SCX strong cation exchange column (E.I. duPont de Nemours) under the following conditions: column temperature, 60 C mobile phase, 0.25 N tetramethyl-ammonium nitrate-0.025 N HNO_3; carrier flow rate, 0.5 ml/min; uv detector, 280 nm. A standard solution of 10^{-4} M MBC, 19.1 ug/ml, in 0.1 M H3 PO4 was on scale at a detector setting of 0.16 absorbance units full scale (AUFS) and had a retention time of 18 minutes. The standard solution was stable for many months at room temperature before any 2-aminobenzimidazole (2-AB), the breakdown product of MBC, was observed eluting with a retention time of 22 minutes. Occasionally, 2-AB was observed in trace amounts from bark of treated trees.

In the second modification, the 2 g of air-dried bark was extracted with 40 ml of water by stirring at room temperature for 30 minutes and filtering. The aqueous extract was then made basic with 6.5 N NaOH and treated as before. In this method, there was usually a residue to be filtered from the final solution in 0.1 M H3PO4. The agar zones of inhibition were extracted by first stirring with 40 ml of 0.1 N HC1 and treating as before.

Recovery factors were determined by diluting 2 ml of 10^{-4} M MBC in 0.1 M H3 PO4 or 20 ,ug MBC/ml in H_2O to 10 ml, stirring with 2 g of air-dried American chestnut bark, and evaporating to dryness in a vacuum oven at 60 C. Extraction with ethyl acetate resulted in the recovery of 68 ± 4 percent of the MBC from the bark based on the combined average of three trials with MBC and three trials with MBC. H_3PO_4. Extraction with water resulted in the recovery of 12 ± 0 percent of MBC and 27 ± 4 percent of MBC. H_3PO_4 from the bark based on two trials each. The 20 *pg* MBC/ml in H_2O solution was prepared by diluting a saturated solution with 107.5 ,ug MBC/ml H_2O at room temperature. The standard was made up to 0.1 M in H_3PO_4 for HPLC analysis by adding 2 drops (0.05 ml) of concentrated H_3PO_4 (14.6 M) to 7.9 ml of aqueous MBC. A saturated solution of benomyl contained 10.6 ug benomyl/ml H_2O.

Calculations of the MBC levels in air-dried bark extracted with ethyl acetate were based on the linear relationship between peak heights (p.ht.) and concentration (Kirkland *et al.,* 1973) and were made as follows:

$$\text{MBC } \mu g/g = \frac{19.1\ \mu g/\text{ml standard}}{0.68 \text{ recovery factor}} \times \frac{2 \text{ ml standard}}{2 \text{ g sample}} \times \frac{\text{AUFS sample}}{\text{AUFS standard}} \times \frac{\text{p.ht. sample}}{\text{p. ht. standard}}$$

Corresponding calculations of the MBC levels in bark extracted with water were made in the same way except that no recovery factor was included since the objective of those experiments was to determine available MBC.

In sampling, bark "patches" were not taken from the lower main stem until the end of the experiments for fear of injuring the tree. Duplicate analyses were not run because of the lengthy extraction procedure and because of the scarcity of samples available from the small trees of Experiment I. Instead, sequential analyses of samples collected at different sampling periods gave confidence in the results by providing the opportunity to compare the relative magnitudes of the MBC levels in the bark (Table 1, tree 1-5; and Table 3). Bark "patches" from the lower main stem were separated into component tissues by peeling the periderm and scraping off the green chlorenchyma to leave the phloem.

Inoculation with E. parasitica. The trees in Experiment I were inoculated in June, 1975, 14 months after soil injection, and those in Experiment II were inoculated in August, 1975, three months after soil

injection, with a pathogenic strain of *E. parasitica* grown on PDA. Inoculations were made to the cambium at three points on each tree by removing a bark plug with a 6-mm cork borer, placing an agar plug of mycelium of *E. parasitica* with the same diameter in the wound, and covering with masking tape to prevent drying. Inoculations were located on the main stem of the trees within 2 m of the ground except for the largest tree, 11-4, which was inoculated on the upper side of a large lateral branch 3 m from the ground. The extent of disease advance was determined periodically by measuring the length and width of the canker. The orange canker margin was more easily identified after first wetting the bark with water.

Results and Discussion

Bioassay of MBC in bark. Bark agar bioassays showed zones of inhibition indicating the presence of a fungitoxicant in seven of eight of the small trees in Experiment I five months after soil injection (Table 1). No correlation was noted between the level of treatment and the size of the zone of inhibition. The fungitoxicant diffusing from the bark into the agar was shown by HPLC to be MBC at 0.1 *µg/ml.*

Using only the two trees with the highest concentration of MBC (1-4, 1-5), bark-agar bioassays showed even distribution of a fungitoxicant in all four quadrants and at the tips and bases of the branchlets six months after soil injection (Table 2).

HPLC analysis of MBC in bark. Analysis by HPLC of the bark from the eight trees in Experiment I (Table 1) and the seven trees in Experiment II (Table 3) showed MBC levels from 2.9-24.2 ug/g at the time of inoculation with *E. parasitica.* Levels of MBC in the bark were generally higher in the summer when transpiration was high (Table 1, tree 1-5; and Table 3), but MBC levels did not drop rapidly during the winter when transpiration was low. The higher MBC levels at the base of trees II-1A, II-5A, and 11-6 (Table 3) suggest that MBC is translocated through the rays into the bark on its way up from the roots to the top. In contrast, Jaynes and Van Alfen (1977) presented bioassay data for American chestnut trees stem injected with MBC•H_3PO_4 that indicated the fungitoxic material was first translocated to the crown and then redistributed downward to the bark of the trunk.

Blight development. Approximately 15 weeks after inoculation, one of the untreated trees in Experiment I was dead from girdling at two inoculation points, while the other untreated tree showed

Table 1
Analysis of MBC in bark of small American chestnut trees soil-injected with benomyl in April 1974.

		Bark-agar Bioassay, September 1974			MBC Analysis by HPLC	
Benomyl µg/ml[a]	Tree No.	Dry Weight of Bark, mg	Net Inhibition Zone[b] Width, mm	Length, mm	Diffused into agar[c]	Extracted by EtOAc[d]
4,000	I-1[e]	38	0	0	—	6.3
4,000	I-2	29	4	0	1.7	10.0
10,000	I-3	49	15	7	1.8	18.1
10,000	I-4	47	22	14	4.1	20.1
20,000	I-5	48	19	13	4.2	24.2[f]
20,000	I-6	42	4	0	1.2	6.0
40,000	I-7[e]	51	2	0	—	3.7
40,000	I-8	42	17	9	—	20.3
0	Control I-1	43	0	0	—	0
0	Control I-2	61	0	0	—	0

[a] Applied 12 1 of benomyl suspension.

[b] MBC present in the inhibition zones at 0.1 µg/ml as determined by HPLC.

[c] MBC levels in µg/g dry bark in September, 1974. Calculated from the volume of agar into which MBC diffused, the weight of the bark tissue sampled, and the average MBC level in the inhibition zones.

[d] MBC levels in µg/g dry bark in June, 1975, at the time of inoculation with *E. parasitica.* EtOAc = ethyl acetate.

[e] All trees except I-1 and I-7 gave inhibition zones in the October, 1974, bark-agar bioassay.

[f] MBC levels, µg/g dry bark, in May, 1976-12.4, August, 1976-59.5, and March, 1977-44.7. Only tree in Experiment I still living in March, 1977.

severe canker development with extensive fruiting of the pathogen on all three cankers. At this time all benomyl-treated trees in Experiment I were alive, with three trees exhibiting good callus development at all three inoculation points and with little or no evidence of infection. No correlation between the rate of benomyl application and disease development was noted, however. Severe canker development with or without pathogen fruiting was noted for one inoculation point for each of three treated trees. Another tree, receiving the lowest concentration of benomyl, was chlorotic and severely infected at all three inoculation points. All other inoculation points of benomyl-treated trees typically showed callus formation with moderate levels of infection. Forty-eight weeks after inoculation, all trees from Experiment I were dead except for tree 1-5 which had the highest MBC level of all the treated trees in the study (Tables 1 and 3). Fifty-nine weeks after inoculation, the stem of this tree was dead above the middle inoculation point.

In Experiment II, small but measurable cankers developed by early fall (two to three months after inoculation) on all but one of the benomyl-treated trees. These cankers continued to expand through the winter months, but greatest canker growth appeared to occur during the following growing season. For example, in tree II-1, the mean canker length increased 0.9 cm per month from October 24, 1975 to May 11, 1976, and 3.1 cm per month from the latter date to October 1, 1976. There was no correlation between mean canker lengths and MBC levels in the bark. However, cankers expanded the least (0.3 cm per month) during the summer months on tree 11-5 which had the highest MBC level of all the benomyl-treated trees in Experiment II (Table 3). Tree 11-4 also showed small canker expansion (0.4 cm per month) but had the lowest level of MBC in the bark of all the treated trees in the study (Tables 1 and 3). This large American chestnut tree (17 cm dbh) may have some resistance to *E. parasitica* as it was the only inoculated stern in Experiment II still alive after 22 months. Four of seven trees in Experiment II were alive 14 months after inoculation. The trees not treated with benomyl died early (within ten months of inoculation) in the course of the experiment.

Evaluation of tolerance to MBC. The development of tolerance to MBC (Dekker, 1976) by *E. parasitica* appeared to offer a reasonable explanation for lack of control of chestnut blight with MBC levels in the barks in excess of the 1 pg MBC/ml. As previously indicated, this MBC level in PDA completely inhibited *E. parasitica* growth. To determine if *E. parasitica* tolerance to MBC had developed in benomyl-treated trees, *E.* parasitica-infected tissues were obtained from the margin of 13 cankers on five benomyl-treated trees. Pieces from these tissues were inoculated on PDA plates, and PDA plates containing 0.5 or 1 pg MBC (supplied as MBC• H_3PO_4) per ml. An *E. parasitica* isolate from a tree not treated with benomyl was also included. The results obtained for isolates from benomyl-treated trees paralleled the previous findings. The fungus grew from only six of 30 tissue pieces (20 percent) on PDA containing 1 pg MBC/ml. Similar results were obtained for 0.5 g MBC/ml, whereas *E. parasitica* grew from 34 of 35 (97 percent) tissue pieces on PDA alone. Interference from undesired fungi and bacteria was slight to moderate on the PDA-MBC plates. Most of the isolates made only scant growth (mostly less than 0.5 cm radial growth) on the PDA containing 1 *pg* MBC/ml after 12 days incubation. Upon subsequent mass transfer of these isolates and 13 other isolates from benomyl-treated trees to PDA plates containing MBC•H_3PO_4 (1 pg MBC /ml), no growth occurred. One isolate, obtained in a separate preliminary experiment, grew slowly at 1 *pg* MBC/ml in repeated trials. Tolerance to 2 *µg* MBC/ml was not observed. Thus, we obtained little evidence that tolerance to MBC could explain canker development on trees containing MBC in bark tissues at concentrations greater than 1 pg/g.

The possibility of a pH-regulated "tolerance" to

Table 2
October, 1974, bark-agar bioassay of benomyl-treated American chestnut trees.

Benomyl ug/ml[a]	Best Trees	Dry Weight of Bark, mg Tip . . . Base	Net Inhibition Zone, mm Width Tip . . . Base	Length Tip . . . Base
10,000	I-4N	40 94	18 . . . 24	12 . . . 15
	I-4S	18 56	20 . . . 22	13 . . . 13
	I-4E	33 91	19 . . . 22	9 . . . 15
	I-4W	55 152	23 . . . 25	9 . . . 16
20,000	I-5N	79 110	34 . . . 23	23 . . . 18
	I-5S	71 83	32 . . . 22	21 . . . 17
	I-5E	54 63	28 . . . 29	16 . . . 25
	I-5W	65 81	20 . . . 23	12 . . . 15

[a] Applied 12 1 of benomyl suspension.

MBC by *E. parasitica* remained. Lambert and Wuest (1976) reported that benomyl-tolerant strains of *Verticillium malthousei* were capable of increasing the acidity of the culture medium. Since American chestnut bark is rich in tannins and since *E. parasitica* shows maximum growth in culture at pH 4 (Puhalla and Anagnostakis, 1971), it appeared conceivable that *E. parasitica* might not be inhibited in bark extract containing 1µg MBC/ml. To evaluate this, filter-sterilized aqueous extracts from American chestnut bark (10 ml H_2O/g bark) were amended to contain MBC (supplied as MBC•H_3PO_4) at concentrations of 1, 5, and 10µ g/ml and inoculated with mycelium from a PDA slant of a highly pathogenic isolate of *E. parasitica.* No growth occurred after seven days incubation on any of the MBC-amended extracts. Good growth occurred on the extract without MBC. The initial pH of the extract without MBC was 4.6 and the final pH was 3.5. Thus, the high acidity of the bark of American chestnut apparently does not explain canker development on trees containing MBC in the bark tissues at concentrations greater than 1 µg/g.

Comparison of MBC levels in trees treated with benomyl by soil injection and with MBC salts by stem injection. The absence of control of chestnut blight with soil-injected benomyl and the reported control of chestnut blight with stem-injected MBC salts (Jaynes and Van Alfen, 1974; 1977) suggested that MBC levels in bark residues might be different for the two different treatment methods. Analysis by HPLC of a terminal branchlet from a stem-injected tree (Connecticut tree 6 supplied by R. A. Jaynes of the Connecticut Agricultural Experimental Station) showed an MBC concentration of 65.9 pg/g, a higher concentration than for any tree in our study and higher than the MBC concentration in the total bark tissue of the lower-stem bark "patch" from Connecticut tree 6 (Table 4). Indeed, Jaynes and Van Alfen (1977) point out the necessity for high treatment levels with MBC•H_3PO_4 to get high enough MBC concentrations into the bark for control. In an attempt to explain the need for such high levels of MBC in the bark, bark "patches" from the lower main stem were separated into periderm, chlorenchyma, and phloem to determine whether the MBC was being partitioned into the lipophilic periderm and chlorenchyma tissues. The results shown in Table 4 indicate a little movement of MBC into the lipophilic tissues from soil injection and greater movement from stem injection. However, a large amount of the MBC (84 percent from soil injection and 55 percent from stem injection) was retained by the phloem which contains the bulk of the bark tissue and is the infection locus of the pathogen. Therefore the MBC is apparently corn-

Table 3
HPLC analysis of MBC in bark of large American chestnut trees soil-injected with benomyl in May, 1975.[a]

Tree No.[b]	August, 1975[c,d]	May, 1976[c]	August, 1976[c]	March, 1977[c]	April, 1977[c]
II-1, Top	9.3	10.0	8.5	—	[e]
-1, Bot	—	—	—	—	4.4
-1A, Top	—	—	—	8.5	8.5
-1A, Bot	—	—	—	—	17.2
II-2, Top	15.9	20.6	36.0	20.0	[e]
II-3, Top	7.9	[e]			
II-4, Top[f]	2.9	0.3	2.5	—	—
II-5, Top	18.8[g]	21.3	24.6	[e]	
-5A, Top	—	—	—	—	8.2
-5A, Bot	—	—	—	—	8.5
II-6, Top	6.9	4.3	7.6	—	3.2[e]
-6, Bot	—	—	—	—	13.1
II-7, Top	10.0	5.6	10.7	[e]	
Control II-1	0	[e]			
Control II-2	0	[e]			

[a] Applied 72 1 of 10,000 µg benomyl/ml in suspension.

[b] Top=terminal branchlet, Bot=at base adjacent to canker, A=associated stem.

[c] MBC levels in µg/g dry bark.

[d] Time of inoculation with *E. parasitica.*

[e] Main stem dead or dying.

[f] Largest tree in study, 17 cm dbh. Only tree in Experiment II still living in April, 1977. Inoculations retarded.

[g] Nut had MBC concentration of 0.7 µg/g with branchlet concentration of 20.0 µg/g in September, 1975.

partmentalized in the phloem in some way so it does not come in contact with *E. parasitica.*

In order to shed light on the mobility of MBC in the bark, the amount of water-extractable MBC was determined. A bark "patch" from the lower main stem was collected in September, 1977, from soil-injected tree II-1A and found to contain 4.3 mg MBC/g by ethyl acetate extraction and 1.1 g MBC/g by water extraction while a terminal branchlet from the stem-injected Connecticut tree 6 was found to contain 65.9 ug MBC/g by ethyl acetate extraction and 34.1 mg MBC/g by water extraction. The lower MBC levels obtained by water extraction could have been predicted from the lower recovery factors for MBC (12 percent) and MBC•H_3PO_4 (27 percent) with water and suggest the possibility that the availability of MBC in hydrophilic bark tissue may be crucial for control of chestnut blight. Still, the concentration of MBC available for diffusion into agar from the bark of benomyl-treated trees in Experiment I was in excess of 1 *μg/g,* with a range of 1.2-4.2 ug/g of dry bark (Table 1) or 0.8-2.9 *Mg* MBC/g of fresh bark. For this reason, water extraction would appear to be the method of choice for determining available levels of fungitoxicants in plant tissues.

Conclusion. The potential for chemical control of bark pathogens by soil injection of fungitoxicants was demonstrated for tap-rooted trees such as the American chestnut. However, a more effective systemic fungitoxicant than benomyl is required for control of chestnut blight by soil injection. The breakdown product of benomyl, MBC, was found in the bark of all treated trees, including one large tree with a dbh of 17 cm and a height of 13 m, at a concentration in excess of that required to inhibit the pathogen *in vitro.* The potential for long-term protection without injury to the trees was demonstrated by the high levels of MBC found in the bark of one tree three years after initial treatment and in other trees for up to two years before they succumbed to the blight. Problems with soil injection revolve around possible deleterious effects on the ecology of the soil (Stringer and Wright, 1973). Pollution from soil injection may not be a problem since benomyl has been found to be essentially immobile in the soil (Janutolo, 1977).

At present, any hope for preserving specimen American chestnut trees by chemical means rests with stem injection with MBC salts. The major problem with stem injection is the potential for injury to the tree from annual injections with high concentrations of MBC salts as has occurred with elms (Shigo and Campana, 1977). Rumbold (1916) has documented the kinds of injuries that can occur to American chestnut upon stem injection of chemicals.

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Table 4
HPLC analysis of MBC in bark tissue of American chestnut trees treated by two methods.

	Soil-injected Benomyl[a]		Stem-injected Elmosan[b]	
Tissue	MBC Concentration (μg/g Tissue)	% of Bark (Dry Wt.)	MBC Concentration (ug/g Tissue)	% of Bark (Dry Wt.)
Phloem	6.8	87.8	17.5	91.6
Chlorenchyma	11.9	4.3	83.4	4.3
Periderm	8.1	7.9	232.6	4.1
Total bark tissue	7.1	100	29.1	100

[a] Applied 72 1 of 10,000 μg benomyl/ml in suspension. Tree II-A sample collected August, 1977.

[b] Applied 3 1 of 1,700 μg MBC•H_3PO_4/ml. Connecticut tree 6 sample collected August, 1977. Bark tissue supplied by Richard A. Jaynes, Connecticut Agricultural Experiment Station.

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