RESEARCH ARTICLE

Enzyme-linked immunosorbent assay (ELISA) based detection of *Fusarium circinatum* for alleviation of pine seedlings wilt

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A major threat to patula pine plantation is *Fusarium circinatum*, the causal agent of pitch pine canker and seedling wilt which are serious diseases of numerous pine species. *F. circinatum* is an ascomycete teleomorphic fungus found within the *Gibberella* complex responsible for many economically important plant diseases. The objective of this study was to develop an enzyme-linked immunosorbent assay (ELISA) for quick detection of *Fusarium* species causing seedling wilt in *Pinus patula*. In this study, an ELISA for quick detection of *F. circinatum* was developed using antibodies raised against mycelium-soluble antigens of this fungus. To validate the specificity of this assay, antibodies to *D. pinea* and *F. oxysporum* were also developed. Antigens developed from different plant organs (root, stem, and leaf) used for ELISA were produced by the inoculation of seedlings with conidia from each pathogen and were monitored by using scanning electron microscopy and the developed symptoms. The ELISA test showed sensitivity to detect infection caused by a conidia concentration as low as approximately 1 conidium/mL two weeks post inoculation. Cross-reactivity was observed, but a positive reaction for the detection of *F. circinatum* and *F. oxysporum* was indicated by an optical density above 0.8. Fungal identity was confirmed by polymerase chain reaction using primer pairs for amplification of a 70, 89, and 360 bp fragment for *F. oxysporum*, *D. pinea*, and *F. circinatum*, respectively. Results also showed that validation of each pathogen was obtained and no cross-infection between seedlings was detected. The cross-reactivity of the ELISA may have occurred due to factors such as common epitopes on fungal antigens. Therefore, ELISA optimization using these antibodies could provide an easy and fast field test to identify fungal pathogens infecting pine seedlings.

Keywords: Antibodies; ELISA; *Fusarium* spp.; Pine seedling wilt; Polymerase chain reaction.

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Introduction

Pinus patula, commonly known as patula pine serves as one of the most important tree resources produced and sold in Africa, particularly South Africa, as the best sawn wood among the 75% of timber species used for structural lumber and fiber for cellulosic pulp. According to Erasmus *et al*. [1], *P. patula* accounts for more than 52.2% of the total softwood area in South Africa. However, this species is also widely cultivated in several other South American countries despite its poor saw log production based on land expectation value and fungal disease attacks that have shown persistence in the forestry industry [2, 3]. *Fusarium circinatum* is one of the most critical pathogens of *P. patula* causing pine seedling wilt

and pine pitch canker on mature pine trees. Symptoms include root and collar rot, tip wilting as well as discoloration beneath the growing tip, which often progresses to the rest of the seedling and cause subsequent deaths of plantlets. On mature plants, pitch canker is marked by branch die back, development of stem resinous canker and resin-soaked wood [4]. *F. circinatum* pose serious challenges in the pine production industry in South Africa and other countries worldwide, leading to serious losses in softwood processing. This pathogen and others such as *F. oxysporum*, *F. sporotrichides*, *F. graminearum*, *Phytophthora*, *Pythium*, and *Rhizoctonia* have been reported in several countries including Japan, Mexico, New Zealand, Australia, Chile, and Brazil [3]. The abovementioned pathogens, particularly *F. circinatum* have huge negative impacts on ecological, economic, and timber production value chain, causing impaired seedling development, damage on tree growth, and reduced yields. Cook and Matheson [5] reported dramatic losses of revenue estimated over 11 million US dollars per year as a result of low seedling survival rates caused by *F. circinatum* infections. However, more pine deaths are associated with the infection of seedlings with *F. circinatum* or any other pathogenic fungi than infections of established trees, as this often emerge as a nursery pathogen posing a threat on new plantations of *P. patula*. Coutinho *et al*. [6] reported that in nurseries, *F. circinatum* readily spreads from contaminated planting pots, irrigation water, and supporting medium, while plant tissue wounding caused by beetles (*Ips conophthorus*, *Ernobium*, and *Pissodes nemorensis*) serve as infection sites in mature trees. In this study, the development of enzyme-linked immunosorbent assay (ELISA) for quick detection of *F. circinatum* using antibodies raised against mycelium-soluble antigens of this fungus was investigated. The assay specificity was validated using antibodies against *Diplodia pinea* and *F. oxysporum*. Reports indicated that fungal pathogens identified using conventional methods such as culturing of pathogens on selective medium, analysis of visible symptoms, and microscopic analysis are ineffective in early detection of causal pathogens [7, 8]. Early detection of the causal pathogen plays a major role in ensuring that proper control and limiting the spread of the disease. The use of nucleic acid based detection methods such as polymerase chain reaction (PCR) and protein based immunochemical techniques are more sensitive, quick, and specific [9-11]. These methods, especially PCR, have been well documented for detection of fungal pathogens [12, 13]. Therefore, an ELISA that uses pathogen-specific antibodies for easier and quicker detection in the quantification of fungal pathogens both in the laboratory and field was investigated to mitigate the adverse effects of *F. circinatum* on pine seeding development.

Materials and methods

Preparation of fungal pathogens and seedling infection

The four months old pine seedlings used in this study were obtained from South African Pulp and Paper Industry (Sappi) Limited, Gauteng, Johannesburg, South Africa. The seedlings were grown using bark potting mix and maintained in a greenhouse at 25±5°C under natural light conditions. Fungal pathogens (*F. circinatum*, *F. oxysporum*, and *D. pinea*) were obtained from Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Gauteng, Tshwane, South Africa. Fungal pathogens were cultured on potato dextrose agar (PDA) (Merck Group, Modderfontein GP, South Africa) at 28°C for 14 days, treated with UV light for two weeks to promote sporulation, and then, used to isolate fungal conidia for use in seedling inoculation. A drenching method was used to inoculate seedlings with the pathogens using different conidia concentrations (1×10^6, 1×10^3, 1×10^2, 1×10^1, and 1 conidia/mL). After inoculation, infected seedlings were rinsed with tap water, and then, longitudinal cut into root, leaf, and stem sections. Sections were then fixed overnight in 2% buffered osmium tetroxide [50% (v/v) of 4% OsO_4 : 25% (v/v) of 0.2 M sodium cacodylate buffer, pH 6.5] and dehydrated in

varying percent of ethanol (30, 50, 70, 80, 90, and 99.9%) before transfer in a pre-cooled Hitachi Critical Point-2 (HCP-2) drier (Hitachi, Gauteng, Johannesburg, South Africa). Dried plant materials were then coated with gold palladium sputter coating by using ESEM coating unit E5100 (Polaron Equipment Ltd, Sacramento, CA, USA) and visualized for infections under a scanning electron microscope (ESEM, Philips FEI XL 30) (Rotterdam, South Holland, Netherlands).

Indirect ELISA for antigen detection

To detect the levels of antigens from different plant parts, conidial infected seedlings for each pathogen were evaluated post-artificial inoculation. Inoculated seedlings were separated into plant organs and dissected into smaller pieces using a scalpel. The samples were homogenized using a Mini-bead-beater (Biospec, Bartlesville, OK, USA) for 5 minutes in phosphate buffered saline (PBS) (0.8% (w/v) NaCl : 0.02% (w/v) KCl : 0.115% (w/v) $Na_2HPO_4.2H_2O$: 0.02% (w/v) KH_2PO_4), and then, centrifuged for 5 minutes at 15,000 g. Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL, USA) was used to determine the concentration of proteins in the supernatant before use in indirect ELISA. For ELISA analysis, micro-titer plates were coated with 100 µL of antigen in PBS and incubated overnight at 4°C. Non-specific binding was blocked using 200 µL BSA (0.5% w/v in PBS) for one hour at 37°C. This was followed by the addition of 100 µg/mL pathogen specific (anti-*F. circinatum*, anti-*F. oxysporum*, and anti-*D. pinea*) BSA primary antibody (0.5% w/v in PBS). The mixture was incubated at 37°C for 60 minutes. The plates were then washed twice with PBS-Tween 20. After washing, rabbit anti-chicken IgY-horse radish peroxidase (HRPO) conjugate (Sigma, St Louis, MO, USA) was added to the wells and incubated for another hour at 37°C. After incubation, plates were washed three times with PBS-Tween 20 and then mixed with 2,2'-azinobis-diammonium salt chromogen/H_2O_2 substrate solution before incubation in the dark at room temperature for 10 minutes. Absorbance was then read at 405 nm using a FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany) with optimal reactivity of the antibodies at 100 µg/mL for indirect ELISA.

HRPO linked detection and cross-reactivity evaluation of antibodies

To increase assay sensitivity and specificity, a sandwich ELISA was developed. The first step involved conjugating primary antibodies developed in chickens against mycelium-soluble antigens from *F. circinatum*, *F. oxysporum*, and *D. pinea* to HRPO as detector antibodies. For conjugation, 4 mg HRPO was dissolved in distilled H_2O and thoroughly mixed with a freshly prepared 100 mM sodium periodate solution. The mixture was dialyzed overnight for 16 hours against four changes of 1 mM Na-acetate buffer at pH 9.5. Following the change in pH, IgY was immediately added to a final concentration of 8 mg/mL and the mixture incubated for 2 hours at room temperature. Free enzymes were reduced by the addition of freshly prepared 4 mg/mL Na-borohydride solution, incubated at 4°C for 2 hours and dialyzed for 16 hours against four changes of 100 mM Na-borate buffer (pH 7.4) at 4°C. An equal volume of 60% (v/v) glycerol in Na-borate buffer (pH 7.4) (Merck Group, Modderfontein GP, South Africa) was finally added and the conjugate was stored at 4°C until use for ELISA [14]. Cross-reactivity evaluation of antibodies were performed using sandwich ELISA prepared as indicated previously for indirect ELISA. Seedling stems that showed clear infection symptoms within two weeks (infected with 1×10^3 conidia/mL) were used for evaluating antibody cross-reactivity. During indirect ELISA, plates were coated with 0.2, 0.6, and 0.2 µg/mL of anti-*F. oxysporum*, anti-*F. circinatum*, and anti-*D. pinea* antibodies in PBS, respectively. These were incubated at 4°C overnight, followed by blocking of unoccupied sites with 200 µL of 0.5% (w/v) BSA in PBS for 1 hour at 37°C. Antigens (200 µg/mL each) were added into the wells coated with antibodies and incubated for 2 hours under a similar temperature. After 2 hour incubation, the plates were washed three times with PBS containing 0.1% (v/v) Tween 20, and then, 1 in 20 diluted HRPO-linked antibodies were added into the respective wells, followed by incubation for 1

hour at 37°C. Plates were again washed with PBS-Tween 20, added with 2,2'-azino-di-3-ethyl-benzthiazoline-6-sulphonic acid (ABTS)/H_2O_2 chromogen substrate solution [0.05% (w/v) ABTS and 0.0015% (v/v) H_2O_2 in 50 mM citrate phosphate buffer, pH 5.0] and incubated in the dark at room temperature. Absorbance was read at 405 nm and the non-infected seedling samples as well as pre-immunization antibodies were used as controls.

Polymerase chain reaction (PCR) analysis in pine seedlings

PCR was used to confirm the identity of pathogens tested in the ELISA and to evaluate the possibility of any cross-reactivity that might have occurred during seedling inoculation in the greenhouse. Seedlings used for isolation of antigens were also used for DNA isolation using hexadecyltrimethylammonium bromide (CTAB method) as described by Murray and Thompson [15]. The DNAs of seedlings stems infected with *F. circinatum*, *F. oxysporum*, and *D. pinea* were extracted in warm extraction buffer [10% (v/v) 10 mM Tris-HCl, pH 8 : 4% (v/v) 20 mM EDTA : 28% (v/v) 1.4 mM NaCl : 20% CTAB : 0.2% (v/v) 2-mercapto-ethanol] (Merck Group, Modderfontein GP, South Africa). A chloroform:isoamyl alcohol (24:1) solution was added into the mixture after 60 minutes incubation at 65°C, followed by centrifugation at 12,000 g for 3 minutes at 4°C. The supernatant was transferred to a centrifuge tube, and 500 µL of cold isopropanol was added, centrifuged at 12,000 g for 5 minutes at 4°C to recover the pellet, which was re-dissolved in 70% (v/v) ethanol. The pellet-ethanol mixture was allowed to stand at room temperature for 20 minutes, centrifuged at 12,000 g for 5 min at 4°C. The DNA pellet was then air-dried. Tris-EDTA (TE) buffer (pH 8) was used to resuspend the pellet first at room temperature for 1 hour followed by an overnight incubation at 4°C. A total of 200 µL of TE buffer was used to resuspend the DNA with the addition of RNase A solution and incubated at 37°C for 2 hours. DNA concentration and purity were determined using the A260/280 ratio determined through a NanoDrop ND-1000 Spectrophotometer (Marshall Scientific, Hampton, NH, USA). The alternative DNA source was prepared by longitudinally cutting stem section from *Fusarium*-infected seedlings and cultured them on a *Fusarium* selective medium containing 1.5% peptone, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4.7H_2O$, 2% (w/v) bacterial agar, and 1% (w/v) quintozene (Merck Group, Modderfontein GP, South Africa). Mycelium growths developing from seedling stems were cut out and sub-cultured on a fresh agar medium to grow new mycelium colonies used for DNA extraction using CTAB method as described above. For DNA amplification, a total volume of 20 µL constituting 5 µL of DNA, 2 µL of 100 µM forward/reverse primers, 10 µL of KAPA universal master mix, and 1 µL of nuclease free water. The primer pair specific for each pathogen was used for the pathogen confirmation step and cross-reactivity evaluation. The oligonucleotide sequences for *D. pinea* were DpHRM_F forward primer (5'-GCTACCTTGGAGTAAGGGACA-3') and DpHRM_R reverse primer (5'-TTTCCATCTAGG AGCGAAAAT-3') [16]; for *F. circinatum* were CIRC1A forward primer (5'-CTTGGCTCGAGA AGGG-3') and CIRC4A reverse primer (5'-ACCTAC CCTACACCTCTCACT-3') [17]; for *F. oxysporum* were PFO3 forward primer (5'-CGGGGGATA AAGGCGG-3') and PFO2 reverse primer (5'-CCC AGGGTATTACACGGT-3') [18]. PCR reaction was initiated by first heating the lid of G-storm thermocycler (Vacutec, Roosevelt Park, South Africa) to 110℃, followed by a hot start temperature of 95℃ for 300 seconds, and then, 30 cycles of 95℃ for 15 seconds, 65℃ or 57℃ for 900 seconds for *Fusarium* pathogens or *D. pinea*, respectively, 72℃ for 15 seconds, and a final elongation at 72℃ for 180 seconds. The PCR products were stored at 10℃. A 2 % (w/v) agarose gel was used to evaluate PCR products. To verify the results, specific fungal sequence in the mitochondrial small subunit ribosome gene (mt SSU rDNA) were analyzed to distinguish between the fungal strains on the basis of size of PCR products. These were determined based on the expected sizes of 60 bp for *D. pinea*, 360 bp for *F. circinatum*, and 70 bp for *F. oxysporum*,

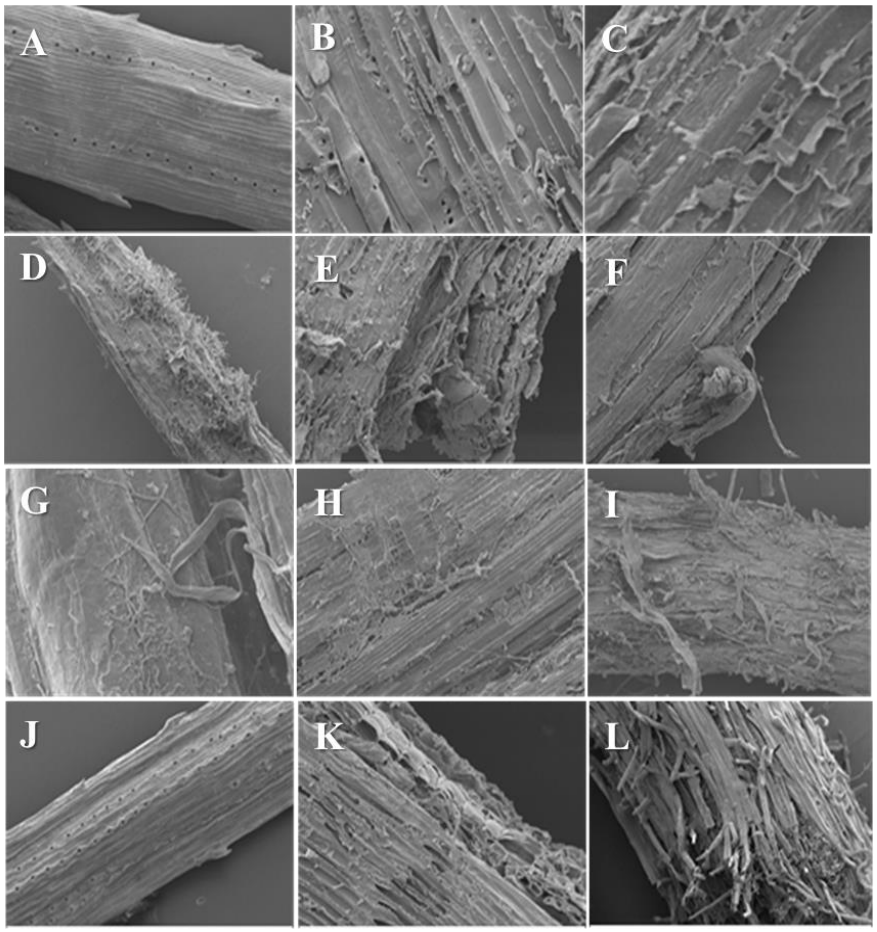

Figure 1. Electron micrographs showing different sections of pine seedlings artificially inoculated with different fungal pathogens. Pine seedling leaf (A), stem (B), and root (C) used as controls. Pine seedlings two weeks post inoculation with *D. pinea* (leaf (D), stem (E), and root (F)), *F. circinatum* (leaf (G), stem (H), and root (I)), and *F. oxysporum* (leaf (J), stem (K) and root (L)).

which are relatively divergent and distinctive of fungal species.

Results

Detection of fungal antigens from different seedling parts using ELISA

According to the findings, pine seedlings inoculated with fungal mycelium showed extensive mycelial growth mostly on the leaf and stem samples. The greatest growth of mycelium was mostly observed on the stems than both roots and leaves of pine seedlings, which was then selected to provide the highest antigen concentration used during ELISA. The observed mycelium growths showed greater distribution in almost all seedling stems, followed by roots and leaves, weeks after *F. circinatum* and *D. pinea* plant infections. However, *F. oxysporum* infected seedlings showed no mycelium growth on the leaves but, infection occurred only on stems and roots. When indirect ELISA was used for the detection of fungal antigens directly isolated from pine seedlings, there was no strong correlation of the levels of mycelia observed under scanning electron microscope (Figure 1) and ELISA readings (Figure 2). The infection patterns observed on scanning electron micrographs and ELISA were strongly expressed with all fungal species used (Figure 1, D–L). Furthermore, the levels of antigen detected in the ELISA method using the corresponding antibodies were much higher in the stems than in

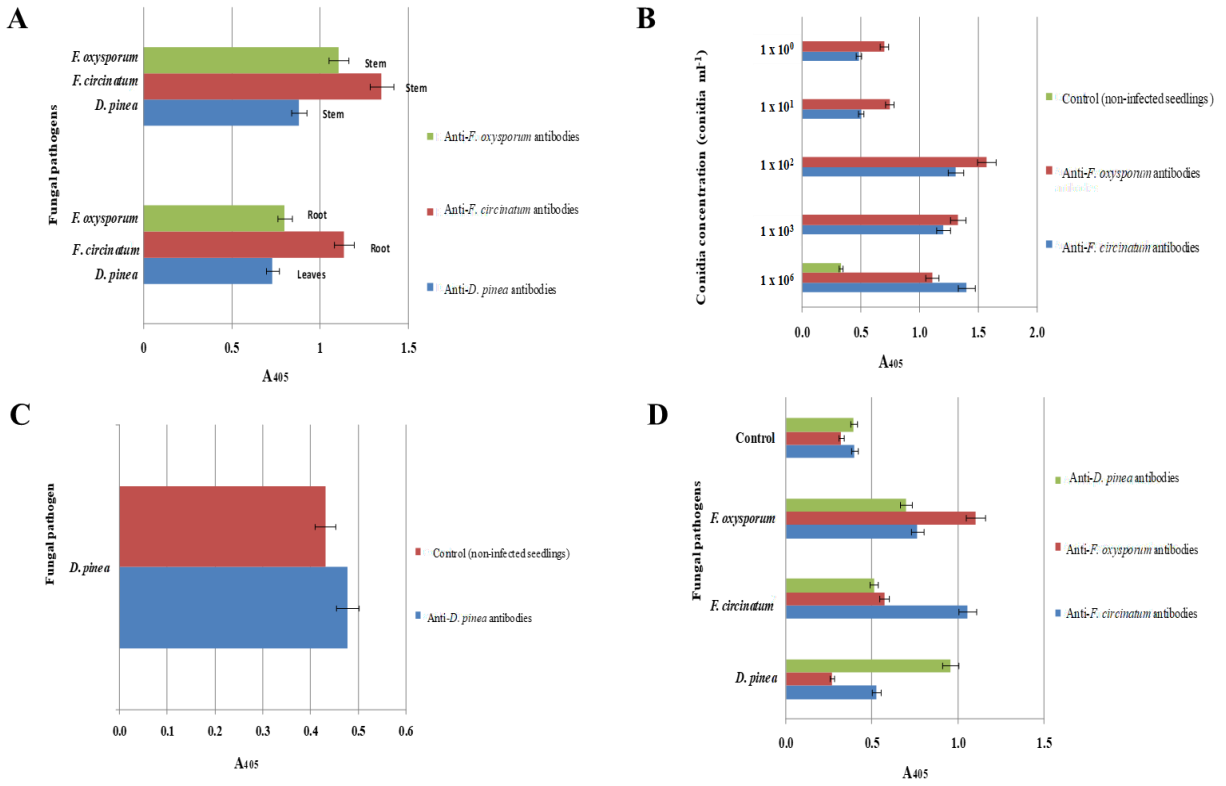

Figure 2. Antigen detection using indirect ELISA of fungal growth on different parts of pine seedling inoculated with fungal pathogen. **A.** Evaluation of antibody sensitivity for the detection of antigens isolated from infected seedlings. **B.** Seedlings inoculated with fungal conidia (concentration of 1 to 1x10^6 conidia/mL) from *F. circinatum* and *F. oxysporum* used for antigen isolation. **C.** Antigens isolated from pine seedlings inoculated with conidia from an agar block of *D. pinea* culture, directly inoculated on seedlings. **D.** Evaluation of antibody cross-reactivity with antigens isolated from *D. pinea*, *F. oxysporum*, and *F. circinatum* infected pine seedlings using a sandwich ELISA.

the roots. Furthermore, the results also indicated that higher antigen levels in stems were increased with the increase in duration of the incubation post-infections.

Sensitivity and cross-reactivity of ELISA in the detection of fungi at different infection levels in pine seedlings

Seedlings infected with various concentrations of fungal conidia ranging from 1 to 1x10^6 conidia/mL using drenching method exhibited varying sensitivities to ELISA. Detection levels on seedlings infected with 1x10^6 – 1x10^2 *Fusarium* conidia/mL were similar, but absorbance values were lower at conidia concentrations of 1x10^1 – 1 conidia/mL (Figure 2A). The ELISA readings obtained for detection of *D. pinea* on pine seedlings was relatively low compared to the detection levels observed for *Fusarium* infected seedlings, indicating the difference in the antibody sensitivity levels (Figure 2A and 2B). Furthermore, there were no major variations between non-infected and *D. pinea* infected seedling samples of *P. patula* (Figure 2C). When sandwich ELISA was used to evaluate cross-reactivity of anti-*F. circinatum*, anti-*F. oxysporum*, and anti-*D. pinea* with antigens isolated from plant extracts, similar reactivity with that of indirect ELISA was observed at a primary antibody concentration less than 10 µg/mL, whereas 100 µg/mL was required to get a similar response (Figure 2D). Anti-*D. pinea* antibodies were more reactive towards *D. pinea* antigens and showed less cross-reactivity with *F. circinatum* than *F. oxysporum*. Anti-*F. circinatum* antibodies were also more reactive with the antigens isolated from *F. circinatum* infected seedlings but, with significant cross-reactivity with *F. oxysporum*. Anti-*F. oxysporum* antibodies were the most reactive antibodies indicated by

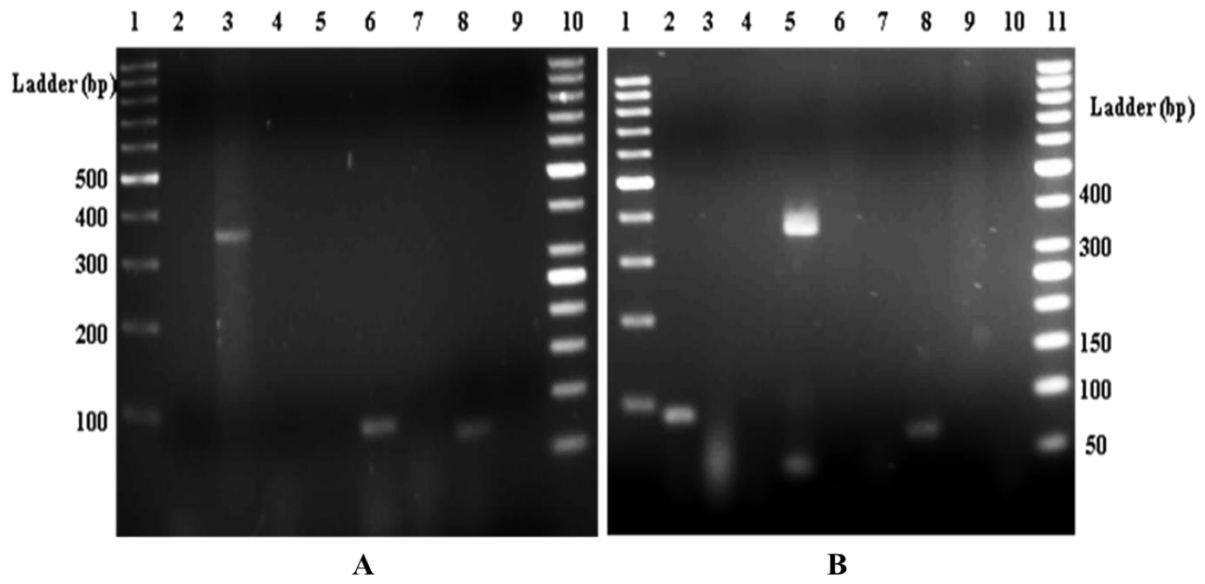

Figure 3. PCR amplification for confirmation of the fungal pathogen isolated from infected pine seedlings, and evaluation of the possible multiple infections in each seedling. **A.** Fungal pathogen isolated directly from an infected plant: lane 1: 100 bp DNA marker; lane 2: control seedlings (non-infected); lane 3: *F. circinatum* infected seedling using FCM; lane 4: seedling infected with *F. circinatum*; lane 5: seedlings infected with *D. pinea*; lane 6: seedlings infected with *D. pinea* using PDA; lane 7: seedlings infected with *F. oxysporum*; lane 8: *F. oxysporum* using FCM; lane 9: distilled water control; lane 10: 50 bp DNA marker. **B.** Evaluation of multiple infections: lane 1: 100 bp DNA marker; lanes 2, 3, and 4: DNA from *D. pinea*, *F. circinatum*, and *F. oxysporum* infected seedlings, respectively, amplified with the DpHRM_F- DpHRM_R primer pair; lanes 5, 6, and 7: DNA isolated from *F. circinatum*, *D. pinea*, and *F. oxysporum* infected pine seedlings, respectively, amplified using the CIR1A-CIR4A primer pair; lanes 8, 9, and 10: DNA isolated from *F. oxysporum*, *F. circinatum*, and *D. pinea* infected seedling, respectively, amplified using the PFO3-PFO2 primer pair.

the highest absorbance values obtained when reacting with antigens obtained from *F. oxysporum* infected seedlings (Figure 2D). These antibodies cross-reacted with the other two fungal pathogens studied as indicated in Figure 2.

Although, cross-reactivity was obtained between fungal pathogens, the multiple ELISA tests carried out using stem and root samples from *Fusarium* infected seedlings indicated an absorbance reading above 0.8 taken as a positive reaction. Furthermore, variations with *D. pinea* were noted, and antibodies developed also showed some reactivity with plant constituents indicated by ELISA reading of approximately 0.4. This was observed when each antibody was reacted with antigen isolated from the non-infected seedlings as indicated in Figure 2D. An absorbance of 0.4 is 50% of the cut-off value for the specific detection of *Fusarium* pathogens, and close to the cross-reactivity of the *D. pinea* with anti-*Fusarium* antibodies.

PCR-based identification of fungal pathogens

To assess and prevent cross-contamination of seedlings kept under similar greenhouse conditions during incubation from other opportunistic fungi, PCR analysis was performed. This technique was used to confirm the identity of the pathogens detected by optimised ELISA. No DNA amplification was observed when samples were directly analysed from infected seedlings without the use of a selective potato dextrose agar medium (PDA), probably due to plant phenolics. Results indicated that the use of PDA media eliminated phenolics from the test materials used for isolation of *Fusarium* species from the infected seedlings. DNA amplification was observed as shown in Figure 3A, lane 3, 6, and 8 where an 89, 360, and 70 base pair products were observed indicating the infectious fungus for each seedling to be *D. pinea*, *F. circinatum*, and *F. oxysporum*, respectively. Since DNA amplification occur from primer pair specific to the isolated DNA, findings made in this study clearly indicated that cross-reactivity observed

with ELISA was due to common epitopes and not multiple infections. Primer pairs DpHRM_F-DpHRM_R, CIR1A-CIR4A, and PF03-PF02 gave DNA products of the expected size from the seedlings infected with fungal species as indicated on Figure 3, which were also 89, 360 and 70 bp for *D. pinea*, *F. circinatum*, and *F. oxysporum*, respectively (Figure 3 B, Lanes 2, 5, and 8).

Discussion

This study presents findings of an optimized ELISA test developed to detect fungal pathogens in pine seedlings, with a broader aim of discriminating between *F. circinatum*, *F. oxysporum*, and *D. pinea* using antibodies established from mycelium-soluble antigens of these fungal pathogens. This study indicated that mycelium scraping serves as a preferred method for mycelial inoculation and alternative DNA isolation source comparing to that of the drenching method. This was clearly demonstrated by higher readings of ELISA absorbance observed when the detected antigens were isolated from plants inoculated by directly placing mycelium scrap on wounded plant tissues comparing to drenching method. Perez-Sierra *et al*. [19] made similar observations using *F. circinatum* inoculum scraped from PDA plates and directly placing the pathogen on the wounds of 8 months old *P. pinaster*, *P. nigra*, and *P. sylvestris* seedlings. As a result, fatal pathogenic symptoms were rapidly induced within 7, 8, and 15 days post-inoculation on *P. nigra*, *P. pinaster*, and *P. sylvestris*, respectively. Results made in this study support several reports indicating that artificial method of inoculating plants through direct placing of pathogen on induced wounds results in a significant and rapid disease development [19-21]. Furthermore, mycelium growth observations made in this study, especially on *F. oxysporum* infected pines, indicated that more mycelium colonized the seedling roots as expected from *Fusarium* species. This observation was similar to earlier findings made by Ricker, *et al*. [22] that some pathogens grew better in certain plant tissue organs. Similarly, *Botrytis cinerea* was also reported to grow better in tissues possessing high levels of photosynthetic sugars (e.g., leaves or assimilates storage organs) while *Fusarium* pathogens continue to indiscriminately invade and colonize the roots [21, 23]. However, testing of isolated antigens using ELISA also indicated that most reactive antigens were observed in stems than roots or leaves of all seedlings infected with *Fusarium*. Moreover, an increase in the level of antigen detection was directly proportional to the increase in conidia concentrations. Detection was made regardless of the antibodies used, with severity of disease observed with time post-infection, and others showing ELISA detection of the fungus without symptoms. Similar asymptomatic seedlings infected with fungal pathogens were reported by Luchi, *et al*. [8] and Maresi, *et al*. [13], which were detected using ELISA and PCR. In contrast, Clausen [24] earlier reported that the use of antibodies is not ideal system for specific and quantitative detection of fungal antigens in infected wood samples. In response to the above report, ELISA test findings in this study showed that the approach is sensitive enough to detect pathogens at very low levels, given reported detection at 1 conidium/mL. Furthermore, results on cross-reactivity indicated a positive reaction above 0.8 OD, with developed antibodies presenting a non-specificity binding with different plant constituents illustrated by the lack of reactivity on non-infected seedlings. But similar cross-reactivity can be observed from species of the same genus as in *Fusarium* spp. or different genera as for *Diplodia* sp. This observation was also made earlier by Bossi and Dewey [25]. The report indicated that fungal pathogens produce similar cross-reactive immunodominant molecules in plants and that species-specific molecules were either not immunogenic, produced in same quantities as those that are genera-specific, or they are not water soluble. However, species-specificity also existed in this study since reactivity of each of the antibodies developed shown to be more specific to its corresponding antigen isolated from the

plant extracts. The PCR results confirmed the presence of each fungus using primers specific for each pathogen. The primers indicated that there was no cross-infection between seedlings, emphasizing that the observed cross-reactivity was due to common carbohydrate epitopes on fungal antigens as reported by Hitchcock, *et al*. [26]. The common nature of biochemical components that make up the mycelium of most fungi is still of greater concern in studies that are aimed at raising species-specific fungal antibodies. This limits antibody specificity to the genus level rather than to species level, where either monoclonal or polyclonal antibodies have been raised against fungal antigens [27, 28]. Nevertheless, common antigens observed between fungal species analyzed in this study through ELISA and western blotting indicated that antibody binding to mycelium antigens is more directed to binding with chitin, hyphal tips, and septa [26, 29]. Thus, to increase the level of specificity, some researchers have proposed using antibodies from the first few weeks post-immunization as these do not have high titer and cross-adsorbing antibodies [22]. But, as indicated in the present findings of this study, no increased antibody specificity was observed when antibodies produced during the first six weeks post the first immunization were used for detection.

Conclusion

ELISA-based field tests for the detection of *Phytophthora*, *Phythium*, and *Rhizoctonia* have been developed previously using sandwich ELISA [30]. There is also a dipstick kit available for the detection of *Fusarium* mycotoxins (T-2) [31]. The ELISA tests prepared in the present study were able to detect 50% of *F. circinatum*, *D. pinea*, and *F. oxysporum* in infected pine seedlings, and discriminated between these pathogens. This can be used directly by nursery staff in the field where plates coated with primary antibodies can be provided in a kit, together with labeled secondary antibody, and the resulting reaction could be measured using a spectrophotometer. Use of an ELISA dipstick or ELISA using these antibodies should provide an easy, fast field test to identify infections of pine, discriminating between *F. circinatum*, *F. oxysporum*, and *D. pinea*.

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