Inoculation of Brazilian Sour Orange Seed with an Endomycorrhizal Fungus

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ABSTRACT

Brazilian sour orange seed pelleted with inoculum of *Glomus fasciculatus* (*Endogone fasciculata*) produced mycorrhizal seedlings in greenhouse and field experiments. In the greenhouse, Arasan treatment of seed retarded and reduced mycorrhizal infection. In a nursery field fumigated with methyl bromide, seed inoculation prevented stunting and chlorosis of seedlings. Seed inoculation appears to be a practical method for obtaining mycorrhizal infection of citrus in fumigated or heat-treated soil.

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Citrus seedlings grown in fumigated nursery soils are often stunted and chlorotic. This problem was attributed to inadequate nutrition brought about by killing of endomycorrhizal [vesicular-arbuscular (VA)] fungi (5). Citrus is highly dependent on mycorrhizae for acquisition of phosphorus and possibly other elements. There is much evidence that plants with VA mycorrhizae absorb larger amounts of phosphate (6) and that the external hyphae absorb and translocate phosphate through the soil to the root (4). Increased absorption of other elements may, at times, also be important (2, 3). Application of fertilizer containing a high percentage of phosphate partially overcomes stunting in fumigated citrus nurseries; however, it is probable that even better growth of seedlings could be obtained without high rates of fertilization if a practical method of inoculation with mycorrhizal fungi could be developed.

At present, it is not possible to produce inocula of VA mycorrhizal fungi in axenic culture. However, inocula can be produced in "pot cultures" or in small field plots on plants grown under carefully controlled conditions to avoid contamination with pathogens. Such inocula, consisting of spores and infected root fragments, can be incorporated into a soil at the time of planting. However, application of inocula directly to seed would have many advantages. No change in present planting practices would be necessary; smaller amounts of inocula would be needed, and possibly seed producers could inoculate seed prior to shipment to growers. This research was done to determine whether inoculation of citrus seed with a VA fungus is a practical method for obtaining mycorrhizal infection.

MATERIALS AND METHODS.—*Greenhouse experiments.*—A soil mixture of loam to sand (2:1, v/v) was used. In previous experiments, similar growth responses to mycorrhizal infection were obtained on citrus seedlings in soils that had been steamed, autoclaved, or fumigated with methyl bromide (5). In the present experiments, the soil mix was steamed for 1 hour at 82 C, and then chemically analyzed by the University of Illinois' Agronomy Department Soil Testing Laboratory which used the Bray method for phosphorus. In the first of two experiments, testing indicated 21 kg/hectare (ha) available phosphorus (P1), 42 kg/ha, available and acid-soluble phosphorus (P2), K 150 kg/ha, pH 7.3. In the second experiment, the soil mix tested P1 21, P2 47, K 170, pH 6.8.

Experiments were done in 15-cm diameter plastic pots with 15 Brazilian sour orange (*Citrus aurantium* L. 'Brazilian') seeds planted in each pot. Experiments were replicated five times and the treatments were arranged in a completely randomized design. Pots were rearranged approximately every 2 weeks. An isolate of *Glomus fasciculatus* (Thaxter) Gerd. & Trappe (*Endogone fasciculata*) originally obtained from the citrus nursery of Willits and Newcomb, Inc., Thermal, California, was used. Inoculum was extracted from pot cultures maintained on sudangrass [*Sorghum sudanense* (Piper) Staph.] by wet-sieving and decanting. Sievings were collected on 233-, 149-, and 74-μm sieves. In addition, roots retained on a 1-mm sieve were fragmented in a Waring Blendor and added to the other sievings. To ensure that contaminating microorganisms in the inoculum were also added to control pots, the inoculum was washed, and the wash water was passed five times through a 44-μm sieve. Ten ml was added to the soil in each control pot.

Seed was inoculated by thoroughly coating 420 Brazilian sour orange seeds with a slurry consisting of about 35 ml of sievings and 5 ml of a 1% solution (w/v) of 400-centipoise methyl cellulose. The seed was dried until it could be handled without appreciable loss of inoculum. It was then planted or stored in plastic bags at 4-5 C until required. Noninoculated seed was stored under comparable conditions.

Mycorrhizal infection, indicated by increased growth rates, was confirmed by wet-sieving for hyphae and spores of the fungus, or by direct examination of roots for attached hyphae and spores. In every instance, increased growth was associated with production of hyphae and spores of *G. fasciculatus*.

Height of plants was measured at regular intervals

starting at the time growth differences were expected or first observed. Dry weights of entire plants were taken at the conclusion of each experiment.

Field experiments.—Three field experiments were established in a randomized complete block design at Willits and Newcomb, Inc., nursery, in soil previously fumigated with methyl bromide. Brazilian sour orange seed without fungicide treatment was used. Experiment I was replicated four times, and each plot contained 95 seeds planted 2 cm deep, 3.8 cm apart, in five rows spaced 7.5 cm apart. Experiments II and III were each replicated six times, with 50 seeds planted at 7.5 × 7.5 cm spacing in each plot. The three experiments were planted on 16 Jan, 21 Feb, and 22 Feb 1974, respectively, and were terminated 1 Nov 1974. Plants were lifted after a cutting bar was passed about 23 cm below the soil surface. Fresh weights were taken and root and soil samples collected at the time of harvest.

The isolate of *G. fasciculatus* used in greenhouse experiments was also used in the nursery. Inoculum was produced in pot cultures on sudangrass or Brazilian sour orange, and in these experiments the cultures on sudangrass were established from surface-sterilized spores. Inoculum from sudangrass was prepared as for the greenhouse experiments. Inoculum from cultures on Brazilian sour orange was collected on 74- and 149-μm sieves. Because of the near absence of root fragments in this soil, virtually all material passed through the 1-mm sieve.

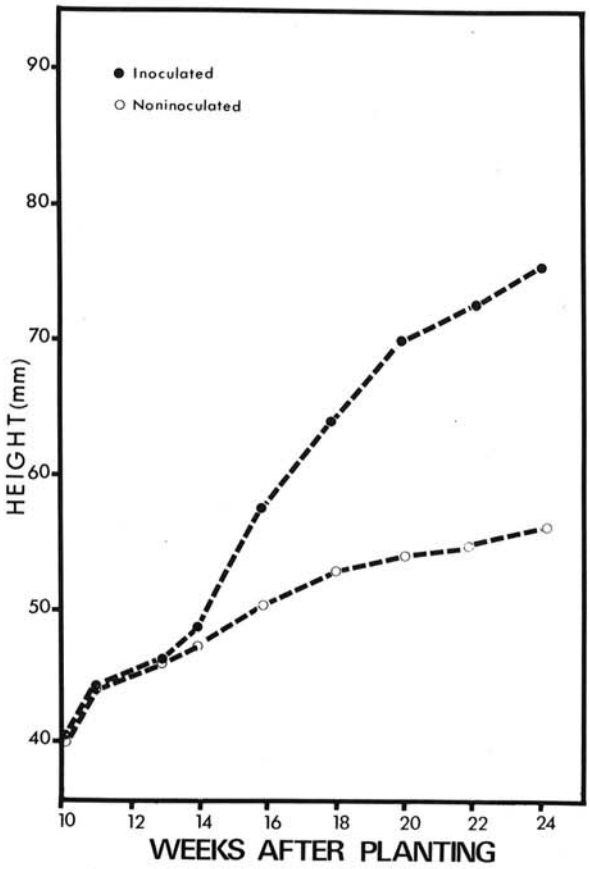

Fig. 1. Growth of Brazilian sour orange seedlings in steamed soil. Inoculated seed was coated with inoculum of the vesicular-arbuscular mycorrhizal fungus, *Glomus fasciculatus,* in a solution of methyl cellulose. Noninoculated seed was planted in steamed soil to which was added a microbial suspension obtained by washing the inoculum.

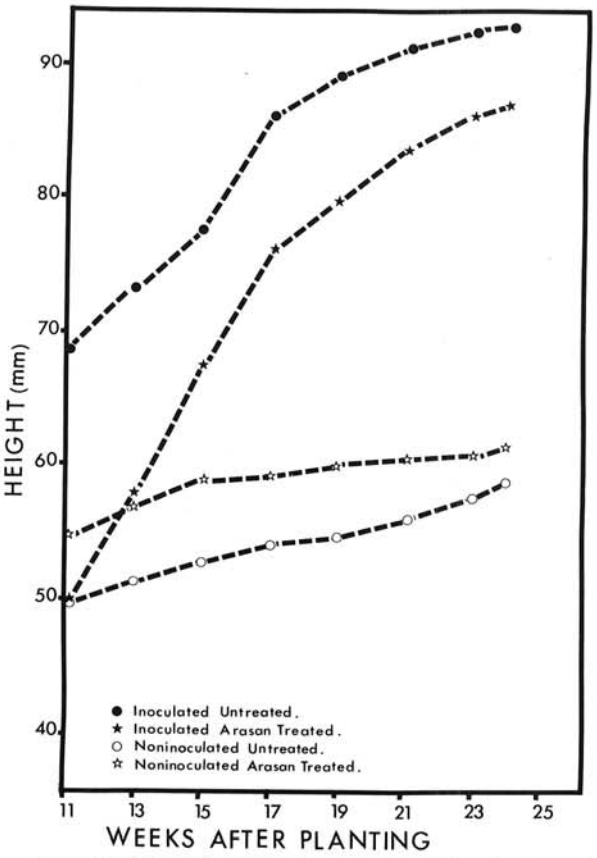

Fig. 2. Growth of Brazilian sour orange seedlings in steamed soil. Inoculated, untreated = Seed without Arasan treatment coated with inoculum of the vesicular-arbuscular mycorrhizal fungus, *Glomus fasciculatus,* in a methyl cellulose solution. Inoculated, Arasan-treated = Arasan treated seed coated with inoculum of *G. fasciculatus* in a methyl cellulose solution. Noninoculated, untreated = Noninoculated seed without Arasan treatment. Noninoculated, Arasan-treated = Noninoculated, Arasan-treated seed. A microbial suspension obtained by washing the inoculum was added to the soil of both noninoculated controls.

In Experiment I, seed was inoculated at the same rate as in greenhouse experiments. In Experiments II and III, a slightly lower rate was used: 40 ml of sievings to approximately 615 seed (100 g). Each experiment had two controls: seed coated with methyl cellulose and nontreated seed. In Experiments II and III, inocula with and without methyl cellulose sticker were compared.

Root samples taken at the end of the experiments were cleared, stained, hand-sectioned, and examined microscopically for mycorrhizal infection. Soil samples were also taken at the conclusion of the experiments. The pH of the soil in all three experiments was 8.5. In Experiments I, II, and III, tests for phosphorus indicated

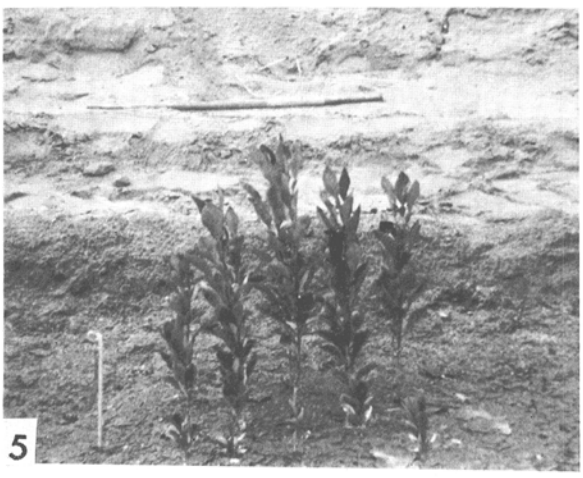

Fig. 3-5. Brazilian sour orange seedlings in Experiment II at the nursery of Willits and Newcomb, Inc., Thermal, California. **3)** Seed inoculated with the vesicular-arbuscular mycorrhizal fungus, *Glomus fasciculatus*, from a culture produced on Brazilian sour orange. **4)** Seed inoculated with *G. fasciculatus* from a culture produced on sudangrass. **5)** Noninoculated seed. Methyl cellulose was used as a sticker in all three treatments.

P1 217, 224, and 223 kg/ha, respectively. P2 in all three soils exceeded 224 kg/ha. The potassium tests were 108, 124, and 105 kg/ha, respectively.

RESULTS AND DISCUSSION.—*Greenhouse experiments.*—A preliminary experiment was done to determine the ability of spores of *G. fasciculatus* to withstand desiccation. Soil from a pot culture was spread in a thin layer and air-dried for 24 hours at 15-21 C and 30% relative humidity. It was stored in an open tray for up to 2 weeks at 15-27 C and 30-50% relative humidity. At the end of the 1- and 2-week storage periods, samples of the dry soil were mixed with steamed soil, and maize (*Zea mays* L.) seed was planted. Mycorrhizal infection was obtained in all inoculated pots, and the controls were free of infection. Thus, it was shown that spores of *G. fasciculatus* are capable of withstanding air-drying in soil for at least 2 weeks.

In the first greenhouse experiment, seed was inoculated with *G. fasciculatus* and stored in plastic bags at 4-5 C. Portions of the seed were planted after 0, 1, 7, 14, and 28 days.

Comparable results were obtained with all dates of planting. Therefore, only the data obtained from seed stored for 28 days is presented. All plants were approximately the same height until about the 14th week. After that, plants inoculated with *G. fasciculatus* grew more rapidly than the controls (Fig. 1). After 24 weeks, the mean dry weight of entire seedlings (including tops and roots) was: inoculated 0.72 g, control 0.46 g. The difference was significant $P = 0.01$. When soil from around the roots was wet-sieved, an abundance of new spores was found in the *G. fasciculatus* treatments. No spores were found in soil from the control pots. The experiment demonstrated that seed inoculation of Brazilian sour orange with *G. fasciculatus* is feasible.

In the second greenhouse experiment, seed that had been previously treated with a fungicide was inoculated. Citrus seed planted at the Willits and Newcomb nursery is customarily treated with Arasan 75 W. P. (thiram, tetramethylthiuram disulfide) by dipping the seed in a slurry consisting of 0.91 kg Arasan in 4.4 liters of water. Therefore, the previous experiment was repeated adding Arasan-treated seed as one of the variables. Arasan-treated Brazilian sour orange seed was obtained from the nursery and inoculated as in the previous experiment. Seeds were planted immediately or after 14 days storage at 4-5 C. Similar results were obtained with both plantings, therefore, only the data from the 14-day planting are reported. Eleven weeks after planting, the seedlings grown from inoculated seed, without Arasan treatment were distinctly taller than the plants from inoculated Arasan-treated seed, or the controls (Fig. 2). High greenhouse temperatures were likely responsible for the early growth response in this experiment. After the 11th week, plants from the inoculated Arasan-treated seed grew rapidly, and by the end of the experiment plants in four out of five pots were as large as the plants from inoculated seed without Arasan treatment. In one pot, plants from inoculated Arasan-treated seed were approximately the same size as the controls, and they proved to be nonmycorrhizal. At 14 weeks, plants exhibited characteristic symptoms of nitrogen deficiency, and on the 15th and 16th week 50 mg of ammonium nitrate was added to the soil in each pot; however, this

TABLE 1. Influence of seed inoculation with *Glomus fasciculatus* on the growth of Brazilian sour orange in soil fumigated with methyl bromide at the nursery of Willits and Newcomb, Inc., Thermal, California

Inoculum source (Pot-culture host)[a]	Mean fresh wt (g)[b]		
	Experiment I[c]	Experiment II[d]	Experiment III[e]
Brazilian sour orange	...	37.2	59.5
Brazilian sour orange + MC	60.2	40.0	56.0
Sudangrass	...	33.2	54.2
Sudangrass + MC	46.3	32.2	53.4
Control (no inoculum)	37.0	9.2	40.1
Control + MC	37.5	11.7	42.3
LSD ($P = 0.05$)	10.3	5.8	8.1
LSD ($P = 0.01$)	14.9	7.8	10.8

[a]MC indicates inoculum was applied to the seed with methyl cellulose used as a sticker.
[b]All seedlings were cut at 23 cm below the soil surface. In general, inoculated seedlings had longer roots and hence they had a greater weight loss than the controls.
[c]Both inoculated and control plants were mycorrhizal.
[d]Inoculated plants were mycorrhizal. No mycorrhizae were found in root samples from controls.
[e]Mycorrhizal status of plants was not determined.

amount was apparently inadequate because all mycorrhizal plants continued to have deficiency symptoms, and after 17 weeks their growth rate declined.

After 24 weeks, the mean dry weights were inoculated Arasan-treated (including one pot containing nonmycorrhizal plants in which the mean plant weight was 0.67 g) 1.23 g; control, 0.66 g (significant $P = 0.05$); inoculated without Arasan treatment 1.28 g, control 0.58 g (significant $P = 0.01$). Plants in all control pots were nonmycorrhizal, whereas plants in all inoculated pots, with the one noted exception, were mycorrhizal. Seed treatment with Arasan apparently retarded, and in one pot prevented, mycorrhizal infection. However, the rate of infection obtained could be satisfactorily high. In a nursery, the close spacing of seedlings should permit the fungus to spread from one plant to another. Further research is required to determine the practicality and desirability of combining seed inoculation with chemical seed treatment.

Field experiments.—Seed inoculation increased the growth of Brazilian sour orange plants in the nursery in all three experiments (Table 1). In Experiment I, all of the controls sampled, as well as the inoculated plants, proved to be mycorrhizal, which indicated that fumigation had not completely eradicated mycorrhizal fungi. Nevertheless, seed inoculated with *G. fasciculatus* from a Brazilian sour orange pot culture produced plants with an average of 65% greater fresh weight than the controls. An earlier and a higher level of infection was probably responsible for the improved growth. Seed inoculated with *G. fasciculatus* from a pot culture of sudangrass produced seedlings that were significantly smaller than those inoculated with the same isolate grown on Brazilian sour orange.

In Experiment II, the controls grew very poorly in comparison with the plants grown from inoculated seed (Fig 3, 4, 5) and differences between treatments were highly consistent. Root samples from inoculated plants were mycorrhizal, and samples taken from the controls were nonmycorrhizal.

In Experiment III, seed inoculated with *G. fasciculatus* from Brazilian sour orange and from sudangrass produced seedlings significantly larger than the controls. In this experiment, the roots were not examined for mycorrhizal infection; however, the relatively good growth of the controls suggests that they were mycorrhizal.

No clear pattern emerges from the comparison of inoculated seed with and without methyl cellulose. If methyl cellulose had an effect, our experiments were not sensitive enough to measure it. If the seed is to be handled to any extent after inoculation, it would probably be wise to use methyl cellulose or some other sticker to avoid loss of inoculum. The present experiments provide no evidence as to why seed inoculated with cultures from Brazilian sour orange consistently performed better than seed inoculated with the same isolate grown on sudangrass. It is tempting to assume the cause to be adaptation; however, the inocula differed in several other respects. Inoculum from sudangrass cultures contained considerable root fragments, whereas sievings from Brazilian sour orange were nearly free of roots. No attempt was made to standardize spore numbers. Also, the cultures produced on sudangrass were established from surface-sterilized spores, whereas the cultures grown on Brazilian sour orange were started from nontreated spores. It is possible that bacteria present in the inoculum obtained from Brazilian sour orange cultures may have had a beneficial effect on seedling growth (1).

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