Factors Affecting Growth of *Ophiostoma ulmi* on Elm Callus Tissue[1]

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Abstract

We examined growth of the Dutch elm disease fungus, *Ophiostoma ulmi*, on callus derived from a susceptible American elm (*Ulmus americana*, selection A), an American elm of intermediate resistance (*U. americana*, selection 8630), and a resistant Siberian elm (*Ulmus pumila*) at 16, 22, and 28°C (61, 72, and 83°F) and inoculation concentrations of 15×10^6, 2×10^6, or 0.3×10^6 conidia/ml. After 72 hours, the rates of fungal growth for all treatments were most rapid on calli from the American 8630 selection followed by the American A and Siberian selections. While fungal growth was more rapid over American 8630, it was more dense on American A. Most rapid fungal growth occurred at 22°C (72°F) and was directly proportional to the inoculum concentration. A significant interaction was noted between callus source and temperature.

Index words: tissue culture, Dutch elm disease, disease resistance

Significance To The Nursery Industry

Dutch elm disease (DED) is a serious vascular wilt disease responsible for destruction of millions of elm trees worldwide. Screening elms for resistance to DED, using conventional techniques, is time consuming and labor intensive. This study was carried out to determine the feasibility of using tissue culture technology to speed up the identification of disease resistant elms. Callus cultures of susceptible and resistant elms were exposed to three different concentrations of the DED fungus. The callus cultures were incubated at three different temperatures. The results indicated that while varying environmental conditions of inoculum concentration and temperature affected growth of the pathogen on the callus, there was consistently less growth on callus derived from resistant than from susceptible elms. This study may serve as a model to identify disease-resistant germplasm in other tree species using tissue culture technology.

Introduction

Dutch elm disease (DED) caused by *Ophiostoma ulmi* (Buisman) Nannf. has caused worldwide destruction of elm trees (14). Host resistance is the most reliable means for controlling the disease. Screening elms for resistance to DED using conventional techniques is time consuming and labor intensive. Tissue culture systems for studying host-pathogen interactions offer advantages over those utilizing intact plants (2). Studies have shown that host-pathogen interaction, expressed in intact plants, correlated with the responses shown using callus tissue (1, 4, 7, 8, 9, 10, 12, 21). Haberlach et al. (6) indicated that tissue morphology, incubation temperature and growth regulator concentration in the medium quantitatively affected colonization. Thus, before a tissue culture system can be useful in studying host-pathogen interactions, it is necessary to identify the level of genetic control and the influence of environmental factors.

In preliminary studies, we determined interactions between *O. ulmi* and callus from American elm (*Ulmus americana* L.) genotypes with varying degree of susceptibility, resistant Siberian elm (*U. pumila* L.) and a non-host petunia (*Petunia* × *hybrida*) to identify criteria that correlated with those of the intact plant. These studies established reproducible techniques and a rating system to measure variables such as inoculum levels, inoculation techniques, optimum ages of fungus cultures and callus, and incubation periods.

This study presents a systematic approach to simultaneously test inoculum concentration, incubation temperature and elm genotypes using a callus tissue system.

Materials and Methods

Stock plants of American elm A, susceptible to DED; American elm 8630, an American elm resistant to DED, and Siberian elm, a species resistant to DED, were propagated in the greenhouse from softwood cuttings from 15–20 year old trees. Callus cultures were initiated from young leaves (16) on full-strength Murashige and Skoog (15) culture medium, (MS), supplemented with 200 mg/l casein hydrolysate, 8 μM 6-benzylaminopurine, 0.5 μM 2,4-dichlorophenoxyacetic acid, 3% (w/v) sucrose, and 0.7% (w/v) Difco Bacto Agar (pH 5.7). These cultures were initiated in May, 1987 and routinely subcultured every 6 to 8 weeks onto fresh half-strength MS medium fortified with the same supplements. Cultures were grown in the dark at 22 ± 1°C (72 ± 2°F). On April 7, 1988 pieces of calli, 10–15 mm (0.4–0.6 in) in diameter, were transferred to 15 × 60 mm (0.6 × 2.4 in) Petri plates containing half-strength, fortified MS medium. After eight days at 22 ± 1°C (72 ± 2°F) the calli were incubated at 16 ± 1°C (61 ± 2°F), 22 ± 1°C (72 ± 2°F), or 28 ± 1°C (83 ± 2°F) for three days prior to inoculation.

Aggressive *O. ulmi* isolate PMP1 (5, 16) was recovered from a diseased American elm at Delaware, Ohio. PMP1 was grown on cellophane-covered potato dextrose agar (PDA:

[1]Received for publication May 5, 1991; in revised form August 9, 1991. Part of this work was funded by a grant from the Horticultural Research Institute, 1250 I Street, N.W., Suite 500, Washington, DC 20005.

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Difco, Detroit, MI) for 5 days at 24°C (75°F). A spore suspension was made by washing the surface of the plates with sterile distilled water and adjusting the spore concentration to 15 × 10^6 spores/ml (high); 2 × 10^6 spores/ml (medium); or 0.3 × 10^6 spores/ml (low). Six replicates of each callus type (American elm A, American elm 8630, and Siberian elm) were inoculated with one of the three concentrations of PMP1 spore suspensions. Three mm (0.12 in) diameter filter paper discs were cut from 6-mm (0.24 in) -diameter concentration discs with a paper punch. Four, 1.5 mm (0.06 in) triangles were cut from each of the 3 mm (0.12 in) discs, sterilized and inoculated with 20 µl of the appropriate concentration of spore suspension. After air-drying, the inoculated filter paper triangle was placed onto the center of the callus. Controls were: (1) an inoculated triangle placed in the center of a fortified half-strength MS medium plate, and (2) an uninoculated triangle placed at the center of the callus piece. After inoculation, the plates were returned to their respective incubation temperature in the dark.

To determine the average diameter of the callus and fungal colony, two axes perpendicular to each other were drawn on the bottom of the Petri plate intersecting where the filter paper triangle was placed on the callus. Seventy-two hr after inoculation, the diameter of the fungal colony was determined by averaging the growth along the two axes. The size of the filter paper triangle 1.5 mm (0.06 in) was subtracted from each diameter reading. Subsequently, univariate analysis was carried out after arc sin transformation and analyses of variance with associated 'P' values were obtained using the software program from the Statistical Analysis System (17). Density of the fungal mycelium was rated subjectively by visual observation.

Results and Discussion

Analysis of variance of fungal growth on calli from three selections of elms, at three temperatures, inoculated with three concentrations of spores is presented in Table 1. The rates of growth of *O. ulmi* for all treatments was most rapid on calli from American elm 8630 followed by American elm A and Siberian (Fig. 1 & 2). The rate of fungal growth increased with increasing spore concentration. Similarly, the rates of fungal growth on half-strength MS medium, in the absence of callus, increased in direct proportion to inoculum concentration (Fig. 3). There was no significant callus × spore concentration interaction.

Optimum temperature for fungal growth on calli from all selections was 22°C (73°F) followed by 16 and 28°C (61 and 83°F). An interaction ($P < .01$) was noted between callus source and temperature. Thus, at 16 and 28°C (61 and 83°F), differences in rates of fungal growth on American elm 8630, American elm A, and Siberian were similar. However, at 22°C (72°F), the relative fungal growth rate on American elm A callus increased in proportion to that on either American elm 8630 or Siberian (Fig. 2). Fungal growth on medium was in direct proportion to the inoculum concentration (Fig. 3). Fungal growth on half-strength MS, in the absence of callus, was most rapid at 22°C (72°F) followed by 16 and 28°C (61 and 83°F) (Fig. 4). Siberian callus was colonized more rapidly at 16 (61°F) than at 28°C (83°F), while fungal growth rates on either American elm A or 8630 were equal at 16 and 28°C (61 and 83°F).

While the rate of fungal growth was more rapid on American elm 8630 than on American elm A, greater fungal density was observed on the American elm A callus (Fig. 5). However, after fungus reached the medium, it grew equally; independent of the genotype. This was the case at all temperatures and inoculum concentrations over the 5 day period. Following inoculation, callus from American elm 8630 turned lighter in color.

We examined the effects of inoculum concentration and incubation temperature on the growth of *O. ulmi* on callus from American and Siberian elms. These genotypes represented the extremes of susceptibility and resistance, re-

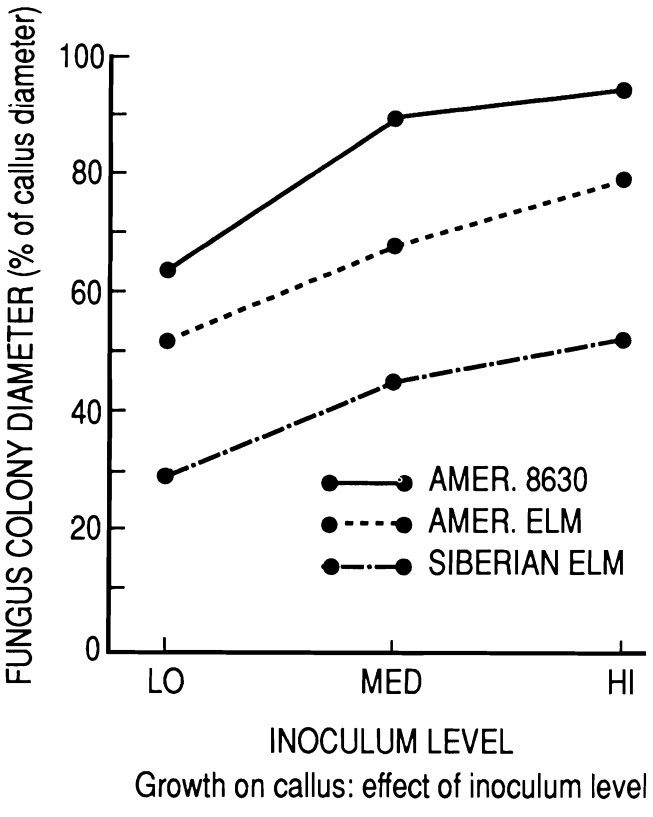

Fig. 1. Effect of three concentrations on the average colony diameters of *O. ulmi* on callus tissue from three elm selections. Calli were inoculated with concentrations of 0.3 × 10^6 (LO), 2 × 10^6 (MED), or 15 × 10^6 (HI) spores/ml. Twenty µl of the appropriate concentration was placed on a 1.5 mm filter paper triangle in the center of the callus. Calli and fungus colony diameters represent an average of measurements along the two random axes. Average colony diameter after 72 hours is expressed as percent of the average diameter of the callus.

Table 1. Analysis of variance (F-Ratios) for fungal development on calli from three elm selections inoculated with *Ophiostoma ulmi*.[z]

Source of Variation	df	F
Selection	2	91.8*[y]
Inoculum conc.	2	43.4*
Temperature	2	41.4*
Selection × inoculum conc.	4	0.8
Selection × temperature	4	3.6*
Error	6	

[z]The data were analyzed using six replicates.

[y]Asterisk (*) indicates differences significant at $P < .01$. SAS version 5.16 (SAS Institute, Cary, NC).

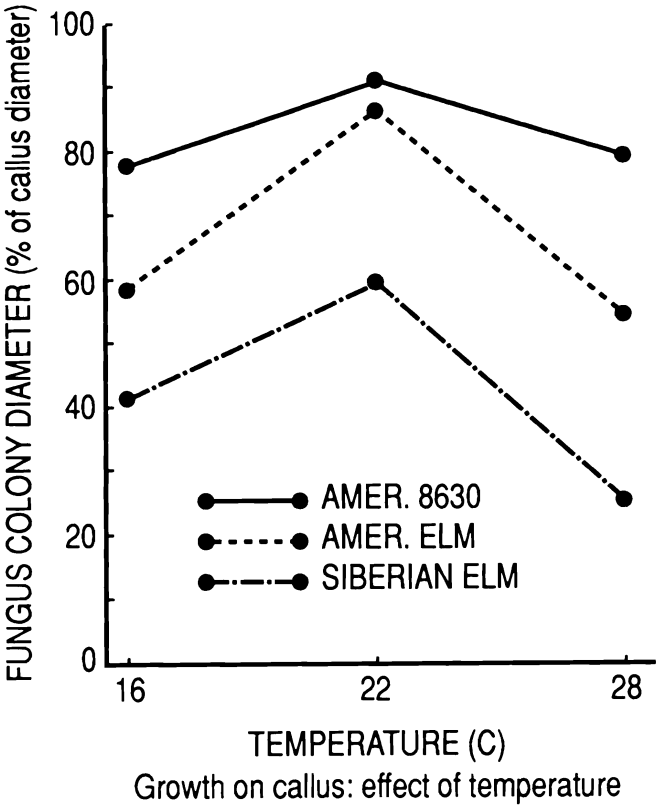

Fig. 2. Effect of three incubation temperatures on average colony diameters of *O. ulmi* on callus tissue from three elm selections. Calli were inoculated by placing a 1.5 mm filter paper containing 20 µl of a concentration of 0.3 × 10^6, 2 × 10^6 or 15 × 10^6 spore/ml in the center of the callus. Calli and fungus colony diameters represent an average of two random axes drawn at right angles to one other. Average colony diameter, after 72 hours, is expressed as percent of callus diameter.

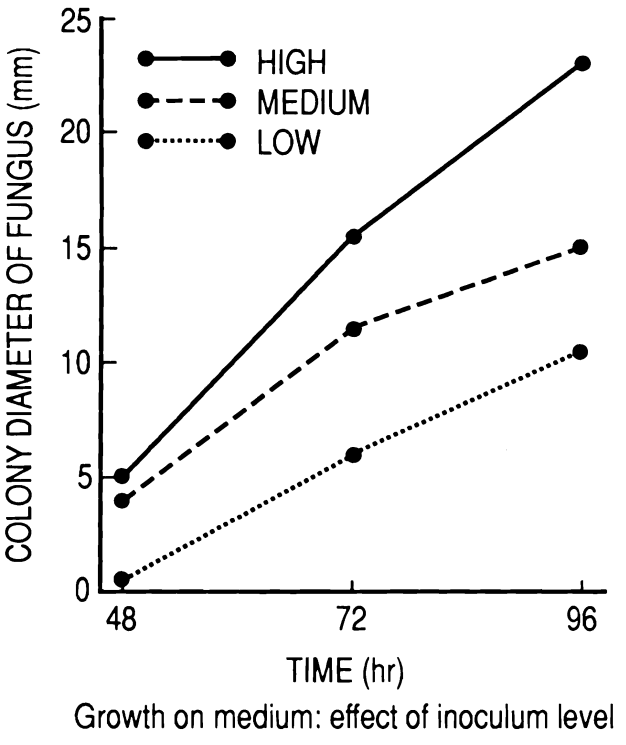

Fig. 3. Average colony diameters of *O. ulmi* on one half strength modified MS medium. The medium was inoculated by placing a 1.5 mm filter paper triangle, containing 20 µl of a suspension of 0.03 × 10^6, 2 × 10^6, or 15 × 10^6 spores/ml, in the center of a 15 × 60 mm (0.6 × 2.4 in) Petri plate. Colony diameters were determined by averaging two random axes at right angles to one other. Data were collected after 48, 72 and 96 hours.

spectively, as demonstrated by inoculations of intact plants. Latunde-Dada and Lucas (11) showed that temperature affected disease reactions in a lucerne-*Verticillium* interaction through influences on both partners without changing the genetic background of either.

O. ulmi colonized the surface of callus tissue of American elm A and 8630 more rapidly than that of the resistant Siberian elm under all conditions of temperature and inoculum concentration. While fungal growth rate was more rapid over calli of the more resistant American elm 8630 than the more susceptible American elm A, fungal density was greater on calli from the latter selection. This would indicate overall more fungal growth on susceptible American elm A than on resistant American elm 8630. These results corraborate those of others that report increased fungal growth on callus from susceptible than resistant hosts (3, 9, 13, 18). These results are consistent with the reported differences in resistance of intact plants (19, 20).

Incubation temperatures similarly affected growth rates on callus from all three selections and on the half-strength MS medium. Most rapid growth occurred at 22°C (72°F) followed by 16 and 28°C (61 and 83°F). Differential effects of temperature were noted particularly at 22°C (72°F). Apparently differences between fungal growth rates on American elm 8630 and A calli were reduced due to accelerated growth on American elm A callus at 22°C (72°F). Extension of the fungal growth rate lines of American elm A and 8630 in Fig. 2 indicate that growth rate differences on these calli sources may disappear between 22 and 25°C (72 and 77°F). Thus, temperature has a differential effect depending on the callus source as well as influencing fungal growth rate. On the other hand, the fungal growth rate on Siberian elm callus would clearly remain below that on the American elm sources at any temperature. This suggests that resistance factors in the Siberian callus are more strongly genetically controlled than they are in American elm A and influenced less by temperature (Fig. 2).

We observed a direct correlation between growth rate and inoculum concentration of *O. ulmi* both on calli from resistant and susceptible sources and on half-strength MS medium. Holliday and Klarman (9) reported similar results with *Phytophthora* on soybean callus. As with temperature, growth rates were most rapid on American elm 8630 followed by American elm A and Siberian. However, there were no callus × inoculum concentration interactions. This would indicate that inoculum concentration affects fungal growth rate but has less influence than temperature on genetic factors controlling resistance.

Since Schreiber and Stipes (18) reported a direct correlation between inoculum concentration and rate of disease development in 8 to 10 year old American elms, we would have expected a callus × inoculum concentration interaction. Such an interaction may occur at higher inoculum levels.

Color changes, as noted in inoculated calli of American elm 8630, were reported by others (6, 11, 13) and have been associated with a hypersensitive, resistant reaction.

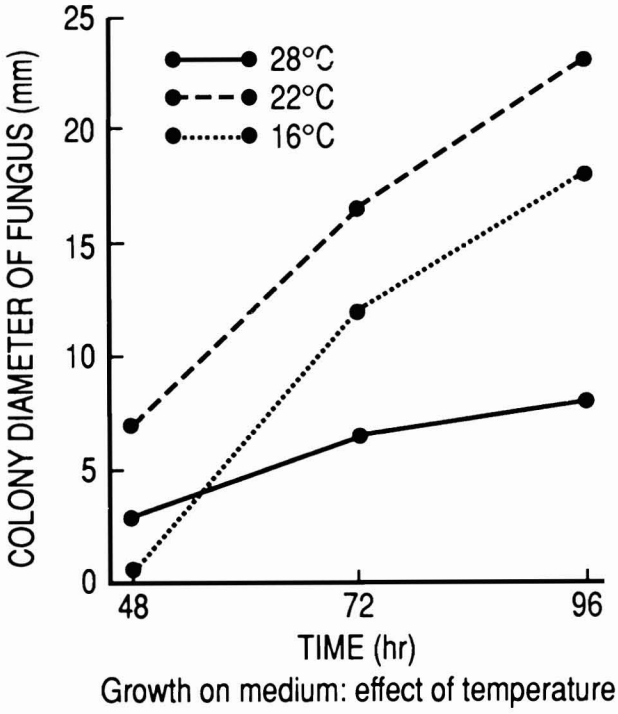

Fig. 4. Average colony diameter of *O. ulmi* on one-half strength MS medium incubated at 16, 22, or 28°C (61, 72, or 83°F). The medium was inoculated by placing a 1.5 mm filter paper triangle, containing 20 µl of a suspension of 0.3×10^6, 2×10^6, or 15×10^6 spores/ml, in the center of a 15 × 60 mm (0.6 × 2.4 in) petri plate. The colony growth was determined by averaging two random diameters at right angles to one other. Data were collected after 48, 72, and 96 hours.

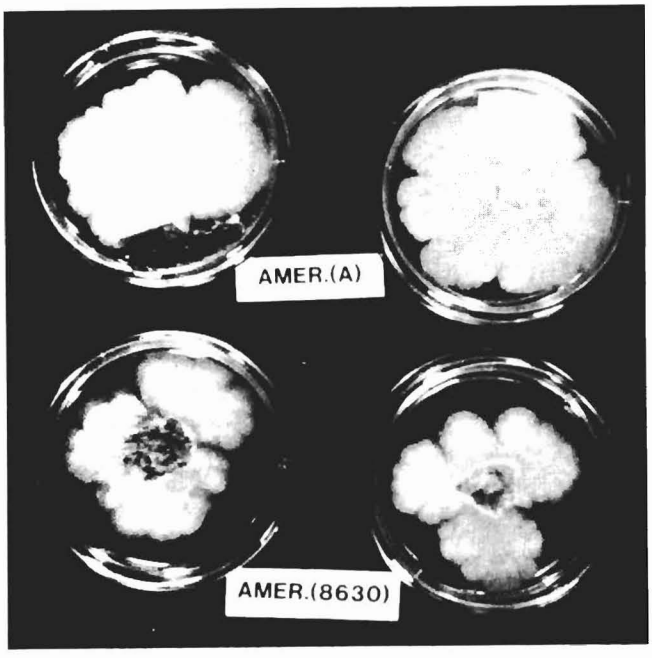

Fig. 5. Comparison of density of fungal growth on callus tissue from susceptible American elm (A) and resistant American elm (8630), 5 days after inoculation.

This study shows that an elm callus culture system may be used to differentiate resistant and susceptible elm germplasm. It may also be useful in determining factors that influence disease expression in elms. The *in vitro* system reflects advantages for identifying resistant germplasm by savings in time, space and cost over current methods. In addition, it may be applicable to other tree species-pathogen interactions. Additional studies, using intact plants and analytical and biochemical analyses, will help determine the mechanisms of disease resistance in elms.

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Leaf Gas Exchange of Eastern Redbud (*Cercis canadensis* L.) Grown Under Sun and Shade[1]

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Abstract

Leaf physiology of eastern redbud (*Cercis canadensis* L.) was assessed under natural photoperiod when grown in 100% sun or under polyethylene shade with a light transmittance of 69%, 47%, or 29% sun. Net CO_2 assimilation rate (A) was similar under 100%, 69%, and 47% sun; A was reduced under 29% sun. Adaptations to shade included a near perpendicular leaf orientation to the sun, reduction in specific leaf weight (SLW), and a decreased chlorophyll *a*: chlorophyll *b* ratio. Conversely, eastern redbud adapted to 100% sun by manifesting an increased SLW and a vertical orientation of leaves that curled inward toward the midrib. Light response curves were similar for sun- and shade-acclimatized plants. When all data were analyzed collectively, A was most closely related to photosynthetic photon flux (PPF) ($R^2 = 0.52$), whereas stomatal conductance to water vapor (gs) was primarily influenced by vapor pressure deficit (VPD) ($R^2 = 0.75$). Hence, A and gs were not well correlated ($R^2 = 0.41$). The lack of strong coupling between A and gs allowed the stomates to remain open under low PPF, resulting in an elevated intercellular CO_2 concentration. Thus, A was stimulated above what might have normally occurred under low PPF.

Index words: net CO_2 assimilation, transpiration, stomatal conductance, chlorophyll

Significance to the Nursery Industry

Eastern redbud (*Cercis canadensis* L.) typically is grown in full sun in nurseries throughout the country. Physiological evidence from our research suggests that eastern redbud performs equally well at moderate to high light intensities in the deep south. Net photosynthetic rate was reduced under heavy shade (29% sun). The only advantage of producing eastern redbud under less than full sun, especially in the south, is the improved foliage display resulting from darker green leaves with a less vertical orientation.

Introduction

Cercis canadensis, the eastern redbud, is widely grown and cultivated as a small tree. In the cooler northern limits of its natural distribution, eastern redbud is found in areas of high light such as open woodlands and along borders of woods, while it is more prevalent under shaded conditions in the warmer southern part of its range (12). Therefore, eastern redbud appears to be a shade-tolerant species (and possibly shade obligate) in the southern part of its range, and possibly farther north. No physiological analyses have demonstrated that these empirical observations are due to physiological responses to varied light availability.

Information regarding the shade tolerance of eastern redbud may prove beneficial to the nursery and landscape maintenance industries. The objective of this study was to determine the sun tolerance of containerized eastern redbud by assessing leaf gas exchange and chlorophyll levels under four light regimes—100%, 69%, 47%, and 29% sun.

Materials and Methods

1987 to 1989 experiments. Eastern redbud seedlings, derived from seeds collected in the southeast (specific location not identified), were obtained from a local nursery. In April 1987, plants were potted into 3.8 l (1 gal) containers with a medium of pine bark: Canadian sphagnum peat: sand (2:1:1 by vol). One cubic meter (1.3 yd^3) of medium was amended with 2.97 kg (6.6 lb) dolomite, 2.97 kg (6.6 lb) superphosphate, 0.89 kg (2.0 lb) Micromax (12S-0.1B-0.5Cu-12Fe-2.5Mn-0.05Mo-1Zn), and 5.9 kg (13.0 lb) Osmocote 18N-2.6P-10.0K (18-6-12). Plants were placed outdoors under shadecloth (69% sun transmittance) until the experiment was started on June 17, 1987.

The experiment, set up as a randomized complete block design, was conducted in an 24.4 × 17.1 × 2.0 m (80 × 56 × 6.6 ft) open-sided structure with the long axis oriented

[1]Received for publication June 3, 1991; in revised form August 9, 1991. Florida Agricultural Experiment Station Series Journal No. R-01363.

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