Sanitation of a South African Forestry Nursery Contaminated with *Fusarium circinatum* Using Hydrogen Peroxide at Specific Oxidation Reduction Potentials

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Abstract

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Pitch canker, caused by *Fusarium circinatum*, was first reported in a forestry nursery in the Mpumalanga Province of South Africa in 1990, and it has since spread to almost all forestry nurseries in the country, where it causes significant economic losses. The aim of the current study was to (i) identify sources of *F. circinatum* contamination in the Karatara forestry nursery in the Western Cape Province and (ii) manage the disease by implementing an oxidation reduction potential (ORP)-based sanitation method using hydrogen peroxide. The irrigation water, planting tray inserts and seeds were screened for fungal contamination. *Fusarium circinatum* colonies were identified morphologically and confirmed by polymerase chain reaction using species-specific primers. Both the irrigation water and planting tray inserts served as sources of inoculum that introduced the pathogen into the nursery. The irrigation water was amended with hydrogen peroxide at an ORP level of 400 mV for an exposure time of 6 h because it was observed that such a treatment effectively killed all *F. circinatum* spores and was not phytotoxic to pine seedlings under laboratory conditions. In addition, the contaminated planting tray inserts were cleaned in water amended with hydrogen peroxide at an ORP value of 360 mV for 6 h, which was shown to efficiently eliminate all inoculum from planting tray inserts. Since the introduction of the ORP-based sanitation method at Karatara nursery, losses of pine seedlings were reduced to insignificant levels, and field losses were minimized.

Pitch canker of pines (*Pinus* spp.) is a disease caused by *Fusarium circinatum* Nirenberg & O'Donnell (teleomorph: *Gibberella circinata* Nirenberg & O'Donnell) (30). The disease was first discovered in the southwestern parts of the United States of America (16), and it has since been reported from Haiti (17), Japan (24), South Africa (37), Chile (39), Spain (25), Italy (7), Portugal (5), Mexico (18), and Korea (27). In forestry plantations, *F. circinatum* causes stem cankers and twig die-back that reduce yields, lower wood quality, and are responsible for tree mortality (38). When introduced into forestry nurseries, *F. circinatum* causes root and collar rot that result in significant losses (37). Seedlings infected in the nursery also can be symptomless and may develop the disease after they are transplanted to plantations (33).

Shoot dieback and root rot were first discovered in a South African forestry nursery in the Mpumalanga Province in 1990 (37). The causal agent, *F. circinatum*, has subsequently spread to almost all pine seedling nurseries throughout the country. The sources of introduction and dissemination of *F. circinatum* in South Africa have not been established but it is generally believed that the fungus was introduced into the country with infected seed (6) and that it spread from one nursery to another by the movement of contaminated plants and planting trays. Potential sources of dissemination within nurseries include airborne inoculum, insect vectors, and irrigation water. The pitch canker fungus currently is considered the most important constraint to pine seedling production in South Africa (40).

The first outbreak of pitch canker in a forestry plantation in South Africa occurred in Tokai near Cape Town in 2005 (9). *Pinus radiata* D. Don trees grown in this plantation were obtained from the Karatara nursery near Knysna in the Western Cape Province, making this particular nursery the most likely source of inoculum (9). This hypothesis is supported by the fact that Karatara nursery was experiencing an outbreak of the disease 10 years earlier, when the trees affected at Tokai were produced. Why the disease remained undetected for 10 years is unknown but this could be attributed to Cape Town's climate, low levels of initial inoculum, absence of effective insect vectors, and lack of wounding agents (38). Since the initial report of pitch canker in Tokai, the disease has been found in pine plantations in George, Sedgefield, and Knysna in the Western Cape Province (A. Van der Hoef, *personal communication*).

Karatara nursery produces about 6 million pine seedlings annually. Production consists primarily of *P. radiata* plants but also includes *P. elliottii* Engelm., *P. taeda* L., *P. tecunumanii* Eguiluz and Perry, and *P. pinaster* Ait. Of these species, *P. radiata* is known to be most susceptible to the pine pitch canker fungus (1,36). In 2005 and 2006, Karatara nursery lost approximately 750,000 (30%) and 600,000 (22%) seedlings, respectively, because of infection by *F. circinatum*. The pitch canker fungus could have been introduced into the nursery by means of infected seed, contaminated seedling trays, growth medium, or irrigation water. Because of its airborne nature, the fungus also could have entered the nursery by means of airborne inoculum that originated from adult pine trees affected by pitch canker in the vicinity of the nursery (14,33). Management strategies introduced to curb seedling losses at Karatara nursery included the use of fungicides, a chlorination system to disinfest irrigation water, soaking seed in hydrogen peroxide after seed stratification, the use of freshly composted pine bark medium for *P. radiata* seedling production, lifting of planting trays off the ground surface, introducing a disease monitoring system to rogue out and destroy unhealthy seedlings, and the strict application of basic hygiene factors (A. Van der Hoef, *personal communication*). Although these disease management practices implemented in the nursery reduced losses of pine seedlings, the quality of seedlings produced was often compromised because mycorrhizal growth on pine roots was reduced and *F. circinatum* was not entirely eliminated from the nursery.

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Current strategies available for sanitation of irrigation water include the use of ozone and chlorine (12,41). Ozone-generating plants, although very effective, are expensive to install and the maintenance costs of these plants are high (41). The use of chlorine is a common method used for sanitation of irrigation water in South Africa, and chlorine levels of 2 to 3 ppm are effective in either reducing or eliminating *F. circinatum* in irrigation water (2). However, high levels of chlorine in the irrigation water can have detrimental effects on mycorrhizal fungi and on seedling roots that, in turn, can lead to stressed seedlings that are more susceptible to diseases (4). Chlorine also is corrosive, effective only within a narrow pH range, and leads to the formation of harmful chlorinated by-products (31,42). An alternative to chlorine and ozone as an irrigation water disinfectant is hydrogen peroxide. Hydrogen peroxide raises the oxidation reduction potential (ORP) of the irrigation water and, in this way, eliminates pathogens that are harmful to plants and animals (22,23,29).

In this study, three potential sources of inoculum at Karatara nursery were screened for the presence of *F. circinatum*. These included irrigation water, pine seed, and planting tray inserts. Irrigation water was considered a potential source of inoculum because a small pond next to the nursery was used for irrigating seedlings in the nursery. Seed were suspected as a potential source of inoculum because they were harvested from a seed orchard next to the nursery where some trees have shown symptoms of pitch canker. Seed planted in the nursery also were obtained from orchards in other locations in South Africa where no pitch canker symptoms had been observed. Pine seedlings at Karatara nursery are grown in plastic trays, each consisting of 98 removable tube-like inserts. When seedlings reach the appropriate age and physiological stage, they are transplanted to plantations. The inserts are then cleaned and reused. The cleaning process for the inserts involves removal of excess compost medium; soaking in a commercial disinfectant containing washed neutral oil, high-boiling tar acids, and methanol called Jeyes Fluid (Jeyes Professional Division Limited, Lancashire, UK); followed by drying. The plant inserts, in which individual pine seedlings are planted, were considered a potential source of *F. circinatum* inoculum because these inserts are reused. The planting medium, composted pine bark, was not considered a potential source of inoculum because fresh medium is obtained from pitch canker-free areas in South Africa, and because the medium is not reused. Composted pine bark medium also often has disease-suppressive properties resulting from colonization by beneficial microbes (19).

The current study was initiated to identify sources of *F. circinatum* that affect pine seedlings in the Karatara nursery. Once identified, an attempt was made to eradicate these sources in an affordable, effective, and environmentally sound way. Despite the limited information available for the use of ORP-based methods involving hydrogen peroxide for the elimination of plant pathogens (29), hydrogen peroxide was tested as a sanitizing agent to disinfect irrigation water and planting tray inserts at the Karatara nursery. Different ORP levels and exposure times were optimized to ensure elimination of *F. circinatum* without causing damage to pine seedlings.

Materials and Methods

Identification of sources of inoculum. *Irrigation water.* The pine seedlings produced in Karatara nursery obtain irrigation water from a medium-size pond (approximately 60 m long by 40 m wide by 5 m deep) that is situated 50 m away from the nursery on the boundary of a pine plantation. From the pond, water is being pumped into two reservoirs built in series: the first reservoir has a diameter of 8.1 m and a height of 1.6 m and the second reservoir has a diameter of 10 m and is 2.2 m deep. Water is sprayed from the second reservoir onto the seedlings in the nursery. At the time of the first collection to identify sources of inoculum, calcium hypochlorite was added between the first and the second reservoir to elevate the concentration in the second reservoir to 5 ppm. Ten samples, each consisting of 1 liter of water, were collected from each of the four points in the irrigation system, including the pond, the first reservoir, the second reservoir, and at a single irrigation point in the nursery. Water samples were then filtered through sterile Whatman number 1 filter papers (Whatman, South Africa) using a vacuum pump assembly (Picolino, South Africa). Each filter paper was placed for 10 s onto *Fusarium*-selective agar (FSA; 33) in order to transfer *Fusarium* spores. After 5 to 7 days of incubation, suspected *Fusarium* colonies were subcultured onto potato dextrose agar (PDA) plates, single-spored, and incubated for 10 days on PDA and carnation leaf agar (CLA) plates for morphological identification (28). If identities were uncertain, identification was confirmed using molecular techniques as described below.

Seed. In a first screening, seed samples representing all *Pinus* spp. grown at Karatara nursery, including *P. tecunumanii*, *P. elliottii*, *P. taeda*, *P. pinaster,* and *P. radiata*, first and second generation, were tested for contamination with *F. circinatum*. *P. radiata* second-generation is the open-pollinated progeny of first-generation *P. radiata* plants that are produced in an effort to improve growth characteristics in the species. Of each batch, 50 untreated and 50 surface-sterilized seeds were placed directly onto PDA plates containing neomycin (0.12 g/liter), with five seeds per petri dish. Surface sterilization was accomplished by dipping seed in 70% ethanol for 1 min, then in 3% sodium hypochlorite for 3 min, and then washing them three times in sterile distilled water (26). In addition, the seed coats of another 50 seeds of each batch were removed and the endosperm and embryo were placed on PDA. In a second screening, 500 untreated and 500 surface-sterilized *P. radiata* first- and second-generation seeds from the same batches were tested further as described above. *Fusarium* colonies that developed from any of the seed were subcultured, single spored, and identified to species.

Seedling tray inserts. Tray inserts were collected from the nursery for testing for contamination with *F. circinatum*. During the first collection, dirty tray inserts were cleaned by nursery employees for reuse by removing excess soil residues present on the inside of inserts and washing with Jeyes Fluid, which is a treatment implemented by the nursery personnel. Fifty seedling tray inserts each were randomly selected before and after they were cleaned. Soil residues that remained on the inside of the inserts after cleaning were scraped off and collected in bottles containing 5 ml of sterile water, and subsequently plated on FSA for identification of *F. circinatum*.

Molecular identification of *F. circinatum*. For molecular identification, approximately 100 mg of fresh mycelia was scraped from the PDA plates with single-spored colonies and transferred to 1.5-ml microtubes. DNA was then extracted using the Wizard SV genomic DNA purification system (Promega, South Africa). The pitch canker pathogen was identified by using species-specific primers CIRC1A and CIRC4A for *F. circinatum* (33). The polymerase chain reaction (PCR) mixture contained 13.5 µl of H_2O, 2.5 µl of 10× PCR buffer, 2.0 µl of 50 mM $MgCl_2$, 0.8 µl of deoxynucleoside mixture (10 mM), 0.5 µl of each primer (50 mM), 0.2 µl of Platinum *Taq* DNA polymerase (Invitrogen, South Africa), and 5 µl of template DNA (corresponding to approximately 250 ng of DNA). PCR amplification consisted of an initial step at 94.0°C for 3 min; followed by 45 cycles of 94°C for 35 s, 68°C for 55 s, and 72°C for 50 s; with a final extension step at 72°C for 12 min. Resulting PCR products were separated by gel electrophoresis, and the gels were stained with ethidium bromide and visualized under UV lights. The *F. circinatum* isolate FCC 3577 (obtained from the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa) was used as a positive control.

Effect of hydrogen peroxide at different ORP levels and different exposure times on *F. circinatum*. *F. circinatum* (reference isolate FCC 3577) was grown on PDA plates for 14 days at 25°C. A spore suspension was prepared from these plates by adding 2 ml of sterile distilled water to each plate and gently loosening the fungal spores with a bent glass rod. The spore suspension was then filtered through four layers of cheese cloth to remove pieces of

mycelium and agar. The final spore concentration was determined with a Bright-Line hemacytometer (Boeco, South Africa) and adjusted to 10^7 spores/ml.

To prepare water with a range of ORP levels, six 500-ml glass beakers containing 200 ml of deionized sterile water were placed on a magnetic stirrer. The control did not receive any hydrogen peroxide and had a final ORP level of 180 mV as measured with a handheld ORP meter (Hanna Instruments, South Africa). The ORP of the water in the other five beakers was adjusted by adding hydrogen peroxide (Hyprox 500; Protea Chemicals, South Africa) in order to raise ORP levels to 300, 350, 400, 450, and 500 mV. The beakers were then left in the laboratory at room temperature, and the ORP was again measured 6 and 24 h after the ORP values had been set.

In order to test the effect of the different ORP levels on *F. circinatum* at different exposure times, 20 ml of the *F. circinatum* spore suspension were added to 180 ml of water representing each of the six different ORP values to obtain a final spore concentration of 10^6 spores/ml. After exposing the spore suspensions to the different ORP levels for time periods of 30 s, 5 min, 30 min, 2 h, 6 h, and 24 h, survival of *F. circinatum* spores was determined. Samples (1 ml) were taken for the each ORP value from the same container at the different time points, and a 10-fold dilution series was prepared in sterile distilled water ranging from 10^5 to 10^1 spores/ml. A 1-ml subsample for each ORP level and time point were then transferred to PDA plates, with five plates used for each dilution point. The plates were then incubated at 25°C and the average number of CFU per milliliter of water of each ORP value and at each time point was calculated after 3 days. The experiment was conducted twice.

Phytotoxicity of hydrogen peroxide-amended water to *P. radiata* seedlings. Healthy 6-month-old *P. radiata* seedlings were irrigated daily for 30 days with hydrogen peroxide-amended water at ORP values of 300, 350, 400, 450, and 500 mV. The control treatment consisted of seedlings that were given water without hydrogen peroxide added (ORP value of 180 mV). The seedlings were arranged in a randomized block design with four replications and 14 seedlings per replicate and, after treatment, they were monitored daily for phytotoxicity. Seedling mortality was documented for 30 days.

Treatment and elimination of sources of inoculum. *Irrigation water.* At the time of the second collection, a hydrogen peroxide treatment had been implemented to treat the irrigation water. Hydrogen peroxide was added to the irrigation water in one of the reservoirs at Karatara nursery to raise the ORP level to 400 mV and was retained in the reservoir for at least 6 h before it was released. When the water was used to irrigate the seedlings in the nursery, the ORP level had dropped to 340 mV. Twelve samples, consisting of 500 ml of water each, were collected from each sampling point, which included the pond, one of the reservoirs, and at a single irrigation point in the nursery. Irrigation water was then tested for the presence of *F. circinatum* as described above.

Seedling tray inserts. During the second collection, planting tray inserts were collected from Karatara nursery after the cleaning process was modified by replacing the commercial disinfectant with an ORP treatment using hydrogen peroxide. Planting tray inserts were cleaned by the nursery personnel by removing all the debris and growth medium from them and soaking them in water amended with hydrogen peroxide with an ORP value of 360 mV for 6 h. Inserts were then dried and tested for the presence of *F. circinatum* as described above. Fifty seedling tray inserts were randomly selected before and after the cleaning process, *F. circinatum* was identified, and the effectiveness of the ORP treatment determined.

Statistical analysis. One-way analysis of variance and Fischer's least significant difference (LSD) ($P = 0.05$) were used to compare the number of CFU found at the different points in the Karatara nursery irrigation system; the number of CFU that survived the different ORP levels and exposure times in the laboratory study; and the number of healthy, wilted, and dead 6-month-old seedlings after being irrigated with hydrogen peroxide-amended water. Fischer's LSD post-hoc test was used to calculate significant differences between the number of CFU at different points in the irrigation system, exposure time, and ORP level treatment combinations, and the effect of irrigating 6-month-old seedlings with water with different ORP levels. A 5% significant level was used as a guideline.

Results

Sources of inoculum. *Irrigation water.* During the first inspection for contamination of irrigation water at Karatara nursery, the average number of *F. circinatum* (CFU per liter of water) from the pond and from the untreated reservoir (reservoir 1) did not differ significantly (Table 1). When the water in the second reservoir (reservoir 2) was treated with chlorine, the average number of *F. circinatum* propagules in reservoir 2 and in the nursery was significantly reduced. However, the pathogen was not entirely eliminated from the water and *F. circinatum* could still be isolated at a rate of 1.3 CFU/liter of water in the nursery.

Seed. In the first screening, seed of *P. tecunumanii*, *P. elliottii*, *P. taeda*, and *P. pinaster* tested negative for the presence of *F. circinatum* whereas, for *P. radiata*, 32% of the seed of the first generation and 22% of the seed of the second generation were contaminated. In the first- and second-generation batches, contamination decreased to 6 and 14%, respectively, following surface sterilization. *F. circinatum* was only isolated from the embryos and endosperm from the *P. radiata* first generation seed at a rate of 14%. In the second screening, seed from the contaminated first- and second-generation batches tested negative for the presence of the pitch canker fungus, whether treated or not treated with surface disinfectants (*data not shown*).

Seedling tray inserts. When first tested, 30% of planting tray inserts were found to be contaminated with *F. circinatum* before cleaning and disinfestation. After the inserts were cleaned and disinfested with Jeyes fluid, 18% were still contaminated with *F. circinatum*.

Effect of hydrogen peroxide on *F. circinatum*. Supplementation of water with hydrogen peroxide at certain ORP levels significantly affected the survival of *F. circinatum* spores (Fig. 1). At ORP values of 300 mV, 33% of fungal spores survived after an exposure time of 24 h. However, for shorter exposure times, there were no significant differences between the 300 mV treatment and the control group (180 mV treatment). In all, 14% of fungal spores survived an exposure time of 6 h at an ORP value of 350 mV. Less than 5% of fungal spores survived after an exposure time of 30 min (450 mV), 2 h (400 mV), and 24 h (350 mV). The following treatments killed all *F. circinatum* spores: 400 mV for exposure times of 6 h and 24 h; 450 mV for exposure times of 2, 6, and 24 h; and 500 mV for all exposure times.

After supplementation of hydrogen peroxide to water, ORP levels were monitored after 6 and 24 h (Table 2). Some of the ORP levels decreased over time. The 400- and 450-mV treatments decreased by 6 and 11.3%, respectively, after 6 h but showed no decrease from 6 to 24 h. The 500-mV treatment decreased by 5.4% after 6 h and by 15.4% after 24 h.

Table 1. Presence of *Fusarium circinatum* in irrigation water at the Karatara nursery during chlorine or hydrogen peroxide treatment

	Treatment applied[x]	
Source	**Chlorine[y]**	**Hydrogen peroxide[z]**
Pond	5.9 a	3.2 b
Reservoir 1	6.4 a	0 d
Reservoir 2	1.6 c	…
Nursery	1.3 c	0 d

[x] Results are expressed as the average number of CFU per liter of water. Numbers followed by the same letter within columns are not significantly different from each other ($P = 0.01$).

[y] Calcium hypochlorite added at 5 ppm in reservoir 2 at Karatara nursery.

[z] Hydrogen peroxide added in reservoir 1 at Karatara nursery to an oxidation reduction potential of 400 mV.

Phytotoxicity of hydrogen peroxide-amended water to *P. radiata* seedlings. Six-month-old *P. radiata* seedlings were significantly affected by hydrogen peroxide-amended water (Table 3). For the control treatment (180 mV), 92.9% of plants remained healthy without any signs of phytotoxicity or wilting symptoms throughout the trial. In contrast, all seedlings irrigated with water with an ORP of 500 mV were dead within 30 days. Most (89.3%) of the seedlings were killed when irrigated with water with an ORP of 450 mV. After 30 days of irrigation, there were no significant differences in the number of dead, wilted, and healthy seedlings between the control treatment (180 mV) and the 300-, 350-, and 400-mV treatments. In addition, none of the plants irrigated with water with these ORP values showed signs of browning or chemically burned needles. Although the differences were not significant, seedlings died in the 350- and 400-mV treatments.

Treatment and elimination of sources of inoculum in Karatara nursery. To disinfest the irrigation water at Karatara nursery, hydrogen peroxide was added to an ORP value of 400 mV and it was retained for 6 h in the reservoirs. These conditions were chosen based on the results obtained under laboratory conditions, showing that this treatment was not phytotoxic for the seedlings (Table 3) and that a treatment of 400 mV for an exposure time of 6 h was sufficient to kill all *F. circinatum* spores (Fig. 1). This treatment completely eliminated *F. circinatum* from the irrigation water in both the reservoirs and the nursery (Table 1), showing that the treatment implemented in this study was very effective in sanitizing irrigation water.

We modified the cleaning process of the planting tray inserts by cleaning them in water supplemented with hydrogen peroxide at an ORP value of 360 mV for an exposure time of 6 h. In all, 18% of the inserts before cleaning were contaminated with *F. circinatum* while none of the inserts after cleaning were contaminated, showing that the cleaning process implemented in this study was very efficient in sanitizing seedling tray inserts.

In addition, no phytotoxicity was observed in the nursery, and establishment of mycorrhizae on *P. radiata* roots and root health returned to normal, based on visual observation, compared with the severe damage observed to pine roots and the detrimental effects on mycorrhizae following chlorine treatment of the irrigation water that was used previously to this study in the nursery.

Clean irrigation water in combination with *F. circinatum*-free planting tray inserts reduced losses in the nursery to insignificant levels, and losses of pine seedlings after field transplanting were minimized by 2008 (Table 4). This is in contrast to the substantial losses experienced in the nursery in 2003, 2004, 2005, 2006, and 2007 (Table 4).

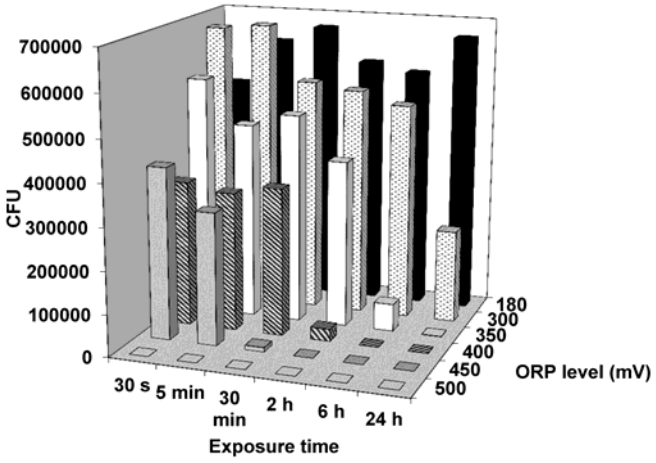

Fig. 1. Effect of different oxidation reduction potentials (ORPs) and exposure times on the survival of spores of *Fusarium circinatum*. Mean values were calculated for the average number of CFU from six plates per treatment.

Discussion

The first introduction of *F. circinatum* into a South African forestry nursery approximately two decades ago left the local forestry industry with a significant challenge: eradicate the pathogen from this nursery or face the possible spread of the fungus to other nurseries and forestry plantations with highly susceptible *Pinus* spp. (37). The pathogen was not eradicated and has since spread to almost all forestry nurseries in the country and, recently, to plantations, presumably because of the movement of infected planting material and equipment between nurseries and into plantations (9). In this study, *F. circinatum* contamination was reduced to insignificant levels in a forestry nursery that was severely affected by the pitch canker fungus. The reduction in disease incidence was the result of a relatively simple strategy: identification of the primary sources of inoculum and elimination of these sources with a sanitation process that is effective, affordable, and environmentally sound.

The irrigation water and planting tray inserts served as important sources of inoculum of *F. circinatum* in Karatara nursery. Despite treatment with chlorine and other disinfectants tested in the Karatara nursery, the pitch canker pathogen was never eliminated from the water and tray inserts and could, therefore, serve as inoculum to infect healthy pine seedlings. In addition, chlorine treatment severely damaged roots and mycorrhizae. By supplementing irrigation water with hydrogen peroxide to raise the ORP level to 400 mV for 6 h, *F. circinatum* was successfully eliminated from irrigation water without damaging the seedlings. In fact, this treatment resulted in normal establishment of mycorrhizae, which may have contributed to the successful establishment of nursery plants in the field, even under conditions of severe drought stress (*unpublished*). Hydrogen peroxide-amended water also was successfully used to eliminate the pathogen from contaminated planting tray inserts when an ORP of 360 mV was used for 6 h. Because the inserts were allowed to dry before planting, this level of ORP did not have any negative effect on newly planted seedlings.

Table 2. Changes in oxidation reduction potential (ORP) of hydrogen peroxide-amended water over time

	ORP level (mV)	
Original ORP level (mV)[z]	After 6 h	After 24 h
Nontreated	183	231
300	302	316
350	344	339
400	376	375
450	399	401
500	473	423

[z] Nontreated water did not receive any hydrogen peroxide and had a final ORP level of 180 mV as measured with a handheld ORP meter. The ORP of the water in the other five beakers was adjusted by adding hydrogen peroxide in order to raise ORP levels to 300, 350, 400, 450, and 500 mV. The beakers were then left in the laboratory at room temperature, and the ORP was again measured 6 and 24 h after the ORP values had been set.

Table 3. Phytotoxicity of seedlings of *Pinus radiata* following seed treatment with hydrogen peroxide-amended water at different oxidation reduction potentials (ORPs) after 30 days

	Incidence on seedlings (%)[y]		
ORP level (mV)[z]	Dead	Wilted	Healthy
Nontreated water	0 c	5.4 a	92.9 a
300	0 c	7.1 a	92.9 a
350	8.9 c	0 a	91.1 a
400	1.8 c	3.6 a	92.9 a
450	89.3 b	5.4 a	5.3 b
500	100 a	0 a	0 b

[y] Numbers followed by the same letter are not significantly different from each other ($P = 0.01$).

[z] Nontreated water did not receive any hydrogen peroxide and had a final ORP level of 180 mV.

The two seed batches (*P. radiata* first and *P. radiata* second generation) investigated in this study initially tested positive for the presence of *F. circinatum*. Because surface sterilization substantially reduced the number of contaminated seed, *F. circinatum* is believed to be more commonly found on the seed surface than inside the seed coat or the endosperm or embryo. Spores of *F. circinatum* on the seed coat are known to be the result of the deposition of airborne spores (34,35). Why no contamination was found when these seed batches were again tested 7 months later is unknown. Although surface disinfestation of *P. radiata* seed from infested seed orchards near Karatara nursery may reduce or destroy *F. circinatum*, it is advised that seed from orchards free of pitch canker be used for planting in the nursery because it is known that affected orchards may be a source of seed contamination (10).

ORP-based methods have been successfully used to kill deleterious microorganisms in different food-processing environments. For instance, chlorine dioxide has been used to adjust the ORP level of water to treat *Salmonella* spp. on alfalfa seed and sprouts (22), and the use of chlorine, bromine, ozone, sodium, calcium hypochlorite, or hydrogen peroxide has been proposed for the elimination of *Pythium* spp. from irrigation water (29). Hydrogen peroxide has previously been used for the disinfestation of human pathogens such as *Escherichia coli* and *Salmonella* spp. (23). The current study, to our knowledge, is the first report on the use of hydrogen peroxide and ORP to disinfest irrigation water and plant inserts in a nursery environment.

The use of hydrogen peroxide to raise the ORP of water provides an efficient, affordable, and safe option for nursery sanitation. Hydrogen peroxide is less expensive than chlorine and measuring the ORP of irrigation water is a simple procedure that can easily be done using a handheld device. Furthermore, ORP is not affected by pH and measures the activity of the specific disinfectant being used without interacting with the water constituents (29). The chemical is noncorrosive and is rapidly broken down to harmless water and oxygen (13,32). The irrigation system at Karatara nursery has the required design for the use of hydrogen peroxide to alter the ORP of the water, in that water can be treated in a reservoir before it is sprayed onto the seedlings. Apart from its antimicrobial properties, hydrogen peroxide is known to induce plant defense responses (21).

The elimination of the pitch canker fungus in the Karatara nursery cannot be attributed only to the introduction of ORP treatment for sanitation purposes but also may involve other integrated disease management strategies implemented over time. The improvement of nursery hygiene and the treatment of fungal gnats and their larvae that can act as potential vectors of *F. circinatum*, or as wounding agents, might have contributed to the lower disease incidence. Recovery of the seedling root systems and recolonization by mycorrhizal fungi after the termination of the chlorine treatment of water also may have contributed to better protection against plant pathogens and better survival during field planting (3,8). A more in-depth study is required to investigate aerial inoculum and the role it plays in the contamination of *Pinus* seedlings in the nursery. Spores could originate from within the nursery itself or they could come from plantations in the surrounding area, where the pitch canker fungus is known to be active. Therefore, a strategy whereby all seedlings and trees with pitch canker symptoms in the vicinity of the nursery should be removed must be considered. There may also be multiple means by which the pathogen survives; for instance, in or on dead plant material and contaminated soil debris in or around the nursery (11,15). The source of the water contamination also should be determined. It is possible that spores were blown into the pond that serves as the main water source to the nursery or into the Karatara River, from which water is pumped into the pond. The Karatara River flows through a forestry area where pine pitch canker is known to occur.

The first step in preventing the widespread introduction of *F. circinatum* into forestry plantations in South Africa will be to eliminate the pathogen from all forestry nurseries in South Africa. This can be achieved by proper sanitation practices and the use of clean seed, water, soil, and planting pots. In this regard, the findings of the current study can make a major contribution to managing the pitch canker fungus in South Africa and elsewhere. In addition, nurseries should neither be near infected trees nor use seed coming from infected trees. The role of fungus gnats in the etiology of the disease should be elucidated, and the insects should be controlled (20). Finally, all *Pinus* spp. grown in South Africa, as well as clonal families and hybrids, should be screened for resistance to *F. circinatum*.

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Table 4. Losses of *Pinus radiata* seedlings in Karatara nursery from 2003 to 2010

	***P. radiate* seedlings[z]**			
Year	**Planted**	**Died**	**Death (%)**	**Remarks**
2003	1,576,428	370,266	23.5	Mortality in 2003 attributed to poor seed germination. No action taken.
2004	2,279,627	415,170	18.2	Mortality in 2004 attributed to poor seed germination. No action taken.
2005	2,543,792	751,650	29.5	Mortality in 2005 resulted in investigation. Causal agent identified a *F. circinatum*. Nursery hygiene upgraded; chlorine introduced as water treatment. Very high field mortality.
2006	2,711,384	595,640	22.0	Continued high mortality in 2006 resulted in further investigation. Nursery hygiene was upgraded, and chlorine concentration increased. Additional investigation on role of fungal gnats. Complaints about field mortality by growers.
2007	298,400	75,950	25.5	High mortality rate in 2007 still contributed to *F. circinatum*. *P. radiata* replaced by *P. elliottii* in nursery. War on fungal gnats. Complaints about field mortality.
2008	2,900,366	Insig.	0	Fewer losses of seedlings in 2008 due to chlorine treatment and growing of *P. elliottii* seedlings. Clients are happier with seedlings, but a significant reduction of mycorrhizae on roots. First report of pitch canker in plantations.
2009	3,038,338	Insig.	0	Change chlorine treatment to ozone and peroxide. Losses of *P. radiata* seedlings very low in 2009. Field losses less than 2%. Clients happy with seedlings.
2010	2,596,164	Insig.	0	ORP treatment optimized in 2010. Very few losses of *P. radiata* in nursery. Clients extremely happy with almost no losses in field, despite severe drought. Significant increase of mycorrhizae on roots.

[z] Notes provided by nurserymen at Karatara nursery, South Africa. Insig. = insignificant losses, indicating that losses were considered by nurserymen to be too few to document.

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