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RESEARCH ARTICLE / *ARTIGO*

Slow sand filtration eradicates eucalypt clonal nursery plant pathogens from recycled irrigation water in Brazil

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ABSTRACT

Contaminated irrigation water constitutes one of the main sources of plant pathogens that can cause disease and lead to potentially significant production losses in forest nurseries. Recycling of contaminated irrigation water increases the risk of spreading diseases. The objective of this study was to evaluate a simple slow sand filtration treatment of irrigation water as a method for eradicating fungal and bacterial pathogens in eucalypt cutting nurseries. Pilot filter units were constructed from PVC pipes containing a 80 cm high layer of sand with grain size varying from 0.50 to 0.75 mm, drainage layers of 10 cm of fine gravel (4 mm) followed by a 15 cm layer of coarse gravel (8 mm), connected to a water outlet. The PVC pipe had space for a 150 mm water column above the sand layer and the filter had a flow rate of 100 to 300 L m^{-2} h^{-1}. Eradication of the bacterial pathogen *Ralstonia solanacearum*, and the fungal pathogens *Cylindrocladium candelabrum* and *Botrytis cinerea* through the use of the filter was evaluated. Detection of bacteria in filter-treated irrigation water was performed through the micro drop method followed by PCR of colonies, while fungal pathogens were detected using a castorbean leaf biological bait method. Evaluations were performed daily 35 times for *R. solanacearum,* 25 times for *C. candelabrum* and 18 times for *B. cinerea* during a period of 12 months. After the last evaluation period, samples from various depths of the sand layer in the filters were taken and analyzed for the presence of the plant pathogens. The slow sand filters were able to remove *B. cinerea* and *C. candelabrum* spores with 100% efficiency while eradication of *R. solanacearum* colonies reached up to 99.6%. Colonies of *Trichoderma* sp. were found predominantly on the all filter layers. The results of this study show that slow sand filters are efficient for recycled water in eucalypt nurseries, especially in eradicating fungal pathogens.

Key words: *Botrytis cinerea, Cylindrocladium candelabrum*, *Eucalyptus* sp., *Ralstonia solanacearum*, eradication.

INTRODUCTION

Water use for production of eucalypt rooted cuttings varies from region to region, depending on the substrate used and nursery management practices. In a recent survey of six forest nurseries in Brazil, it was found that average daily water consumption was 18 m^3 for each million eucalypt cuttings produced (unpublished data). Another survey in two nurseries in Minas Gerais State showed that 49 m^3 of water were consumed per day for each million rooted cuttings produced (Rodrigues, 2007). Discrepancies in reported water use values are partly due to the methods used to estimate them. Irrigation water sources include rivers, streams, rain water reservoirs, recycled nursery wastewater and artesian or semi-artesian wells. Depending on the source, irrigation water may contain plant pathogen inocula which constitute an efficient form of unintentional spread of plant disease (Thomson & Allen, 1974; Strong et al., 1997; Pettit et al., 1998; Mafia et al., 2006). This risk is especially high if recycled irrigation water is used (Mafia et al., 2008).

Water consumption for cutting production and discharge of wastewaters containing plant pathogens and agrochemicals causes environmental impacts (Bahri, 1999). Therefore, efforts have been made to optimize the rational use and reuse of nursery water. Water used in spray or hydroponic irrigation must be collected and treated prior to reuse. Although water reuse can result in substantial water economy, the increased potential for inoculation and spreading of fungal and, or bacterial pathogens in the nursery caused by recycling of water must not be ignored.

Given the increasing scarcity of adequate water sources, safe water reuse is highly desirable from both economical and environmental standpoints. However, treatment prior to reuse to remove plant pathogens is necessary. Water may be treated by physical, physical-chemical or biological methods (Brand & Wohanka, 2001; Ehret et al., 2001; Murtha & Heller, 2003; Newman, 2004; Déniel et al., 2004). Conventional physical-chemical water treatment consists of coagulation-flocculation, sedimentation, rapid filtration, and chlorination (Ehret et al., 2001).

An alternative to conventional water treatment is treatment through the use of slow sand filters or other filter systems (Wohanka et al., 1992; Van Os et al., 1996; Londe & Parteniani, 2003; Ochieng et al., 2004). Water

treatment using slow sand filtration is based on the biological conversion of toxic chemical substances like ammonia to non-toxic substances like nitrate. Furthermore, plant pathogen antagonists can colonize the filter surface, forming a biofilm that is highly efficient in eliminating plant pathogens. In slow sand filtration, the physical barrier is considered of secondary efficiency (Dennis & Webster, 1971; Elasri & Miller, 1999). Such treatment is also efficient in removing herbicide residues and microorganisms that may cause human diseases. Slow sand filtration is a simple and unexpensive method for obtaining potable water, especially in small communities in developing countries (Murtha & Heller, 2003) and can potentially be used for forest nursery irrigation water treatment.

Production of eucalyptus cuttings in nurseries is carried out under environmental conditions that favor multiplication and dissemination of pathogens. Among the nursery diseases, cutting rot and leaf blight caused by *Botrytis cinerea* Pers. and *Cylindrocladium* spp. are, currently, that the most frequent cause of losses. Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi et al. in eucalyptus clonal nursery in Brazil is also very damaging and has led to losses of approximately from US$2.7 (Alfenas et al., 2006) up to US$27 million (Alfenas et al., 2009). In clonal minihedges, the disease is characterized by foliar necrosis, partial or complete xylem discoloration, wilt and death of rooted-cuttings. Leaf symptoms are similar to those observed during the gradual death of rooted-cuttings subjected to drastic pruning or with malformed root systems. In the rooting phase, infected minicuttings can present reddening of leaf blade veins and cutting rot (Alfenas et al., 2006).

Considering the potential for recycling irrigation water in forest nurseries, the present study aimed to evaluate the efficiency of slow sand filtration for eradication of *Cylindrocladium candelabrum* Viégas, *B. cinerea*, and *R. solanacearum*, plant pathogens commonly associated with *Eucalyptus* spp. rooted cuttings in Brazil.

MATERIAL AND METHODS

Slow sand filtration

The filter units included a water reservoir (Figure 1A), water feed system (Figura 1B), inlet valve flow (Figure 1C), water column (Figure 1D), sand filtering column (Figure 1E, F), gravel drainage column (Figure 1G), water outlet and flow meter with control valves to regulate the sand filter flow rate. The filters were constructed from PVC pipes (2.15 m long x 150 mm diameter). A constant water column (Figure 1D), about 100 cm high, was maintained resulting in a flow of of 100 to 300 L m^{-2} h^{-1}. An 80 cm sand (0.50-0.75 mm) bed was used (Figure 1E). The drainage column was composed of a 10 cm layer of fine gravel (4 mm) (Figure 1F) over a 15 cm layer of coarse gravel (8 mm) (Figure 1G). The base of each filter was sealed with PVC caps (Figure 1H), previously treated with adhesive. A PVC pipe (25 mm diameter) was placed connecting the end of each pipe to the drainage system to collect treated water (Figure 1I). The flow valve (Figure 1C) was fitted at the inlet of each filter and measurements were performed daily to confirm the flow rate in the outlet of each filter. The pipes of water outlet were attached to the outlet box (Figure 1J) with a float (Figure 1K) to keep the water level. The filtered water was pumped (Figure 1L, M e N) to the inlet box (Figure 1A). Water was added to the inlet box to maintain the volume of 100 L.

Preparation of plant pathogen inoculum and water infestation

All plant pathogens tested belong to the culture collection of the Laboratório de Patologia Florestal/Bioagro of the Universidade Federal de Viçosa, Minas Gerais, Brazil. The filter system was built in an area of the Departmento de Fitopatologia at the same university.

Ralstonia solanacearum isolate (isolate PF-3) was grown in 523 medium (Kado & Heskett, 1970) for 48 h at 28ºC in the dark. After incubation bacterial colonies were transferred to sterile saline solution (0.85% NaCl). One liter of bacterial suspension was added, monthly, to each 100 L of treated water for human consumption to reach a final concentration of 10^7 to 10^8 CFU mL^{-1}. After three months the bacterial suspension was reduced to 500 mL 100 L^{-1} of water to avoid filter saturation.

Botrytis cinerea spores (isolate PF-10) were scraped from PDA (potato dextrose agar) plates after five days of incubation at 25ºC, light regime of 12 h (30 µmols s^{-1} m^{-2} light intensity), and transferred to sterile saline solution (0.85% NaCl) at a final concentration of 10^6 conidia mL^{-1}. Spores of *C. candelabrum* (isolate PF–22) were produced on disinfested (NaClO, 0.1% Cl_2) twigs of eucalyptus, inoculated with 5 mm diameter mycelium plugs, incubated in a moisture chamber for 5-8 days at room temperature (25ºC ± 5ºC). After incubation, the infested twigs were placed in Erlenmeyers containing sterile water and manually agitated to release the spores. The suspension was filtered in Whatman no. 1 filter paper and subsequently the spore suspension was adjusted to 10^6 conidia mL^{-1}. Water infestation with fungal spores was performed as described for the bacterial suspension.

Detection of *Botrytis cinerea* and *Cylindrocladium candelabrum*

Inoculum of the pathogens was added, weekly, in the inlet box. Water samples were collected daily (25 days for *C. candelabrum* and 18 days for *B. cinerea*) at the filter inlet and outlet to evaluate the effect of filter aging on pathogen eradication. *Botrytis cinerea* (PF-10) and *C. candelabrum* (PF-22) were detected using castorbean leaf disks (*Ricinus communis* L.) as biological bait (Mafia et al., 2006). Fifty mL of each inoculum suspension were used to moisten 25 g of medium grain sterile vermiculite in plastic gerboxes (13 x 13 x 4 cm). Thirty 15 cm diameter

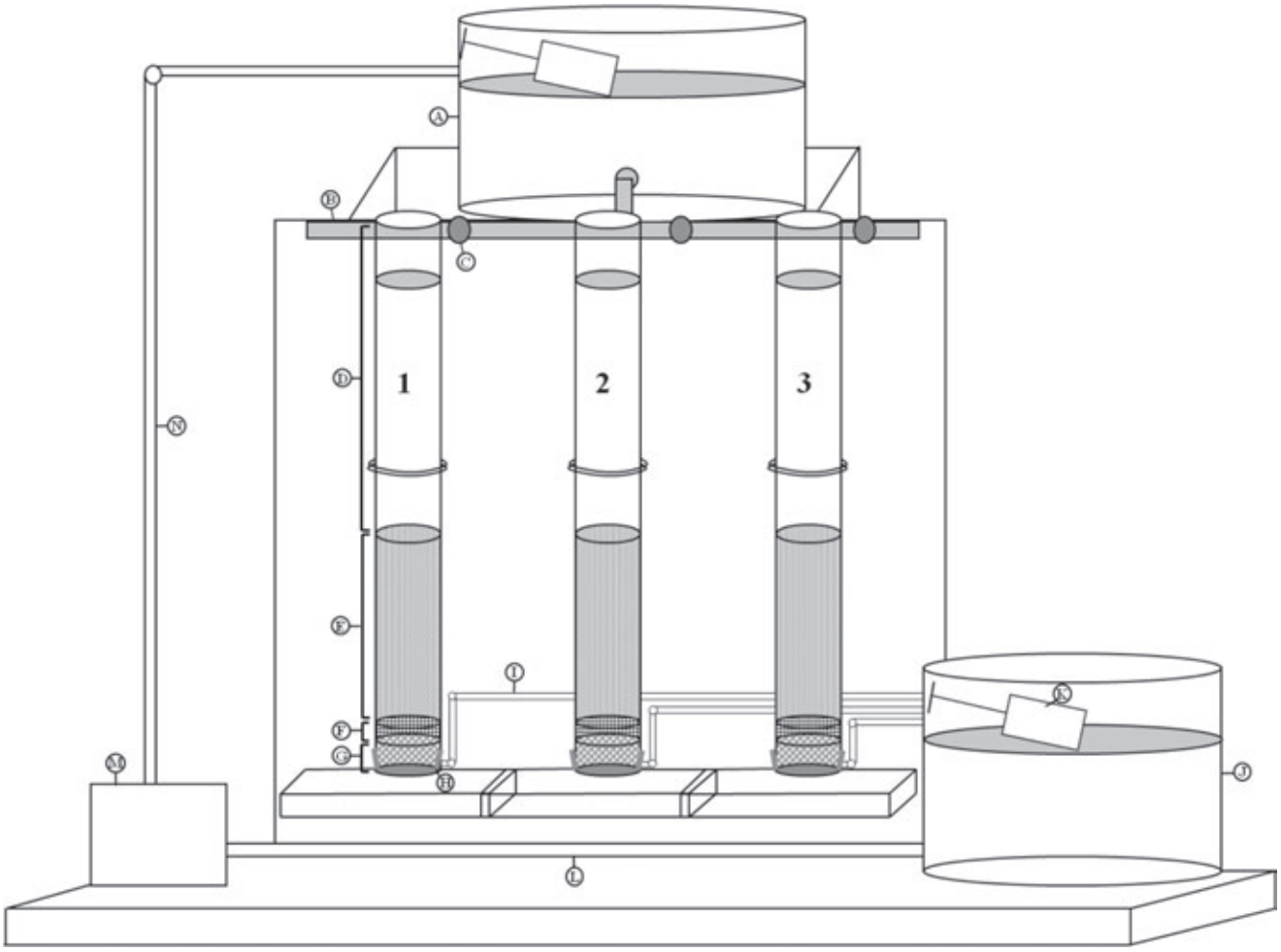

FIGURE 1 - Scheme of filters (1, 2 and 3) used for slow sand filtration. **A.** Reservoir for pathogen infested water; **B.** Pipe connected on inlet for each filter; **C.** Inlet flow valve; **D.** Water column (1 m); **E.** Sand bed (80 cm); **F.** Fine gravel layer (10 cm); **G.** Coarse gravel layer (15 cm); **H.** PVC cap; **I.** Pipe connected to drainage system on outlet of filters; **J.** Reservoir for treated water; **K.** Water level control float; **L.** Returned water from the system outlet to the inlet; **M.** Hydraulic pump; **N.** Infested water reservoir feed system.

castorbean leaf disks were disinfested with 70% ethanol for 30 s, 5% hypochlorite (NaOCl) for 3 min and washed twice in sterile distilled water. The castorbean leaf disks were placed on the moistened vermiculite containing different inoculum suspensions using sterile tweezers and then the boxes were kept at 25ºC in the dark. After 48 h the baits were transferred to acidified (pH = 5.0) PDA plates and incubated under the same conditions. Identification of the plant pathogenic fungi recovered was based on their respective typical morphological characteristics. The percent bait colonization per treatment was calculated using three repetitions per sample of each tube on inlet and outlet during the experimental period. Inlet and outlet sample means were compared statistically using the F test ($p<0.05$).

Detection of *Ralstonia solanacearum*

Water samples were collected from each filter on inlet and outlet and the subsamples were diluted by transferring 100 µL of sample to Eppendorf tubes with 900 µL of sterilized water. Non infested filter feed water was used as control. Ten µL aliquots were transferred to each of ten plates containing 523 medium (Kado & Heskett, 1970), using the microdrop method (Romeiro, 2001). After 48 h incubation at 28ºC in the dark, colonies similar to *R. solanacearum* were quantified and treatment efficiency was determined by comparison with the control. Filter inlet and outlet sample means were compared statistically using Dunnett's test ($p<0.05$).

All bacterial colonies morphologically similar to *R. solanacearum* were identified. For identification, 16S rDNA fragments were amplified by PCR using oligonucleotide primers specific for the *Ralstonia* genus (PS1/ 5'- AGTCGAACGGCAGCGGGGG -3'and PS2/ 5'- GGGGATTTCACATCGGTCTTGCA -3') (Pastrik & Maiss, 2000). The reaction was carried out in a final volume of 25 µL, containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each

deoxynucleotide (dATP, dTTP, dCTP and dGTP), 0.4 mM of each primer, 1 unit of Taq DNA polymerase and sterile water (Milli-Q). After initial denaturation at 95ºC for 10 min, amplification was carried out in 40 cycles of denaturation for 1 min at a 94ºC, annealing for 30 s at 68ºC and extension at 72ºC for 1 min and 30 s. A final extension step was run at 72ºC for 7 min. The PCR product was analyzed by electrophoresis on 1.6% agarose gel in Tris acetate EDTA (TAE) stained with ethidium bromide (0.5 µg mL^{-1}) and photographed. To evaluate reaction specificity, a *R. solanacearum* isolate from the Forest Pathology Laboratory collection was used (Alfenas et al., 2009). Specificity of the amplified PCR product with the PS1/PS2 primers was confirmed by restriction enzyme analysis with the TaqI enzyme (Pastrik & Maiss, 2000). Ten µL of the PCR product were digested with 5 units of TaqI at 65ºC for 45 min. The restriction products were analyzed by electrophoresis on 2% agarose gel.

Evaluation of plant pathogens and fungal antagonists in filter layers

After 12 months of operation, the filtering and drainage columns were divided into ten sub layers (10 cm each) to evaluate the presence of both added pathogens and naturally grown antagonists. The fine and coarse gravel layers were also examined. The bait method was used for both fungal pathogens and antagonists and the microdrop method for bacteria as previously described elsewhere.

RESULTS

Ralstonia solanacearum eradication in the slow sand filter varied in average from 87.5 to 100% (Table 1). In the first months of filter operation eradication of this pathogen varied from 71% to complete elimination (100% eradication). Increased bacterial eradication was observed after five months of operation (April). During the sixth month of operation one of the filter units clogged and the cleaning process used to unplug the filter caused a reduction in pathogen eradication efficiency. Data for this period (and July) was then excluded from evaluations. After seven months (June), average eradication was 99% and reached 100% after nine months (August, Table 1). *Ralstonia solanacearum* presence was confirmed by PCR in all samples.

Cylindrocladium candelabrum and *B. cinerea* were removed with 100% efficiency for all samples evaluated (Table 2). A thick microbial mat was observed in the uppermost layer of the sand bed after 12 months of operation and no inoculated plant pathogens were detected in any sand bed layer at that time. However, colonies of *Trichoderma* sp. were present in all sand and gravel layers.

DISCUSSION

In the present study, average eradication efficiency of up to 99% for the bacterium *R. solanacearum* and 100% for the fungi *C. candelabrum* and *B. cinerea* were observed. Similar efficiencies were reported for *Xanthomonas axonopodis* Starr & Garces emend. Vauterin et al. (Déniel et al., 2004; Wohanka et al., 1999). Eradication efficiencies for viruses and nematodes are lower (Van Os et al., 1999; Berkelmann et al., 1995). For fungal pathogens structure size is an important factor for cell retention in filters, and according to some authors the elimination of fungal pathogens depends as much on sand grain size distribution as on biological processes. Van Os et al. (1999) observed 100% elimination of *Phytophthora cinnamomi* Rands in a hydroponic water treatment system when fine grained sand (0.15-0.3 mm) was used. Equivalent efficiency was observed for *C. candelabrum* and *B. cinerea* in this study.

Slow sand filtration is a popular method for treatment of recycled nutrient solutions in seedling production in greenhouses and hydroponic systems (Van Os et al., 1999). Sand filtration efficiency depends on various factors, including concentration of organic matter and other impurities that may require installation of pre filters. In general, forest industry nursery wastewater meets limits established in federal water quality discharge limits (CONAMA, 2005), and treatment is aimed more at removing pathogens than reducing levels of physical-chemical pollutants.

Slow sand filtration combines both a physical barrier and biological antagonism for pathogen control. The size of sand grains utilized in this work was also considered appropriate by Ehret et al. (2001) for controlling diseases caused by *Phytophthora* spp., *Pythium* spp., and *Fusarium oxysporum* E.F. Sm. & Swingle. Biological antagonism has also been cited as fundamental for efficient control of plant pathogens in hydroponic systems (Wohanka et al., 1992).

In slow sand filtration a layer of antagonistic microorganisms denominated the 'schmutzdecke' colonizes the sand bed surface. This layer is formed by a combination of organic and mineral matter adhered to sand particles that serve as nutrients for microbial growth (Brand & Wohanka, 2001). The presence of *Trichoderma* sp. detected in all sand gravel layers suggests that this fungus may have played an important antagonistic role against the tested pathogens. *Trichoderma* is an efficient mycoparasite in the control of many fungal pathogenic species, including *Rhizoctonia* spp. (Mafia et al., 2003), *Fusarium solani* (Mart.) Sacc. (Rojo et al., 2007) and *B. cinerea* (Elad, 1994), among others.

Advantages of slow sand filtration as compared to other water treatment methods include efficient eradication of suspended solids and microbial pathogens, no involvement of noxious sterilizing substances such as chlorine and instrumentation as microfiltration, low energy consumption, minimal maintenance and relatively low cost (Newman, 2004). However, the space required to ensure the

TABLE 1 - Average number of *Ralstonia solanacearum* cfu's in the filter inlet and outlet and eradication efficiencies

Time (h)	Inlet (x 10^3 cfu mL^{-1})	Outlet (x 10^3 cfu mL^{-1})	Eradication (%)	Mean (%)
		December/2006		
24	146.67	0.00	100.00	
96	95.56	0.00	100.00	
192	116.67	5.22	95.53	
288	135.56	16.11	88.12	95.9
		January/2007		
24	2788.89	218.89	92.15	
96	1877.78	0.00	100.00	
156	2344.44	0.01	100.00	
216	2111.11	322.22	84.74	
276	2722.22	500.00	81.63	91.7
		February/2007		
24	3800.00	1096.67	71.14	
184	788.89	4.37	99.45	
244	21.48	0.37	98.28	
304	5.33	1.01	81.05	87.5
		April/2007		
0	0.67	0	100.00	
7	1.33	0	100.00	
24	0.9	0	100.00	
72	0	0	100.00	
120	0	0	100.00	
168	0.01	0	100.00	100.0
		May/2007		
0	1.33	0.00	100.00	
7	1.50	0.56	62.67	
48	1.33	0.33	75.19	
96	0.50	0.00	100.00	
144	1.17	0.00	100.00	
192	0.00	0.00	100.00	
240	0.37	0.00	100.00	
288	0.02	0.00	100.00	92.2
		June/2007		
0	18.33	0.00	100.00	
7	2.33	0.03	98.71	
24	30.00	0.83	97.23	
48	18.33	0.00	100.00	99.0
		August/2007		
0	27.11	0.00	100.00	
7	21.11	0.00	100.00	
24	3.56	0.00	100.00	
48	0.00	0.00	100.00	
60	0.00	0.00	100.00	100.0

low filter flow rates (7.2 $m^3 d^{-1} m^{-2}$) necessary for pathogen eradication, limit slow sand filter use to small units such as greenhouses and mini-clonal hedges. For daily needs of water of 420 to 592 $m^3 d^{-1}$ as common in eucalypt nurseries in the state of Minas Gerais (Rodrigues, 2007) use of filters in different sectors of the mini-clonal hedges and greenhouses would require filter units having only 2.5 to 4.8 m^2 of surface area. Furthermore, construction costs for slow sand filters are lower than for other treatment systems.

TABLE 2 - Percentage of castorbean leaf biological baits colonized by *Botrytis cinerea* and *Cylindrocladium candelabrum* after one to six days weekly addition of conidial suspensions

Evaluation	Colonized baits (Inlet %)		Outlet (%)	Average eradication (%)
	C. candelabrum	*B. cinerea*		
1	100	90	0	100
2	13	50	0	100
3	15	15	0	100
4	7	2	0	100
5	7	3	0	100
6	10	2	0	100
7	100	100	0	100
8	40	75	0	100
9	16.7	16.3	0	100
10	22	9	0	100
11	30	15	0	100
12	15	15	0	100
13	100	100	0	100
14	40	60	0	100
15	30	60	0	100
16	30	25.5	0	100
17	15	36	0	100
18	7	24.3	0	100
19	100	2	0	100
20	88	5	0	100
21	96	*	0	100
22	17	*	0	100
23	13	*	0	100
24	10	*	0	100
25	7	*	0	100

*Not analyzed.

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