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Evaluation of bioassays to quantify *Cylindrocladium* inocula in soil

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Leaf discs provided better recovery of *Cylindrocladium candelabrum* from soil than stem or twig segments. Leaf discs of eucalypt (*Eucalyptus grandis*), azalea (*Rhododendron* sp.), and geranium (*Pelargonium* sp.) were the best of seven plant baits evaluated. Twig segments of azalea and eucalypt also provided a high percentage of the pathogen recovery, whereas stem segments of pine seedlings (*Pinus elliottii*) proved unsatisfactory. Although slightly less effective, twig segments were easier to handle than leaf discs which were quickly decomposed in soil. Colonization of eucalypt twig segments by *Cylindrocladium* spp. varied with inoculum level, soil moisture content, and incubation period. The highest percentage of recovery of *C. candelabrum* (approx. 95%) was calculated at a field capacity moisture level of 155.9% after 75 h of incubation.

Key Words——bioassay; *Cylindrocladium*; plant pathogen; soil baiting.

The genus *Cylindrocladium* Morgan has several species that are well-known pathogens of *Eucalyptus* spp. (Ferreira, 1989; Crous and Wingfield, 1994). Rot of rooted eucalypt cuttings caused by *Cylindrocladium* spp. is considered one of the main diseases affecting production in Brazil (Ferreira, 1989). The primary source of inocula is cuttings from the infected shoots in clonal hedges or orchards (Alfenas et al., 1997; Ferreira, 1997). Shoots from clonal hedges, that are used to make cuttings, can be contaminated by fungal propagules in the soil that are disseminated via rain or irrigation water splash (Alfenas et al., 1997; Ferreira, 1997). Data on the distribution and inoculum density of the pathogens in clonal hedge soils are therefore essential to establish preventive control measures against such diseases.

Several methods are available for quantification of *Cylindrocladium* spp. in soil. Most of them involve preparation of relatively complex culture media (Krigsvold and Griffin, 1975; Almeida and Bolkan, 1982; Hunter, 1992), are time consuming to prepare (Thies and Patton, 1970; Phipps et al., 1976), and do not allow fungal recovery at low inoculum density (Morrison and French, 1969; Hwang and Ko, 1975; Almeida and Bolkan, 1982).

The use of plant baits (Nash and Snyder, 1962; Sneh et al., 1966; Huang and Kuhlman, 1989; Dhingra et al., 1976; Menge and French, 1976) has been generally proposed as simple, effective and practical methods to quantify fungal inocula of pathogens with a high competitive saprophytic capacity in soil (Campbell and Benson, 1994). Several baits such as alfalfa seedlings (*Medicago sativa* L.) (Thies and Patton, 1966), azalea leaf and stem pieces (Linderman, 1972), castor bean leaves (Almeida et al., 1978; Ferreira, 1989) and geranium leaves (Hunter et al., 1980) have been used for qualitative detection of *Cylindrocladium* spp. in soil. However, only one, namely alfalfa seedlings (Menge and French, 1976) has been proposed for the quantitative detection of *Cylindrocladium*. Although this method has been found to be effective, these baits are not always readily available, which also proves problematic if large numbers of samples have to be treated. Therefore the purpose of this study was to evaluate several different, readily obtainable plant baits for the quantitative detection of *Cylindrocladium* spp. from soil.

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Materials and Methods

Evaluation of plant baits *Cylindrocladium candelabrum* Viégas was analysed as it was the most common species (90% of isolations) in the genus *Cylindrocladium* detected in soil samples from 2-yr-old *E. grandis* clonal hedges (maintained at 40 cm height by frequent pruning) (unpublished data). Several baits were evaluated to quantify the inoculum density of *C. candelabrum* in soil, namely leaf discs obtained from mature leaves (12 mm diam) of azalea (*Rhododendron* sp.), eucalypt (*Eucalyptus grandis* Hill; Maid.), geranium (*Pelargonium* sp.), castor bean (*Ricinus communis* L.) and twig segments (approx. 3 mm diam × 15 mm long) of eucalypt and azalea, and stem segments of pine (*Pinus elliottii* Engelmann) seedlings (120-d-old). A steam treated clay-sand soil mix (38% coarse sand, 14% fine sand, 7% silt and 41% clay) con-

taining 61% organic matter was manually infested with microsclerotia of a *C. candelabrum* isolate (UFV 117) at 8.4×10^{-2}, 3.4×10^{-1}, 1.35, 2.70, 5.40, 10.8 or 21.6 viable propagules per gram of soil (dry weight) (pH = 5.0). A sample of 200 g of infested soil was placed in plastic containers (11 × 11 × 3 cm), and moisture adjusted to 85% of field capacity. Microsclerotia were produced using the methodology described by Garcia et al. (1995). Propagule viability was assessed on potato-dextrose-agar (PDA) containing Rose Bengal (250 mg/L) after 3 d incubation at 25°C in the dark.

Baits were washed with tap water, surface sterilized with NaOCl (1000 ppm active chloride) for 1 min, and rinsed twice with sterile water. They were then half buried equidistantly in the soil in a 5 × 5 grid arrangement, 1 cm apart, immediately after soil infestation. Each replication consisted of a container with 25 baits of each plant material tested in a complete randomized block design with three replications per treatment. Containers were maintained at 25°C under a 12 h photoperiod and after 5 d baits were examined visually for *Cylindrocladium* sporulation. Baits without visual signs of colonization were surface sterilized as described above and placed on Petri dishes containing peptone dextrose agar (PEDA) plate acidified with 25% lactic acid (pH 4.0) (Hwang and Ko, 1975). After 10 d of incubation, the plates were examined under a dissecting microscope for *Cylindrocladium* colonies. An equal number of surface sterilized baits not buried in the soil were placed onto PEDA as controls.

Colonization of eucalypt twig baits in soil under different moisture regimes Two hundred gram samples of loam sandy soil (LSS) (60% coarse sand, 18% fine sand, 6% silt, 16% clay and 4.6% organic matter, pH = 5.0), which were naturally infested with approximately 10 microsclerotia/g soil (dry weight) of *Cylindrocladium* spp., were collected from an *E. grandis* clonal hedge in Belo Oriente-MG. The LSS samples were distributed in containers (11 × 11 × 3 cm) and the moisture content was adjusted to 33, 66, 99, 132 or 165% of field capacity (-0.1 atm). Thirty surface sterilized twig segments (15 × 3 mm) of eucalypt were distributed equidistantly 1 cm apart in a 5 × 6 grid arrangement. A completely randomized design with four replications for each moisture content was used. Incubating conditions, assessment of colonization, and the control treatment were as previously described.

Colonization of eucalypt twig baits in soil at different incubation periods The colonization of baits by *Cylindrocladium* spp. over time was investigated after 24, 48, 72, 96, 120, 144 and 168 h of incubation. The LSS samples previously described were placed in containers, and the moisture content were adjusted to 20% (w/dw) (137% field capacity). As in the previous trial, 30 surface sterilized twig segments were inserted in each container. A completely randomized design with five replications per treatment was used. The control treatment, incubating conditions, and assessment of colonization were as previously described.

Results

Evaluation of plant baits Leaf discs were more effective baits than twig and stem segments (Fig. 1). At 5.6

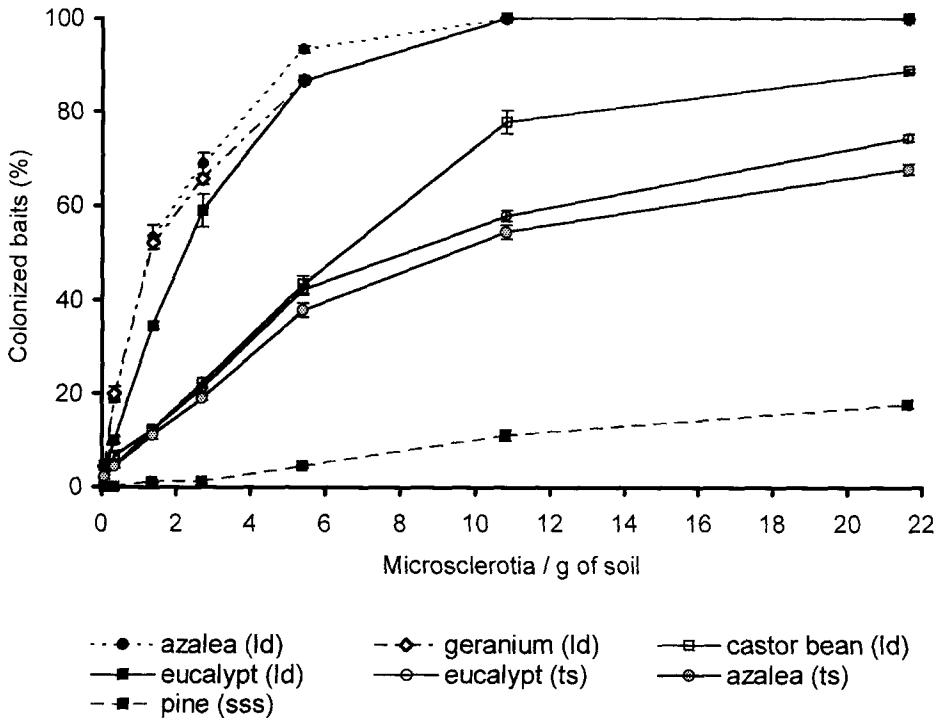

Fig. 1. Colonization of plant baits by *Cylindrocladium candelabrum* from pasteurized soil infested with different microsclerotial inoculum densities; (ld = leaf discs, ts = twig segments, and sss = seedling stem segments). Bars are standard error of the mean.

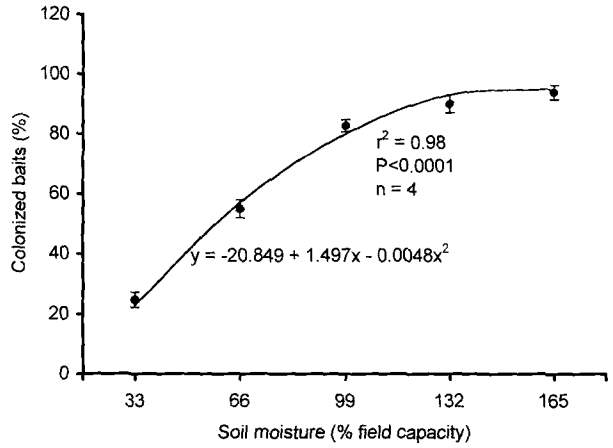

Fig. 2. Recovery of *Cylindrocladium* spp. from naturally infested soil incubated at different moisture levels. Bars are standard error of the mean (●).

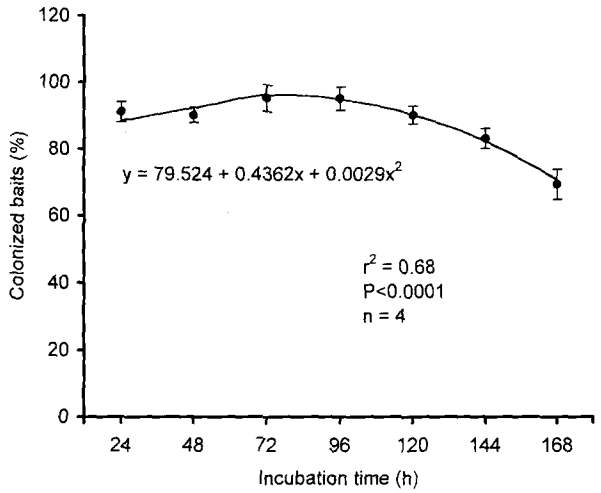

Fig. 3. Recovery of *Cylindrocladium* spp. from naturally infested soil at different periods of incubation. Bars are standard error of the mean (●).

propagules per gram of soil, approx. 90% recovery was achieved with azalea, eucalypt and geranium leaf discs, whereas castor bean leaf discs achieved around 42%. *Cylindrocladium candelabrum* was not recovered from any of the baits used as controls.

Geranium and castor bean leaves were decomposed in the soil, making them unsuitable as baits. In contrast, azalea and eucalypt discs remained intact and, therefore, could be used for pathogen quantification. The maximum colonization of eucalypt and azalea twig segments was approximately 74% and 67%, respectively. However, this was only achieved at high inoculum levels.

Colonization of eucalypt twig baits in soil under different moisture regimes The percentage of baits colonized by *Cylindrocladium* spp. (*C. candelabrum*, *C. quinqueseptatum* Boedijn & Reitsma and *C. variabile* Crous et al.) increased with increased soil moisture content (Fig. 2). Linear regression of the percent of colonized baits as a function of soil moisture was modelled. According to this model, maximal recovery (approx. 95% colonization) was at 155.9% field capacity. Contaminants, especially an *Aspergillus* sp., sporulated profusely on baits at the lowest moisture content.

Colonization of eucalypt twig baits in soil at different incubation periods Colonization of baits at 24–96 h was high, with low levels of contamination from other fungi and nematodes (Fig. 3). Baits incubated longer than 120 h were extensively colonized by other saprophytic fungi and nematodes that hindered assessment. Linear regression of the percentage colonized baits as a function of incubation hours was modelled. The pattern of residuals with this model was considered satisfactory. According to this model, maximal recovery was at 75.2 h of incubation.

Discussion

Azalea and eucalypt leaf discs were as effective as geranium leaves in recovering *C. candelabrum* from soil. This result is in contrast with those of Newhouse and Hunter (1980), who reported that geranium leaves were superior to azalea leaves for baiting *Cylindrocladium* spp. (not including *C. candelabrum*) from soil because abundant sporulation and internal tissue colonization was better on the former. Although castor bean leaf discs also had a high recovery efficiency, and were excellent baits for *Cylindrocladium* spp. (Almeida et al., 1978), the leaf discs were decomposed within 5 d in soil. In general, leaf discs were more efficient in recovering *C. candelabrum* than twig and stem segments under different inoculum densities, especially under low inoculum densities. This could possibly be due to the greater surface area in contact with the soil, the greater amount of solutes that diffuse into the soil, stimulating microsclerotial germination, or the fact that leaf tissue is softer and easier to colonize than woody stem tissue.

Eucalypt and azalea twig segments also yielded satisfactory recovery of *C. candelabrum* (maximum approx. 70%). However, stem segments from pine seedlings resulted in low pathogen recovery (maximum was less than 20%). Eucalypt twig segments were chosen as a standard for the estimation of inocula of *Cylindrocladium* spp. in soil, because they were satisfactory in recovering these species from soil, easier to obtain in great numbers, were more resistant to decomposition, and easier to handle than other baits.

The increase in soil moisture content resulted in an increase in the percentage of baits colonized by *Cylindrocladium* spp. Temperature and moisture levels regulate the diffusion of root exudates into soil (Raney, 1970). Therefore, higher moisture contents may have favoured the solute diffusion from eucalypt twigs and stimulated their colonization by the pathogen.

The incubation period also affected the recovery of *Cylindrocladium* spp. from eucalypt twigs. After 24 h, approx. 90% of the baits were already colonized by these species. The maximum colonization (approx. 95%) was calculated from the regression model to occur at 75 h. The longer the incubation period, the greater the degree

of colonization of baits by other fungi, which seriously impedes the recovery of *Cylindrocladium*. In fact, bait contamination with other fungi was very low at the shortest incubation period. Hwang and Ko (1976) efficiently recovered *C. parasiticum* Crous et al. from soil with papaya stem baits after 48 h of bait incubation.

This study reports the efficiency of the plant bait method in quantifying *Cylindrocladium* from soil under different inoculum levels and incubation regimes. Further work must be done to determine superior baits for recovering *Cylindrocladium* species from other soil types under low inoculum densities to further extend the possible applications of this method.

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