Table 5. Effects of seaweed sprays on mineral composition of Citrus leaves.

Treatments	N	Mg	Mn	Zn	Cu	Fe	B
Greenhouse*	%	%	ppm	ppm	ppm	ppm	ppm
Sour orange seedling							
1. Control	2.11 b	0.17	8 b	21 b	8	76 b	53 b
2. BM-86 (2%)	2.55 a	0.18	6 b	23 b	18	104 a	386 a
3. MZ-63 (2%)	2.30 ab	0.15	32 a	33 a	14	66 b	33 b
Young trees							
Osceola/Carrizo							
1. Control	3.25 b	0.32	26 b	31 b	12 b	59	—
2. MZ-63 (2%)	3.39 a	0.31	67 a	77 a	33 a	73	—
Bearing trees							
Washington Navel orange							
1. Control	2.51 b	0.59	23	20 b	53	61	86
2. BM/MZ/BM (64 oz/A @)	2.58 a	0.61	24	24 a	60	65	98
Ruby Red grapefruit							
1. Control	2.31	0.60	36	21 b	99	60	76
2. BM/MZ/BM (64 oz/A @)	2.30	0.60	38	24 a	111	64	88

Means not having the same letters are significantly different at 5% level. Absence of letter after means indicates the differences are not significant.
*A nutrient solution consisting of 0.053% KNO_3; 0.125%/Ca $(NO_3)_2 \cdot 4H_2O$; 0.52% $MgSO_4 \cdot 7H_2O$, and 0.014% KH_2PO_4 was used on all plants.

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AN ASSAY TO ESTIMATE CITRUS ROOTSTOCK RESISTANCE[1]
TO BURROWING NEMATODES

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Abstract. **Seedlings of 5 different citrus rootstock cultivars germinated from seeds that had their seed coats removed and then surface sterilized were grown in autoclaved glass culture tubes filled with sterile sand. Each tube was inoculated with one of five different burrowing nematode strains 30 days after the seeds were planted. Thirty days later, nematodes were extracted from each seedling and median number of nematodes per treatment was determined. The data suggests that this assay may be used to estimate the relative susceptibility of citrus germplasm to the burrowing nematode or to determine the host range of uncharacterized burrowing nematode populations. The assay requires less time, physical, fiscal and human resources than conventional greenhouse studies while yielding similar information.**

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Losses attributed to spreading decline of citrus caused by the burrowing nematode, *Radopholus citrophilus* Huettel (Suit and DuCharme, 1953), have been reduced in many Florida citrus groves through the use of nematode-resistant rootstocks. However, use of resistant rootstocks does not always afford reliable nematode control. Breakdown of resistance has been associated with the detection of resistance-breaking nematode races (Kaplan and O'Bannon, 1985; O'Bannon and Ford, 1979). Variation in the inheritance of resistance among some rootstocks has also been associated with poor rootstock performance (Kaplan, 1986). Furthermore, none of these rootstocks has a broad range of desirable horticultural characteristics, i.e., resistance to other nematodes, to soil-borne diseases such as Phytophthora foot rot, and tolerance to cold.

Previously, spreading decline had been controlled through the Push and Treat Program and by the creation of chemically maintained fallow barriers to prevent nematode spread to adjacent uninfested citrus groves (Poucher et al.,

1967). Both programs are presently obsolete because they are dependent upon the use of fumigants that are no longer available (Kaplan, 1988). To date, the state nursery certification program (Poucher et al., 1967) continues to provide a highly effective means of preventing long-distance spread of burrowing nematodes. The importance of effective, environmentally safe, and economically feasible control strategies for burrowing nematodes will increase as the nematodes migrate unabated to neighboring citrus groves and thus increase their distribution due to the loss of traditional control programs. Resistant rootstocks could provide an economic and environmentally safe alternative to the use of nematicides if durable resistance can be identified. However, the first stage of screening for resistance is a long and arduous process involving greenhouse studies and subsequent field testing. Initial greenhouse evaluations require 6-9 months. Since burrowing nematodes are strongly influenced by environmental conditions (Duncan and Cohn, 1989), each test plant is grown in an 8" diameter clay pots to minimize fluctuations in moisture and temperature (Kaplan, 1990) in an attempt to ensure that conditions favor nematode-plant interactions that emulate the field. This requires considerable amounts of greenhouse bench space. Long-term experiments are subject to contamination by pests that may adversely influence the outcome of greenhouse studies. Furthermore, diminishing economic, physical, and human resources also limit the extent of replication and the number of nematode isolate-germplasm interactions that may be evaluated.

We report progress toward development of a reliable, time and resource efficient assay system to estimate burrowing nematode-resistance in citrus germplasm. Conversely, this assay is also useful to estimate the ability of burrowing nematode populations to break citrus rootstock resistance.

Materials and Methods

Glass culture tubes (24 x 150 mm) were filled with approximately 55 cm³ of air-dried Astatula sand (hyperthermic, uncoated typic quartsipsamments) and placed in stainless steel racks enclosed in autoclave bags. The racks containing the sand-filled culture tubes were autoclaved for 1 hour at 20 p.s.i. and 125C on a liquid sterilization cycle. The tubes were allowed to cool overnight and were then autoclaved again under the same conditions.

The seed coats of citrus seeds were peeled from the hilum end downward with a fine-tip forceps. The seeds were then soaked in 0.26% sodium hypochlorite for 30 minutes, rinsed 4 times with sterile distilled water, and incubated overnight in a small amount of sterile distilled water that just exceeded the height of a monolayer of seeds in a sterile beaker. Seeds of the tomato cultivar Sunny (*Lycopersicon esculentum* L.) were used as controls. Tomato seeds were surface sterilized with 0.52% sodium hypochlorite for 10 minutes and then rinsed 3 times with sterile distilled water. Tomato seeds were immediately placed beneath the surface of the sand in each tube.

Prior to planting in each sand-filled tube, 3 ml of sterile distilled water was added to moisten the sand. Then 2.0 cm deep depressions were made in the center of the surface of the sand in each tube into which a single seed was placed. The seeds were covered with sterile sand and the tubes were maintained at 25C +/- 1.5 on a shelving unit in a laboratory that was adjacent to a window with a southern exposure. The racks received 9.5 hours of supplemental fluorescent light 5 days per week with intensity ranging from 3200 - 4000 ft. candles. Soil moisture was maintained at ca. 3% w/w of soil through the addition of sterile distilled water.

Beginning 30 days after seeding, the sand in each tube containing a young seedling was infested with approximately 200 burrowing nematodes (mixed life cycle stages) that were extracted from carrot disk culture (Kaplan and Davis, 1990). The nematode suspensions (100 nematodes per ml of sterile distilled water) were administered twice to the sand in each tube at 5-day intervals. The burrowing nematode strains used in the 2 sets of experiments were identified according to Bird and Riddle, 1994; as 1) *Radopholus similis* from Florida (DK6) which reproduces in banana, but does not attack citrus; 2) two *R. citrophilus* isolates from Florida (DK4 & DK8) that reproduce on burrowing nematode-susceptible rootstocks such as rough lemon and sour orange, but which do not reproduce on the rootstocks Milam lemon (*Citrus limon* L.), Ridge Pineapple (*Citrus sinensis* [L.] Osbeck), or Kuharski Carrizo (Citrus sinensis (L.) Osbeck × Poncirus trifoliata (L.) Raf.); and 3) two *R. citrophilus* isolates from Florida which reproduce in the roots of select rootstocks generally considered resistant to burrowing nematodes (DK1 & DK5) (Kaplan and O'Bannon, 1985; Kaplan, unpublished).

Root systems were harvested individually, 30 days after initial nematode infestation, by submerging each tube in a plastic box filled with tap water. The root system and sand were gently removed from the tube and the root system separated from the sand. Roots were then cut into small pieces and returned to their original tube after it was thoroughly rinsed free of sand. Each tube was fitted with a cap and approximately 30 tubes were placed upright in sealed polyethylene bags containing 50 ml of water in the bottom to maintain atmospheric humidity. The tube-filled bags were incubated at 25C +/- 1.5C for 7 days. Then, 2 ml of sterile distilled water was added to each tube and the tubes were mixed on a Vortex Genie set at full speed for 10 seconds. Each resulting nematode suspension was transferred to a counting dish and the number of nematodes extracted from each root system was determined using an inverted microscope at 160 X. Data were expressed as nematodes per tube and median values are reported. The Kolmogorov-Smirnov test statistic (SAS-NPAR1WAY procedure, SAS Institute, Inc., Cary, N.C., Version 6.04) was calculated to identify differences ($P = 0.05$) among nonparametric rating data for burrowing nematode strains by host and for host by nematode strain.

Experiment One. To determine if assay conditions were conducive to burrowing nematode population increase, tubes were seeded with burrowing nematode-susceptible tomato, sour orange, or rough lemon. The burrowing nematode strains DK1, DK4, DK5, DK6 and DK8 were introduced to individual tubes as indicated above and each treatment (nematode strain × plant) was replicated five times. The experiment was repeated twice. The relative rankings and extent of differences between treatment medians were similar in both experiments. Representative data from one of the experiments are presented.

Experiment Two. To determine if the assay could be used to identify nematode-resistant germplasm or rootstocks, the relative change in population densities of five burrowing nematode strains (DK1, DK4, DK5, DK6 and DK8) were determined in roots of rough lemon, Milam lemon, Kuharski Carrizo citrange, and Ridge pineapple; rootstocks known to differ in their suitability as hosts of burrowing nematodes.

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Each treatment was replicated 5 times and the experiment was repeated twice. The relative rankings and extent of differences between treatment medians were similar in both experiments. Representative data from one of the experiments are presented.

Results and Discussion

Population densities for the citrus-parasitic burrowing nematode strains DK1, DK4, DK5, and DK8, one month after infestation of each assay, indicated that experimental conditions were conducive to nematode development in roots of rough lemon, sour orange, and tomato seedlings (Table 1). Similar median values were detected in roots of tomato for all burrowing nematode strains tested (Table 1, 4). Population densities of DK6 (*R. similis* strain that does not attack citrus) declined in all citrus-DK6 treatments. Therefore, bioassay conditions did not alter the host-parasite relationship in a manner which enabled *R. similis* to parasitize citrus.

Although variation in the numbers of nematodes recovered from individual seedling root systems for each burrowing nematode susceptible treatment was considerable, plant treatments that differed with respect to their ability to restrict nematode population size were apparent despite limited replication. The median numbers of burrowing nematodes in

susceptible citrus seedlings were very high compared with initial inoculum densities and with the median numbers of nematodes from resistant citrus seedlings or non-hosts in the case of the interaction of DK6 with citrus (Table 1, 4). This assay appears to be well suited to identify nematode-plant relationships that differ qualitatively rather than quantitatively. Thus, data were handled in a nonparametric manner and were presented as the median number of nematodes per assay tube. Increased replication might enable the assay to be used to estimate quantitative differences in host status, but intermediate levels of resistance to burrowing nematodes are not considered useful for field application.

Assay data (numbers of nematodes) were not standardized on the basis of gram root weight. This was because of the small size of the seedling root systems grown under assay conditions. Root systems of test plants averaged 2.0×10^{-2} g of root dry wt per seedling. Reporting population densities on a root weight basis would yield artificially high values (e.g. 195,000 nematodes per g dry root wt). Thus data derived from the assay were best expressed as the number of nematodes detected per seedling since all root systems were of comparable size (data not shown).

The growth habit of the root systems of rough lemon and sour orange seedlings differed under assay conditions. Rough lemon seedlings had branched root systems whereas sour orange seedlings had a single tap root with limited branching. Preliminary studies involving staining (Kaplan and Davis, 1991) of the two types of root systems 30 days after infestation of test chambers indicated that although burrowing nematodes infected both sour orange and rough lemon, burrowing nematodes were more evenly distributed in the highly branched root system of the rough lemon seedlings. For strain DK5, the number of burrowing nematodes extracted from sour orange root systems were markedly lower than that recovered from roots of rough lemon (Table 3). On the basis of this observation, rough lemon was used as a susceptible control treatment in subsequent experiments. The growth habit of root systems of the test plant material included in the second experiment were similar to that of rough lemon. Sour

Table 1. Median number of burrowing nematodes detected in tube assay 30 days after infestation with 200 nematodes per tube.

Nematode Strain[x]		Tomato	Rough lemon	Sour orange
RC[y]	DK 1	382	2253	1599
RC	DK4	292	1405	937
RC	DK5	221	5922	1012
RS	DK6	299	1	0
RC	DK8	288	508	831

[x]Strain designations for burrowing nematode lines maintained by the Subtropical Plant Pathology Research Unit in Orlando.
[y]RC = *Radopholus citrophilus*; RS = *Radopholus similis*

Table 2. Comparison of burrowing nematode population levels thirty days post infestation of assay tubes.

	Nematode Strain[x]	DK4	DK5	DK6	DK8
Tomato	DK1	0.275[y]	0.087	0.627	0.137
	DK4	—	0.627	0.627	0.720
	DK5	—	—	0.627	0.358
	DK6	—	—	—	0.358
Rough lemon	DK1	0.329	0.329	0.013	0.818
	DK4	—	0.081	0.013	0.329
	DK5	—	—	0.013	0.818
	DK6	—	—	—	0.013
Sour orange	DK1	0.329	0.329	0.013	0.329
	DK4	—	0.818	0.013	0.818
	DK5	—	—	0.013	0.818
	DK6	—	—	—	0.013

[x]Strain designations for burrowing nematode lines maintained by the Subtropical Plant Pathology Research Unit in Orlando.
[y]Values represent the probability (*P*) of a greater KSa (Kolmogorov-Smirnov Asymtotic test statistic) for data presented in Table 1. The KSa is a measure of discrepancy between two comparisons. Values of *P*<0.05 are indicative of significantly different nematode population levels and suggest rejection of the null hypothesis: H_O: A = B in favor of H_1: a≠ B; where A and B represent strains of DK_x vs DK_y.
— Reciprocal Test reported above.

Table 3. Comparison of the host suitability of tomato, rough lemon, and sour orange thirty days post infestation of assay tubes.

Nematode Strain[x]		Rough lemon	Sour orange
DK1	Tomato	0.004[y]	0.004
	Rough lemon	—	0.818
DK4	Tomato	0.004	0.039
	Rough lemon	—	0.818
DK5	Tomato	0.004	0.039
	Rough lemon	—	0.013
DK6	Tomato	0.004	0.004
	Rough lemon	—	0.081
DK8	Tomato	0.008	0.061
	Rough lemon	—	0.329

[x]Strain designation for burrowing nematode lines maintained by the Subtropical Plant Pathology Research Unit in Orlando.
[y]Values represent the probability (*P*) of a greater KSa (Kolmogorov-Smirnov Asymtotic test statistic) for data presented in Table 1. The KSa is a measure of discrepancy between two comparisons. Values of *P*<0.05 are indicative of significantly different nematode population levels and suggest rejection of the null hypothesis: H_O: A = B in favor of H_1: A ≠ B; where A and B represent strains of DK_x vs DK_y.
— Reciprocal Test reported above.

orange might be useful as a control for test plants that did not develop branched root systems under assay conditions.

Burrowing nematode population densities in tubes containing Milam lemon, Kuharski Carrizo citrange, and Ridge pineapple (Table 4, 5, & 6) were consistent with previous observations (Kaplan and O'Bannon, 1985). Large numbers of nematodes from DK1, DK4, DK5 and DK8 were detected in roots of rough lemon and tomato seedlings, whereas the median population densities for DK6 (not a citrus parasite) were negligible for citrus (Table 4). The median values for DK4, DK6, and DK8 were similar to one another for seedlings of burrowing nematode-resistant citrus rootstocks (Table 6). Burrowing nematode strains DK4 and DK8 which developed to significant levels in roots of rough lemon and tomato, were not detected in appreciable numbers from roots of Milam

Table 4. Median number of burrowing nematodes detected in tube assay 30 days after infestation with 200 nematodes per tube.

Nematode Strain^x		Tomato	Rough lemon	Milam lemon	Ridge Pineapple	Kuharski Carrizo
RC^y	DK1	768	744	12	216	154
RC	DK4	608	560	4	2	0
RC	DK5	840	1680	690	0	360
RS	DK6	408	4	0	0	0
RC	DK8	936	1944	12	8	16

^{x}Strain designations for burrowing nematode lines maintained by the Subtropical Plant Pathology Research Unit in Orlando.
^{y}RC = *Radopholus citrophilus*; RS = *Radopholus simlis*

Table 5. Comparison of host influence on burrowing nematode population densities.

Nematode Strain^x		Rough lemon	Milam lemon	Ridge Pineapple	Kuharski Carrizo
DK1	Tomato	0.988^y	0.023	0.116	0.081
	Rough lemon	—	0.036	0.116	0.400
	Milam lemon	—	—	0.521	0.400
	Ridge pineapple	—	—	—	0.818
DK4	Tomato	0.819	0.013	0.013	0.013
	Rough lemon	—	0.619	0.013	0.013
	Milam lemon	—	—	0.999	0.999
	Ridge pineapple	—	—	—	0.818
DK5	Tomato	0.819	0.321	0.014	0.213
	Rough lemon	—	0.321	0.081	0.013
	Milam lemon	—	—	0.329	0.329
	Ridge pineapple	—	—	—	0.329
DK6	Tomato	0.047	0.047	0.047	0.047
	Rough lemon	—	0.999	0.999	0.999
	Milam lemon	—	—	0.999	0.999
	Ridge pineapple	—	—	—	0.999
DK8	Tomato	0.329	0.013	0.013	0.013
	Rough lemon	—	0.013	0.013	0.013
	Milam lemon	—	—	0.999	0.999
	Ridge pineapple	—	—	—	0.999

^{x}Strain designations for burrowing nematode lines maintained by the Subtropical Plant Pathology Research Unit in Orlando.
^{y}Values represent the probability (*P*) of a greater KSa (Kolmogorov-Smirnov Asymtotic test statistic) for data presented in Table 4. The KSa is a measure of discrepancy between two comparisons. Values of *P*<0.05 are indicative of significantly different nematode population levels and suggest rejection of the null hypothesis: H_O: A=B in favor of H_1: A ≠ B; where A and B represent strains of DK$_x$ vs DK$_y$.
— Reciprocal Test reported above.

Table 6. Comparison of burrowing nematode population size thirty days post infestation of assay tubes on each host.

	Nematode Strain^x	DK4	DK5	DK6	DK8
Tomato	DK1	0.999^y	0.329	0.925	0.818
	DK4	—	0.329	0.808	0.818
	DK5	—	—	0.808	0.818
	DK6	—	—	—	0.375
Rough lemon	DK1	0.988	0.999	0.023	0.512
	DK4	—	0.818	0.013	0.818
	DK5	—	—	0.013	0.329
	DK6	—	—	—	0.013
Milam lemon	DK1	0.512	0.040	0.164	0.948
	DK4	—	0.013	0.818	0.818
	DK5	—	—	0.013	0.329
	DK6	—	—	—	0.818
Ridge pineapple	DK1	0.013	0.013	0.013	0.329
	DK4	—	0.816	0.818	0.818
	DK5	—	—	0.818	0.032
	DK6	—	—	—	0.818
Kuharski Carrizo	DK1	0.048	0.329	0.013	0.048
	DK4	—	0.048	0.818	0.048
	DK5	—	—	0.048	0.329
	DK6	—	—	—	0.818

^{x}Strain designations for burrowing nematode lines maintained by the Subtropical Plant Pathology Research Unit in Orlando.
^{y}Values represent the probability (*P*) of a greater KSa (Kolmogorov-Smirnov Asymtotic test statistic) for data presented in Table 4. The KSa is a measure of discrepancy between two comparisons. Values of *P*<0.05 are indicative of significantly different nematode population levels and suggest rejection of the null hypothesis: H_O: A = B in favor of H_1: A ≠ B; where A and B represent strains of DK$_x$ vs DK$_y$.
— Reciprocal Test reported above.

lemon, Ridge pineapple, or Kuharski Carrizo (Table 4, 5). In contrast, median population values for the resistance-breaking strains DK1 and DK5 (Kaplan and O'Bannon 1985), confirmed the ability of these nematodes to reproduce in roots of citrus rootstocks considered to be burrowing nematode-resistant. Numbers of burrowing nematode DK1 were low in roots of Milam lemon but were detected at significant levels in roots of Kuharski Carrizo citrange and Ridge Pineapple, whereas significant numbers of DK5 were recovered from roots of Milam lemon and Kuharski Carrizo citrange, but were not detected in roots of Ridge pineapple (Table 4 & 5) in both experiments. This suggests that physiological mechanisms responsible for burrowing nematode resistance in the rootstocks differ or this may reflect differences between the nematode strains with respect to the manner in which they overcome citrus rootstock resistance (Table 6).

Conventional greenhouse studies designed to evaluate citrus germplasm response or to characterize the host range of burrowing nematodes require 6-9 months. The prolonged placement of these experiments under greenhouse conditions renders them vulnerable to contamination by fungi, bacteria, bacteriophagous nematodes, and insects that may be predators, parasites, or antagonists of burrowing nematodes (Walter et al, 1993). Citrus pathogens may also destroy citrus fibrous roots and thereby limit nematode development. In 1992 and 1993, greenhouse evaluations of citrus germplasm for nematode resistance were destroyed by *Thielaviopsis basicola* (Kaplan, unpublished), a fungus with airborne spores which destroys citrus fibrous roots (Graham and Timmer, 1991; Tsao and Van Gundy, 1962).

The bioassay system described here may be conducted in a laboratory setting or in a growth chamber, with one month required to produce suitable test plants and with a test duration of one month. This reduced time requirement and ability to place tests in confined space renders the assay more amenable to maintenance of stringent cultural conditions that reduce the likelihood of assay colonization by deleterious organisms. Use of autoclaved materials, and the capability to regulate the ambient temperature more carefully, also favor the likelihood that the assay will be reliable and data will reflect only the interaction of nematode and plant, being unaffected by extraneous factors. Furthermore, replication requirements are minimal relative to traditional greenhouse pot studies, variability in data is reduced and fewer counts are necessary. Median values were used conservatively and may obscure some trends, but appeared warranted given the limited number of replications.

In preliminary studies, we attempted to germinate citrus seeds under greenhouse conditions and to transplant the seedlings to the culture tube system. We found that the seedling root system subsequently rotted in the tubes. The studies conducted as described in the materials and methods involved the use of 375 tubes over a period of 6 months. Throughout that period our pest problems were minimal. We detected *Fusarium* sp. in 3 tubes and found bacterial feeding nematodes in two tubes. Fungal gnats were controlled through the use of Sticky Aphid/Whitefly Traps (Seabright Laboratories, Emeryville, CA). The use of microwave, rather than autoclaved soil might also provide an improved rooting medium (D. J. Mitchell, personal communication).

The bioassay has enabled us to characterize the host-range of burrowing nematode populations from Central America imported and maintained under quarantine restrictions because nematodes do not escape in water draining from the bottoms of pots as is the case in traditional greenhouse studies. The system could also be useful for the testing of nematode interactions with transgenic plant material. Furthermore, the bioassay could be used to study the effects of chemicals, biological, or physical treatments on nematode population development when a high degree of control over environmental conditions is required.

In summary, the tube assay system required far less time, personnel, and space than did traditional greenhouse pot studies. Data indicated that nematode-rootstock interactions were comparable to previous greenhouse observations (O'Bannon and Kaplan, 1985; Kaplan, 1986). Placement of the assay in the laboratory in conjunction with the relatively short duration of each test improves the capability to maintain constant conditions and to reduce the likelihood of assay contamination. This assay can provide researchers *working* with other plant parasitic nematodes with a reliable means of determining the relative effect of plant germplasm on nematode development. We obtained similar results with the root knot nematodes, *Meloidogyne arenaria* (Neal) Chitwood and *M. incognita* (Kofoid and White), Chitwood on tomato, and with the citrus nematode, *Tylenchulus semipenetrans,* Cobb on roots of citrus seedlings.

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