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Vol. 13(29), pp. 2922-2929, 16 July, 2014
DOI: 10.5897/AJB2013.13451
Article Number: B67665646089
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Immunodiagnostic potential of a 27 kDa protein of *Fusarium xylarioides,* the cause of coffee wilt disease in Robusta coffee in Uganda

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Received 7 November, 2013; Accepted 20 June, 2014

Several *Fusarium* species infect Robusta coffee; these *Fusarium xylarioides* Steyaert (*Gibberella xylarioides* Heim and Saccas) are the most virulent and responsible for the destructive Robusta coffee wilt disease in Uganda. To date, *F. xylarioides* has not been isolated directly from soil, though the pathogen can persist in soil for a short time. In this study, a promising diagnostic target which can be developed into a serological test for *F. xylarioides* in coffee plants and soil has been identified and validated for identification. Water-soluble extracts of mycelia from six *Fusaruim* species were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The different protein profiles from the other five *Fusarium* species were compared and contrasted with that of *F. xylarioides*. Protein bands that appeared peculiar to *F. xylarioides* were cut and injected into rabbits to produce polyclonal antibodies. Dot blot and Western blot analyses showed one immunodominant antigen (27 kDa) common to all *F. xylarioides* isolates analyzed. No cross-reactivity of anti-27 kDa antibodies were observed in the entire test *Fusarium* species. The results suggest that polyclonal antibodies raised against the endoantigens from *F. xylarioides* of 27 kDa, is a promising tool for the rapid, sensitive, and accurate detection of pathogen in soil and plant parts.

Key words: *Gibberella xylarioides*, coffee wilt disease, antigen, antibodies, Uganda.

INTRODUCTION

One of the major constraints of Robusta coffee (*Coffea canephora* Pierre) production in Uganda is the coffee wilt disease (CWD). Coffee wilt disease, also commonly referred to as tracheomycosis or sometimes

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carbuncularioses, is caused by a typical fungal vascular pathogen known as *Fusarium xylarioides* Steyaert. The sexual (perfect) form is *Gibberella xylarioides* (Heim and Saccas, 1950). *F. xylarioides,* which is the conidial (imperfect or anamorph) state of this species was isolated and described by Steyaert from diseased specimens of Excelsa coffee (*Coffea excelsa*) in 1939 (Flood, 1996). Although, Fusarium species have a wide host range, some are quite specific. *F. xylarioides* is a specific coffee pathogen which is rarely found elsewhere (Waller and Holderness, 1997). Tracheomycosis was first reported in *C. excelsa* in the Central African Republic; on robusta coffee (*C. canephora*) in Côte d'Ivoire in 1948, and Congo causing major damage. The disease affects arabica coffee in Ethiopia, while in Uganda it has been attacking only robusta coffee (Adugna et al., t2005). Rutherford (2006) in his inter-simple sequence repeat (ISSR) analysis of *F. xylarioides* isolates from coffee wilt disease infected robusta or arabica coffee observed that these plants were affected by distinct strains. In addition, a study using infection tests in Ethiopia showed host-strain specificity where isolates from Arabica coffee plants were pathogenic only to seedlings of *Coffea arabica* (Adugna et al., 2005). The pathogen survives in the soil. It is difficult to control the pathogen by fungicides. However, the pathogen may be controlled by antagonistic biological control agent such as Trichoderma species which has been reported to control the pathogen by up to 71% *in-vitro* (Alemu, 2012).

Typical symptoms of the disease include curling and yellowing of leaves, defoliation and die-back, blue-black streaks on the wood under the bark, general wilting and death of infected plant (Heim and Saccas, 1950). Unusual symptoms due to other coffee diseases and pests or physiological disorders such as root rot, bark disease; root mealy bug, termites, stem borer; effect of drought and nitrogen deficiency can sometimes be confused with CWD symptoms (http://aces.nmsu.edu/desertblooms/nmsugardening/docs/chap_3/chap3.a.pdf). This can result in misdiagnosis of the disease in the field. It is generally known that symptom expression is a product of the host-pathogen interaction, and host colonization is a prerequisite for disease manifestation (Gaumann, 1950). Existing procedures for recording the incidence of CWD is based only on the expression of the symptoms. In addition there is no indicator that plants can be used to show the presence of pathogen in soil. At present, identification can only be confirmed in the laboratory by plating out infected, surface-sterilized material on appropriate media; while traditional detection from soil is based on baiting techniques since isolation directly from the soil on artificial medium has so far been unsuccessful (Adipala-ekwamu et al., 2001). The agar plate test is lengthy since sporulation is needed for accurate identification. Unfortunately, the identification of fungi based on traditional cultural, morphological and metabolic characteristics may take days and weeks and are laborious, time consuming and require significant technological expertise. This is because different isolates often display characteristics from more than one species (Bowen et al., 1996). For a more complete understanding of the disease, a specific detection technique for *F. xylarioides* in symptomless but infected hosts is essential.

In recent years, molecular and serology techniques of plant disease detection have been well established (Sindhuja et al., 2010). The molecular methods, which are usually PCR based is where a specific base sequence of the genetic material of the pathogen is utilized, while in serological or immunological methods, the microbial protein (antigen) associated with a pathogen is introduced into an animal that produces specific antibodies against the antigen. The techniques are gaining importance due to their specificity, sensitivity and rapidity. Such advanced plant disease detection techniques can provide rapid, accurate, and reliable detection of plant diseases in early stages for economic, production, and agricultural benefits (Sindhuja et al., 2010). In the present paper, we report the identification of the *F. xylarioides* immunodiagnostic target protein that has produced specific polyclonal antibody in rabbit. The specific antibodies can be packaged into a immunodiagnostic procedure and integrated with the current culture based-techniques for better detection of the pathogen both in soil and coffee plant parts. The outcome of the work will be rapidity and simplicity of the test (Sally and Robert, 1988), early detection of CWD pathogen in the host when the infection levels are very low, in-planta proliferation of pathogen, determination of fungal biomass in infected tissues, characterisation of genetic races of the pathogen (Bhuvanendra et al., 2010), based on their protein banding pattern and immuno-reactivity patterns.

The purpose of the study was therefore, to identify a target antigen and validate its corresponding polyclonal antibody for specificity to formulate an immunoassay technique that is simple, fast, highly specific and sensitive for detection of *F. xylarioides* in the soil and plant parts.

MATERIALS AND METHODS

Sample collection

Samples of CWD infected Robusta coffee trees were collected in duplicates from each of the 8 districts in the four agro-ecological farming systems in Uganda where coffee is grown and CWD has been reported. The farming systems were; 1) banana/Coffee System from the districts of Bundibugyo, Mubende, Mukono, and Kalangala; 2) banana/millet/cotton System from Kamuli district; 3) Montane system from Rukungiri and Kabarole districts; 4) pastoral system from Rakai district. The specimens were transported to the laboratory wrapped and labeled individually in paper bags. *F. xylarioides* isolate obtained from a wilted coffee tree at the Coffee Research Institute, Kituza, Mukonoin Uganda was used as a reference.

Fungal isolation and identification

The bark of the stem specimen was carefully removed and small sections (0.5 x 0.5 cm) excised from the intervening regions between discolored wood (lesion tissues) and white healthy wood using a sterile scalpel. The sections (5 to 6) were transferred to plastic Petri dishes and surface sterilized by immersing in 1% v/v sodium hypochlorite solution for 1 to 2 min and rinsed twice in sterile distilled water. The sterilized tissue was then blotted dry by pressing with sterile tissue paper, trimmed to size and placed on tap water agar (TWA) that comprised 20% agar, and incubated at 25°C for three days (Booth, 1971). Emerging fungal hyphae from the wood pieces were sub-cultured on fresh Synthetic Nutrient Agar (SNA) with four pieces of sterile filter paper (1 x 1 cm) placed around the periphery of the set agar to induce sporulation (Nirenberg, 1976) and then on potato sucrose agar (PSA) to encourage development of pigmentation (Booth, 1971). The cultures were incubated under 12 h fluorescence light and dark cycles at 25°C. After 10 days, different *Fusarium* species were identified based on the typical cultural and morphological characteristics of the species as described by Booth (1971). *F. xylarioides* isolate used as gold standard was the one identified at the Coffee Research Institute under the microscope and confirmed at the Global Plant Clinic, CABI Bioscience, UK Centre, England.

Preparation of mycelial homogenates

The different isolates were grown separately in 100 ml Erlenmeyer flask containing 20 ml of potato dextrose broth. The medium was inoculated with four (4 x 4 mm) plugs taken from the periphery of young fungal cultures (5 day old) on PDA/SNA medium. The cultures were incubated stationery in the dark at 20°C for six days. The mycelium was harvested by pouring the culture broth containing fungal hyphae into sterile steel tea strainer. The mycelial mats were finally rinsed thoroughly with de-ionized water blotted dry and then frozen in liquid nitrogen and immediately ground with a mortar and pestle to a fine powder. The powder was suspended in 2 ml of Phosphate Buffered Saline (PBS) containing 0.05% pentylmethyl-sulphonyl fluoride (PMSF) which was added per gram of mycelium ground and centrifuged at 30,000 *g* for 30 min at 4°C, and the supernatant was stored at 20°C until use. The protein content in the supernatant was estimated by the method of Brayford and Flood (1997) using Bovine serum albumin (BSA) as a standard.

Protein analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess the purity of the samples, locate the required protein antigen (s) and to prepare proteins for transfer to nitrocellulose membrane for immunobloting (Laemmli, 1970). Soluble protenacious components from mycelial extracts were adjusted to 2 mg/ml in PBS. They were diluted (v:v) in denaturing buffer containing 10% glycerol, 16 of 10% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 12.5% 0.5 M Tris-HCl (pH 6.8) and 5% of 1% v/v bromophenol blue then 47.5% distilled water (Laemmli, 1970). Samples were boiled for 5 min and loaded immediately. Leftovers were stored at -20°C till use. Extracts (10 to 30 μg) were placed in each well. Extracts were stacked in a 4% polyacrylamide gel and resolved according to their molecular sizes on a 10% polyacrylamide gel. Electrophoresis was carried out on a Mini-PROTEIN 11 Electrophoresis Cell (BIO-RAD®, USA). Migration took place at a constant intensity of 120 V and 400 mA at room temperature, until the dye marker had reached the bottom of the gel. The resolved bands were revealed on the gel by staining with 1% Coomassie blue R 250 in fixative (40% methanol, 10% acetic acid) for 2 h at room temperature. The gel was de-stained with three changes of de-staining solution consisting of 40% methanol and 10% acetic acid until the background had been removed and the bands were clearly seen. For better viewing, gel was placed against white light provided by transilluminator (VILBER LOURMAT®, France). Gel was documented by photographing using a digital camera (OLYMPUS D-490 ZOOM®, Germany). The different protein profiles from the other 5 *Fusarium* species were compared and contrasted with that of *F. xylarioides*. Three protein candidates that appeared unique to *F. xylarioides* were of molecular weight 66, 50 and 27 kDa. These were excised from the gel, solubilized in PBS by crushing in chilled mortars, centrifuged and supernatant stored at -20˚C until required.

Production of polyclonal antibodies against selected bands

Female New Zealand White rabbits 6 months old were purchased locally and reared in cages. Every selected band extract was adjusted to 100 µg protein in 0.5 ml of PBS to be used per rabbit. An equal volume of Freund's complete adjuvant (FCA) was added and the 1 ml mixture emulsified, and injected by subcutaneous injections into a rabbit at four sites on the back to elicit immune response to produce polyclonal antibodies (Harlow and Lane, 1988). Each rabbit received two or more intramuscular booster immunizations of 50 µg protein/ 0.5 ml PBS emulsified in 0.5 ml Freund's incomplete adjuvant (FIA) after every fortnight. This continued until a required titre was attained. Bleeding was 10 days after boosting. Blood was obtained from the marginal ear vein following the shaving around its posterior edge and cleaning with a cotton wool soaked in 70% ethanol. The blood collected was allowed to clot at room temperature for 1 h then stored in the fridge (4°C) overnight then centrifuged at 2,500 *g* for 30 min to separate the serum. The separated sera were stored in 200 µL aliquots in 0.5 ml tubes at -20°C until required for antibody analysis.

Dot blot analysis

This procedure was performed as a quick check for the positivity and specificity of the antisera from rabbits. Samples of all the soluble protein extracts from seven *Fusarium* species were prepared in PBS pH 7.4 to a final concentration of 2 mg protein per ml. Ten microlitres of this solution was spotted onto strip of nitrocellulose membrane (0.45 mm pore size; Bio-Rad Laboratories, Richmond, California) and air dried for 5 min. Non-specific binding sites were blocked with 5% skimmed milk in PBS by incubating for 2 h at room temperature while rocking. After washing three times, by shaking for 5 min during each wash in PBS-Tween 20 (PBS containing 0.05% tween 20), the membrane was incubated for 1 h in the test rabbit immune sera at a dilution of 1:100 as primary antibodies and again washed as described above. The washed membrane was then probed with peroxidase-conjugated goat-anti rabbit IgG as secondary antibodies for 45 min and washed 3 times with PBS-Tween 20 as above. The positive reaction were visualized by use of diaminobenzitine (DAB) solution (1.3 mM DAB in 0.01M Tris-HCl containing 0.004% H_2O_2) as the substrate. After the appearance of the colour, the membranes were washed with distilled water for several times to stop the reaction. The development of well-defined brown dots on the nitrocellulose membrane was considered as positive.

Immunoblotting (Western blot) analysis

This was done to confirm the specificity of the antibodies: 20 uL of crude protein extracts from the eight *Fusarium* species were first resolved over 10% SDS-PAGE as described above in duplicate, one for Commasie blue staining for contrasting with the Western

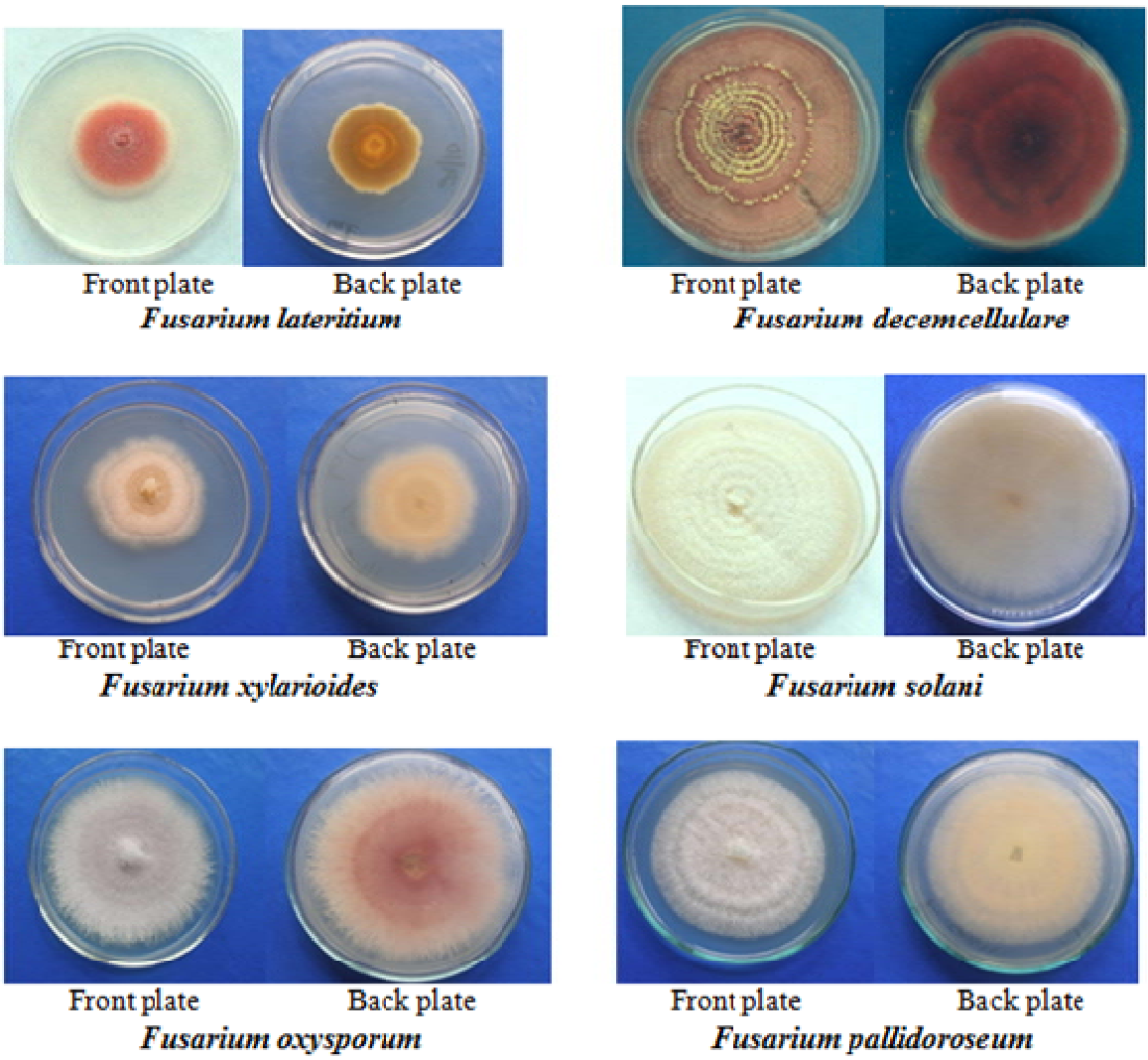

Figure 1. Culture pigmentation of different *Fusarium* species. Cultures grown on Potato Dextrose Agar (PDA) and induced pigmentations peculiar to each *Fusarium* species and used as an aid to correct identification. (Booth,1971). Culturing was done at room temperature at 12 h light / 12 h dark cycles for 10 days.

blot result while the other one was for protein transfer to nitrocellulose membrane. Resolved proteins were transferred onto nitrocellulose paper for 1 h at 100 V and 250 mA current flow using transfer buffer that comprised 25 mM Tris (3.03 g), 192 mM glycine (14.4 g), 20% v/v methanol (200 ml and distilled water 800 ml) and a pH 8.5. Bio-ice unit (Bio-Rad, USA) was included to provide cooling during the transfer that was carried out with continuous stirring using a magnetic stirrer. After the transfer, non-specific binding sites on the membrane were blocked with blocking buffer (5% skimmed milk powder in PBS) for 2 h at room temperature with constant rocking or kept overnight at 4°C. Washing followed 3 times with PBS containing 0.05% Tween 20. Primary antibody (immune serum) was added at a dilution of 1 part immune serum to 100 parts PBS and incubated 45 min at room temperature as above to probe the blot. Washing was as described above. Secondary antibody (Horseradish peroxidase conjugated goat anti-rabbit IgG at a dilution of 1:8000 in PBS and incubated for 1 h at room temperature as above. Washing followed. Colour development to reveal positivity and specificity of the immune serum (antibody) was done by adding 10 ml of 0.01 M Tris-HCl containing 6 mg of diaminobenzitine (DAB) and 10 uL of 30% pre-mixed sodium peroxide (H_2O_2). Brown colour development took place within a few seconds and reaction was stopped by washing the membrane with distilled water. The development of well-defined brown bands on the nitrocellulose membrane was considered as positive.

RESULTS

Culture pigmentation of different *Fusarium* species

The isolates used in the study were successfully obtained mainly from diseased coffee plants. Each *Fusarium* species exhibited peculiar culture pigmentation (Figure 1) that was made; use of correct identification alongside spore morphologies (Booth, 1971). Different *Fusarium*

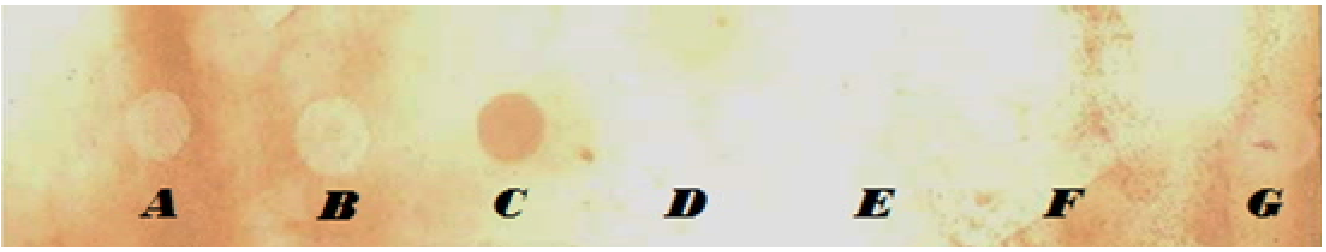

Figure 2. Antibody specificity testing by Dot blot analysis. A, *Fusarium oxysporum* extract spot; B, *Fusarium solani* extract spot; C, *Fusarium xylarioides* extract spot; D, *Fusarium lateritium* extract spot; E, *Fusarium decemcellulare* extract spot; F, *Fusarium moniliforme* extract spot; G, *Fusarium pallidoroseum* (*Semitectum*) antigen spot. The blot was developed with antiserum diluted 100 folds. The positive reaction were visualized by use of diaminobenzitine (DAB) solution (1.3 mM DAB in 0.01 M Tris-HCl containing 0.004% H_2O_2) as the substrate. The development of well-defined brown dots on the nitrocellulose membrane indicated positive reaction.

species produced mycelial mat with pigments peculiar to a species and of varying densities. *F. xylarioides, Fusarium solani* and *Fusarium oxysporum* produced soft mycelial mat and hence eased protein extraction. *Fusarium decemcellulare, Fusarium semitectum* and *Fusarium lateritium*, however, produced tougher mycelium that was more difficult to homogenize using both chilled mortar in liquid nitrogen and ultra-sonicator and produced less amount of protein compared to the soft mycelia.

Reactivity and specificity of the immune sera

Antisera that rose to soluble mycelial proteins or candidate proteins in selected bands of *F. xylarioides* were tested for cross reactivity with antigens from five other *Fusarium* species. Antisera of 50 and 66 kDa band proteins were dropped due to inconsistence in reactivity with different protein extraction batches and antibody titre being low resulting in weak bands and non-specific reactions. In contrast, antisera to proteins in the 27 kDa band reacted positively only with *F. xylarioides* isolates. Pre-immunization serum was included as a negative control. As expected, the pre-immune serum did not recognize any of the test antigens.

Dot blot analysis

This was done to quickly assess the production and specificity of antibodies. The antibodies rose against 27 kDa protein which recognized only the *F. xylarioides* spot and not any of the other *Fusarium* species as shown in Figure 2.

Immunoblotting (Western blot)

Western blot was performed to confirm the specificity of the immune sera. The results confirmed that polyclonal antibody raised against the 27 kDa antigen indeed recognizes only the target CWD pathogen, *F. xylarioides* (Figure 3B). Western blot analysis, therefore, revealed no cross-reactivity with non-target antigens of other *Fusarium* species.

Diagnostic potential of the 27 kDa antigen

Antigenic variations among *F. xylarioides* isolates was evaluated using anti -27 kDa protein anti-serum against eight *F. xylarioides* isolates taken from four farming agro-ecological systems in Uganda where CWD exists. The antiserum recognized all the isolates tested with prominent bands proving its potential to detect the pathogen from any part of the country (Figure 4).

DISCUSSION

The aim of the study was to identify and validate a diagnostic immunogen (antigen) from *F. xylarioides* mycelial extracts that can be used to generate specific polyclonal antibodies for the detection of the pathogen in test materials. Accurate and specific antigen to the pathogen has been identified. The potential of such immunodiagnostic tools to detect colonization of roots by several soil-borne fungal pathogens has been demonstrated (Priestley and Dever, 1993; Srivastava and Arora, 1997). This is the first report describing the use of a serological technique for the detection of *F. xylarioides.* Six *Fusarium* species common to coffee as saprophytes or true pathogen (Hakiza and Webesa, 1997; Serani, 2000) namely *F. xylarioides, F. solani, F. oxysporum, F. decemcellulare, Fusarium Palldoroseum*, and *F. Lateritium* identified according to Nirenberg (1976) and Booth, (1971) were used to identify the target antigen in this study. Soluble proteins, crude cell component, fungal homogenate, culture fluids, and ribosomal proteins from fungal pathogens have been used to raise antibodies (Jamaux and Spire, 1994; Priestley and Dever, 1993;

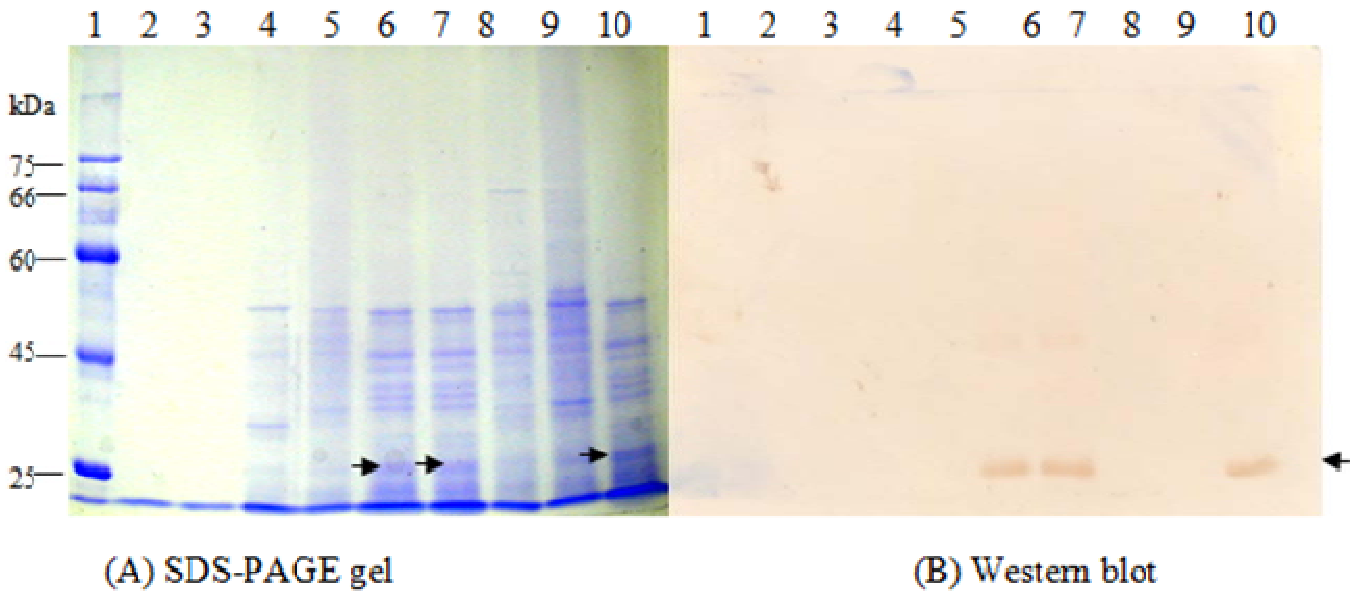

Figure 3. Antibody specificity confirmation by immunoblotting. Lane 1, molecular mass standard; lane 2 and 3, *Fusarium oxysporum*; lane 4, *Fusarium lateritium*; lane 5, *Fusarium pallidoroseum* (*semtectum*); lane 6, *Fusarium xylarioides*; lane 7, *Fusarium xylarioides*; lane 8, *Fusarium decencellulare*; lane 9, *Fusarium solani*; lane 10 *Fusarium xylarioides*. (B) SDS-Polyacrylamide electrophoresis and blotted. The blot was developed with antiserum diluted 100 - fold and conjugate 8000-fold. Column on the left listed the molecular masses obtained for the various bands. Arrowed is the 27 kDa protein (A) with its corresponding Western Blot (B).

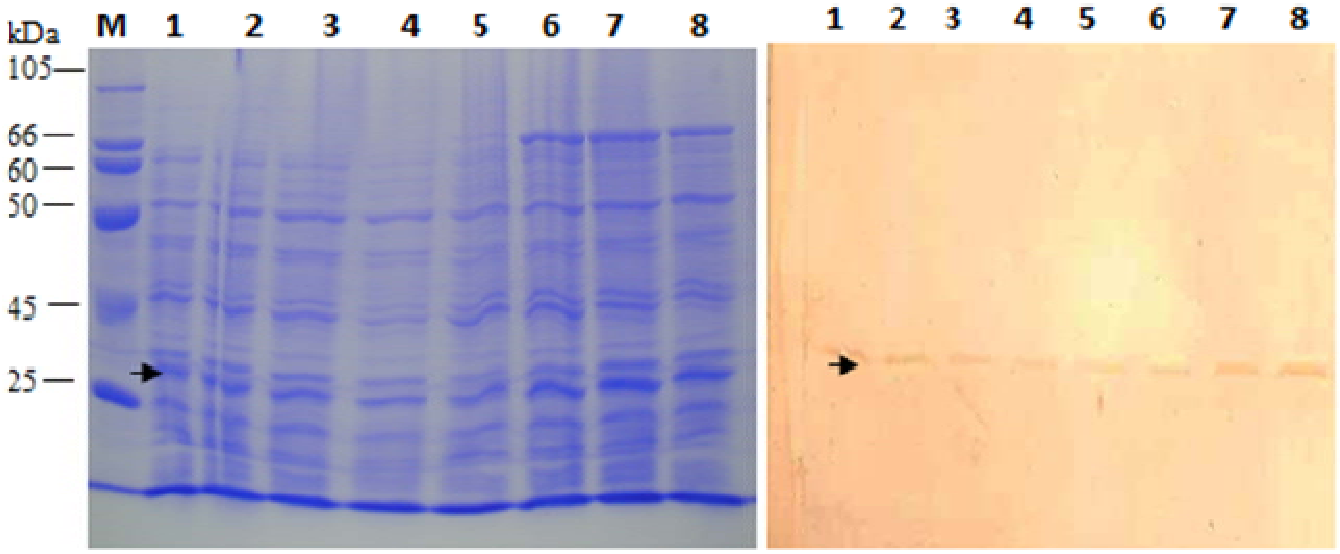

Figure 4. Recognition of a wide range of isolates by the anti- *F. xylarioides* 27 kDa band antiserum. Origin of different *Fusarium xylarioides* isolates. Lane M, Molecular marker; lane 1, Bundibugyo; lane 2, Mubende; lane 3, Mukono; lane 4, Kalangala; lane 5, Kamuli; lane 6, Rukungiri; lane 7, Kabarole; lane 8, Rakai. Samples were separated by SDS-polyacrylamide electrophoresis and blotted. The blot was developed with antiserum diluted 100-fold. Arrows indicate bands of apparent molecular mass 27 kDa (A) and corresponding blot (B).

Srivastava and Arora, 1997). In this study, part of the soluble mycelial extract was used as the antigen for antibody production. The result of analysis of the total mycelial extract from the six *Fusarium* species by SDS-PAGE enabled the identification of protein bands peculiar to *F. xylarioides*. The protein bands that appeared peculiar to the pathogen were those of molecular masses 27, 50 and 66 kDa. Protein bands of 50 and 66 kDa were

dropped from the study due to their inconsistency in reactivity with different protein extraction batches.

In addition, they induced low antibody titre and weak bands on western blots. The study therefore identified mycelial extract fraction of 27 kDa as the best antigen to induce antibodies for specific detection of *F. xylarioides*. The result is in agreement with a finding that electrophoresis of proteins can be a useful tool for identification and characterization of the genetic differences among *Fusarium* species (Bhuvanendra et al., 2010, Heidi et al., 2011). The work by Heidi et al. (2011), however, identified protein of 45.2 kDa as specific to *F. xylarioides* isolated from sugar beet and wheat. This variation in protein markers for the same species could be explained by observed variation in the pathogen races (Rutherford, 2006) host - race specificity (Adugna et al., 2005) or geographical area impact on the genome regardless of the host (Vitale et al., 2011). Since protein polymorphism has been noted to reflects the genetic background of the microorganisms (Shaw, 1965), those specific proteins could be linked to host - race specificity.

No attempt was made to further purify the protein by cross - absorption technique since some workers have reported that the procedure rarely improves the specificity of the antiserum raised to such protein (Srivastava and Arora, 1997) or even it specificity is improved, sensitivity is reduced (Jamaux and Spire, 1994). The specificity of the antiserum to the 27 kDa protein band was evaluated by dot blot and western blot analyses. The antiserum reacted strongly only with *F. xylarioides* and no cross-reaction with any test antigens was observed (Figure 3). The antibodies to this antigen, therefore, provide an ideal probe for the detection of the fungus that causes CWD. It is unlikely that the 27 kDa antigen was a simple protein but a glycoprotein since previous studies has demonstrated that fungal antigens that were specific have always been found to be glycoprotein (Deway et al., 1990). The work also indicated that immunization of rabbits with high-molecular-weight proteins induce antiserum that is non-specific and recognize all test antigens. A possible explanation for this is that the high-molecular-weight protein/glycoprotein molecules are immune-dominant and block the development of antibodies to specific molecules as suggested by Priestley and Dever (1993). Immunization of rabbits with a low-molecular-weight (<30 kDa) reduced considerably the number of cross-reacting antigens. The result indicates that the cross-reactive antigens lie mainly in the high-molecular-weight protein/glycoprotein fraction of mycelial extracts. The present study has produced a specific anti-27 kDa protein antiserum and provides a significant breakthrough in the development of immune-assay procedure for sensitive, specific and rapid diagno-sis of CWD. Species specificity of the anti-27 kDa protein antiserum, therefore, disagrees with earlier work done by some workers which indicated that polyclonal antibodies cannot differentiate between species and more so between *Fusarium* species (Srivastava and Arora, 1997); and that polyclonal antisera raised to crude mycelial extract of one species, have often been found to be generally genus-specific (Jamaux and Spire, 1994). Cloning and expressing the 27-kDa protein can undoubtedly improve the procedure of antigen preparation, thereby making it more cost-effective. Further studies will be concerned with the evaluation of its practical application for epidemiological studies.

The use of immunoassay for identification is based on specific epitopes carried on specific protein/glycoprotein. Fungal proteins/glycoprotein elicits an immune response when introduced into a higher animal, typically a rabbit, resulting in antibody production. Dot blot and Western blot analyses were used to evaluate the specificity of the antibodies produced in this study. The dot-immunobinding assay, a modification of the ELISA using a nitrocellulose membrane as a test matrix, was rapid, specific, sensitive and easy to perform for quick screening for no sophisticated electrical equipment is required as a positive reaction and which is observed by eye with reliability. With Western blotting analysis, some workers have used it to detect either antibodies or antigens and has been used successfully for immunodiagnostic of a variety of parasitic infections (Eamsobhana et al., 2004). The test has been noted as being more specific than the traditional colorimetric ELISA using crude antigens because the band pattern on the immunoblot can be interpreted as positive (reactive) or negative (non-reactive) (Eamsobhana et al., 2004) hence the specificity of the immune sera produced was evaluated with reliability.

For the 27 kDa protein to be considered as species-specific, its presence in all *F. xylarioides* isolates across the country was evaluated. Western blot analysis against 8 *F. xylarioides* isolates from four agro-ecological zones in Uganda where CWD exists using anti- *F. xylarioides* 27 kDa protein confirmed that the protein antigen is indeed species specific (Figure 4). There is therefore no antigenic variation with respect to the 27 kDa antigen in the pathogen. The result is in agreement with DNA characterization work by Janzac et al. (2004); Rutherford (2006) showing no interspecies variations in the pathogen causing CWD on Robusta coffee in Uganda, Tanzania and the DRC. This suggests that the antiserum can be used universally to detect CWD pathogen on Robusta coffee.

Conclusion

In conclusion, the species specificity of the anti- *F. xylarioides* 27 kDa protein is very encouraging for the production of a monospecific antiserum. On the basis of the result obtained in the present study, we can conclude that the 27 kDa *F. xylarioides* protein is species specific to CWD pathogen and is present in all isolates so far

studied in Uganda; and secondly, the higher the molecular mass of the protein, the less specific the antisera produced becomes. Development of monoclonal antibodies against the 27 kDa protein would enhance specificity, homogeneity and production of the antibodies. There is also need to develop double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for quick screening of large samples.

Conflict of Interest

The author(s) have not declared any conflict of interest.

ACKNOWLEDGEMENTS

We are grateful to Uganda Coffee Development Authority (UCDA) and Government of Uganda (NARO Development funds) for their financial support. We are grateful to the management of Molecular Biology Laboratory, College of Veterinary Medicine, Animal Resources and Biosecurity for offering the facility to do the laboratory work. We thank the District Agricultural officers and the coffee farmer groups for their cooperation during sample collection.

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