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Morphological and Molecular Identification and Characterization of Dry Bean Fungal Root Rot Pathogens in Zambia

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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF DRY BEAN FUNGAL ROOT ROT PATHOGENS IN ZAMBIA.

By

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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF DRY BEAN FUNGAL ROOT ROT PATHOGENS IN ZAMBIA.

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Dry bean is among the most important food legume crops for direct human consumption in Latin America and Africa. Recently, root and crown rot (RCR) has emerged as an important production constraint. Root and crown rot often involves fungal complexes. Thus, the straw, detached leaf, cup and stem tests were compared on their ability to detect the most common pathogens reported to be associated with RCR: *Fusarium solani, Fusarium oxysporum, Pythium ultimum, Rhizoctonia solani* and *Macrophomina phaseolina.* Significant differences ($P<0.001$ at 0.05 significance) among the methods in detecting pathogenicity were observed. The straw test detected pathogenicity in all four of the test pathogens, was relatively easy to perform and required less.

Next Generation Sequencing (NGS), conventional Polymerase Chain Reaction (PCR), and classical fungal culturing methods were compared in identification of the primary pathogens causing RCR of dry bean in Zambia. Analysis of DNA from plant tissue and ground tissue extracts spotted on FTA cards by NGS identified *F. oxysporum, F. solani* and other species of the *Fusarium* complex as the most abundant reads and Operation

Taxonomic Units (OTU's). *Fusarium* spp. were also detected in over 70% of the samples analyzed by conventional PCR using specific primers and also had the highest frequency of recovery (>0.8) of which over 90% were pathogenic. Thus it appears that, in Zambia, the *Fusarium* complex causes RCR in dry bean. Significant correlations at 0.05 level and high intermethod agreements >0.7 observed between plant tissue and FTA cards support the hypothesis that FTA cards can substitute for sampling plant tissue and preserving DNA for RCR pathogen identification. Significant correlations also were observed among the different methods of identification of primary pathogens responsible for RCR where *F. oxysporum* is the most likely target for initial screening for resistance in Zambia.

Identification of a primary pathogen associated with RCR of dry bean in Zambia provides evidence to direct intial breeding for RCR resistance to breeding for resistance to *Fusarium oxysporum*.

DEDICATION

To my family for their unconditional love and supporting me. Without you guys I wouldn’t be the person I am today.

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LITERATURE REVIEW

Zambia

Zambia is a land-locked country in sub-Saharan Africa. It is bordered by eight countries. To the North and North West are Democratic Republic of Congo and Tanzania respectively, Malawi and Mozambique to the east, Zimbabwe and Botswana to the south, and Angola and Namibia to the west and south west, respectively. Zambia lies between latitude 8°S and 18° S and longitudes 22°E and 35° E. It has a total area of about 750,000 km^2 divided into 10 provinces and 74 districts. Zambia's population has been estimated at 14.5 million (Central Statistical Office *et al.*, 2014, Zambia development agency and Ministry of agriculture and livestock, 2011)

The four key economic sectors in Zambia are mining, manufacturing, tourism and agriculture. The mining sector is the largest contributor to foreign exchange and has been the backbone of the country's economy since its independence. In 2005, Zambia investment reported that mining contributed about 65% of export earnings and projected its contribution to the gross domestic product (GDP) to grow to USD 1.35 billion by 2015. In an effort to diversify the economy, and meet some of the country's millennium development goals, the government recently increased its focus on agriculture.

Agriculture

Zambia has a large land resource base of approximately 75 million hectares of which only 1.5 million hectares are cultivated every year (Zambia development agency and Ministry of agriculture and livestock, 2011). Although 58% of the land in Zambia is classified as medium to high potential for agriculture, only about 14% of the land is

cultivated (Bigsten and Tengstam, 2008). This leaves a huge amount of fertile land that is not used but has potential for crop production.

Zambia has a mixed economy consisting of a rural agricultural sector and a modern urban sector along the line of rail. Currently, construction contributes 14% of the gross domestic product (GDP), agriculture contributes 9% of the GDP, manufacturing and mining each contribute 8% of the GDP (Central Statistical Office *et al.*, 2014). The Agriculture sector employs some 67% of the total labor force and supplies raw materials to agricultural industries, which account for 84% of manufacturing in the country (Ezekwesili *et al.*, 2009).

Zambia is divided into three major agro-ecological zones. The zones have been formed based upon the amount of rainfall that each area receives. The amount of rain received in an area has influenced the adaptation of certain crops in specific areas since most of the crops grown in the country are rain fed.

Agro-ecological region I encompasses the southern and eastern river valleys of the country and is characterized by low rainfall of less than 700 mm annually. Predominant agriculture activities in the region include production of bulrush millet (*Pennisetum glaucum*), sorghum ((*Sorghum bicolor*), and livestock (Aregheore, 2009).

Agro-ecological region II encompasses most of the western, southern, part of central and part of eastern regions of the country. It receives mean annual rainfall between 800 to 1,000 mm and is split into two subsections. Region IIa which constitutes the central plateau and region IIb which covers the western plateau. Major crops grown include sorghum, maize (*Zea mays*), groundnuts (*Arachis hypogaea*), cowpeas (*Vigna*

unguiculata), tobacco (*Nicotiana tabacum*), sunflower (*Helianthus*), irrigated wheat (*Triticum aestivum*) soybean (*Glycine max*), rice (*Oryza sativa*), bambara nuts (*Vigna subterranean*) bulrush millet, horticultural crops and a range of other cash crops (Aregheore, 2009).

Agro-ecological region III constitutes areas receiving mean annual rainfall above 1000 mm, usually up to 1500 mm. This is a high rainfall region and most of the soils are leached and acidic. This region includes the northwest, part of central and the northern parts of the country. The major crops grown in this region include finger millet (*Eleusine coracana*), bean (*Phaseolus vulgaris*) and cassava (*Manihot esculenta*). Cash crops include maize, sunflower, coffee (*Coffea Arabica*), tea (*Camellia sinensis*), tobacco, irrigated wheat and soybean (Aregheore, 2009).

Zambian agriculture is characterized by three major categories of farmers; small, medium and large-scale. According to the 2009 World Bank report, there are 1,145,829 smallholder households growing crops in Zambia and about 1,500 large-scale commercial farmers. Of the smallholder households, 96% are classified as small-scale farmers with holdings of 5 hectares or less. The rest are medium-scale farmers with holdings of 5-20 hectares (Ezekwesili *et al.*, 2009)

The majority of agricultural production in Zambia is smallholder production. Crop production can be looked at in term of yields or area under cultivation. In most cases, a country's staple accounts for the highest area under production. Zambia is not an exception to this as maize is the staple and accounts for about 40 percent of cultivated land and contributes about 40 percent to the country's agricultural GDP (Ezekwesili *et al.*, 2009). The crop forecast surveys conducted by the Ministry of Agriculture and

Livestock for 2009/2010 and 2010/2011 seasons, showed the top five crops in Zambia in terms of area under cultivation and volume were maize, cassava, groundnuts, cotton and mixed bean. Although counted among the top-five crops produced in Zambia, mixed bean are relatively minor compared to maize and cassava, but with the appropriate policy environment, mixed bean may be elevated as a cash crop in certain parts of the country to contribute significantly to poverty reduction and food security (Mwansa, 2013).

Dry bean

Dry bean (*Phaseolus vulgaris* L.) is an important food legume and has been referred to as the most important legume or pulse crop for direct human consumption in the world (Akibode and Maredia, 2011). Dry bean is very versatile in its adaptation and growth. It is produced in a range of crop systems and environments in regions as diverse as Latin America, Africa, the Middle East, China, Europe, the United States, and Canada (Jones, 1999). Its centers of origin are Central and South America. The centers of origin for the crop have been classified as the Mesoamerican (Mexico and Central America) and the Andean gene pool in South America (Singh, 1999; islam *et al.,* 2002). Generally accepted archeological evidence for dry bean indicates that beans were domesticated more than 6,900 years before the present (BP) in Middle America and about 7600 years BP in the Andean region (Singh, 1999).

Dry bean was introduced to Africa through Mozambique by Portuguese traders around the 16th century (CIAT, 2001; Greenway, 1945). To date, more than 40,000 varieties of common bean are recorded (Jones, 1999) and the major repository and distributor is the International Center for Tropical Agriculture. (CIAT) in Cali, Colombia (Duke, 1983). In

2010, global bean production was approximately 23,816,123 ton, with 24.4 and 17.7% of the world production in Latin America and Africa, respectively (FAO, 2014).

In Africa, beans are grown on more than four million hectares annually and provide dietary protein for over 100 million people in rural and poor urban communities (Buruchara *et al.,* 2011). Although most production and consumption of dry beans is in Latin America, the highest annual per capita bean consumption is in Eastern Africa where it has been estimated at 50 - 60 kg person^{-1} (ISAR, 2011).

In Africa, beans are consumed at various stages of plant development, and thus, offer a food supply over a long period of time. The parts of the plants eaten include the leaves, green pods, fresh grain, as well as dry grain. Beans also have many health benefits including a low glycemic index, being rich in protein (about 22%) and providing a good source of iron and zinc (both of which are key elements for mental development) and fiber (Buruchara *et al.,* 2011). Furthermore, bean consumption reportedly reduces the risk of developing colon and breast cancer, and heart diseases (Shawn McGuire and Louise Sperling, 2011).

Bean production in Zambia

Production of dry bean in Zambia, like most of Sub-Saharan Africa, is done by small-scale farmers. These resource-poor farmers have little or no farm inputs and usually do not adhere to good agronomic practices. This, coupled with disease/pest susceptible local varieties, and low soil fertility, leads to very poor yields. The average yields of local cultivars are in the range of 0.30 to 0.50 ton ha^{-1}. Improved varieties with an acceptable seed size, good color and taste, yield potential of up to 2.0 ton ha^{-1} and some resistance to

most common pests and diseases have been developed, but seed is scarce and in most cases expensive beyond the reach of most smallholder producers in Zambia (Ministry of Agriculture Food and fisheries Information Pack, 2000)

Beans are grown/cultivated in all the provinces in Zambia, however, the major growing areas are Northern, Northwestern, Muchinga, Eastern and Luapula Provinces (Mwansa, 2013). The crop forecast survey of the season 2010/2011 reported the total number of households growing beans as 190,000 with 71,544 ha planted and 68,239 ha harvested. The Northern Province accounted for more than 60% of the total households growing beans, over 70% of the area cultivated and harvested, and over 72% of the expected production and sales (Crop Forecast Survey Report, 2010/2011). Dry bean in Zambia is grown as a mixture of landraces and improved varieties. The most commonly grown are the bush type. Shawn McGuire and Louise Sperling (2014) have reported local markets and recycled seed as the major seed sources of most subsistent farmers in Africa.

Importance of bean

Dry bean is considered to be a very important food legume crop mainly due to its nutritional value. It plays an important dietary role supplying; proteins, carbohydrates, essential elements and vitamins to both rural and urban households. It is estimated that the crop meets more than 50% of dietary protein requirements of households in Sub-Saharan Africa (SSA) (Broughton et al., 2003; Wortmann .C., Kirkby, A. R., Eledu, A. C., and Allan J. D. 2004) and thus compliments the staple crops such as maize, and other crops that are rich in carbohydrates like cassava and cereals. At levels of consumption commonly found in people of restricted economic means (15–20 $kg^{1}yr^{-1}$.), beans provide10–20% of the adult requirement for a number of nutrients (Broughton *et al.*,

2003). Most mineral elements in cereals are lost during processing, but for crops that are eaten whole like bean, they are retained. With the rising interest in iron(Fe) rich foods, especially for people who are immunocompromised, beans are an important source of iron, phosphorus(P), magnesium(Mg), manganese(Mn), and to a lesser degree, zinc(Zn), copper(Cu) and calcium(Ca). This becomes especially important for pregnant women and more so in areas such as Zambia where the rates of HIV/AIDS are high.

In Zambia's major bean growing areas, beans can be marketed early compared to other crops and thus act as bridging source of income before the main crops mature and can also be sold at different stages such as green leaves, fresh pods and dry grain (Broughton *et al.*, 2003). Beans in some parts of Zambia are even considered a high income crop relative to maize especially when yields are high (e.g. 3 ton ha^{-1}). Bean are increasingly becoming an important source of income, in some cases for up to 45% of the households (Broughton *et al.*, 2003).

The most economic part of beans is the dry grain. Most households in the major bean producing areas in Zambia sell part of their beans after harvest especially in Northern and Northwestern Provinces. Producers receive an average price of about K3.9 (USD 0.39) kg^{-1} dry bean grain. Most of the households that produce bean do not have a ready market in their resident villages and sell their produce at the farm gate, accounting for 65.5% of the transactions (Hamazakaza *et al.*, 2014). Hamazakaza *et al*., (2014) reported that only 14.5% of the households have a market within their village and that middle men, usually with 20 brokers, account for 52% of the purchases, followed by consumers 31.4% and then urban wholesalers 13.9%.

Bean Production Constraints

Root and crown rots of dry bean

Dry beans are affected by biotic and abiotic factors which limit their production. Biotic factors include field and post-harvest pests and diseases while abiotic factors include drought, excessive rain/flooding, poor soil fertility, heat and cold stress. Disease and insect infestations as well as weather-related production constraints are becoming more frequent and increasing with change in climate (Hamazakaza *et al.*, 2014, Allen *et al.*, 1989). In Africa, significant yield losses have been reported and attributed to each of these constraints. For example, Wortmann et al. (1998) estimated that yield loss due to drought, N deficiency and P deficiency were 396,000 ton 389,900 ton and 355,900 ton respectively; while losses due to angular leaf spot, anthracnose and bean stem maggot were 384,200 ton, 328,000 ton and 297,100 ton, respectively (Buruchara *et al.*, 2011). Yield losses due to individual soil-borne pathogens have been difficult to ascertain, however data attributed to a complex of pathogens estimated over time amounts to 400,000 tons per year lost to root rots for Eastern, Central and Sub-Saharan Africa altogether (Wortmann, 1998)

The most important soil-borne disease of dry bean is a disease complex referred to as Root and Crown Rot (RCR). The main soil-borne pathogen groups that have been associated with RCR in dry bean production include *Fusarium solani*, *Fusarium oxysporum*, *Pythium ultimum*, *Rhizoctonia solani* (Scandiani *et al.*, 2011) and *Macrophomina phaseolina* (Clare *et al.*, 2010, Kerr, 1963). In dry bean, these soil-borne pathogens are responsible for Fusarium root rot (FRR) Fusarium yellows/wilt, Pythium

seed rot/damping off, Rhizoctonia root rot and Macrophomina charcoal rot. The severity of RCR is a factor of the environment, soil conditions and the density and species of pathogens present. Because RCR affects the roots, crown and vascular bundle of the plants, they indirectly affect the uptake and efficient use of nutrients (Allen *et al.*, 1989) leaving the plant weak and more susceptible to other stress factors. The opposite is also true, where plants are stressed due to drought and extreme temperatures they become weak and are more vulnerable to RCR.

Fusarium root rot

The genus *Fusarium* was defined by Link in 1809, and is now in its third century as a genus that contains many plant-pathogenic fungi. The members of this genus can directly incite diseases in plants, humans, and domesticated animals (Leslie *et al.*, 2006). Fusarium root rot (FRR), caused by the fungus *Fusarium solani* f. sp. *phaseoli* (Fsp) W.C. Snyder & H.N. Hansen, has a cosmopolitan distribution; it is found wherever dry beans are grown. It is one of the most widely distributed diseases of dry beans in Latin America, where most of the production and consumption is concentrated (Nicoli *et al.*, 2012). In Africa, it has been reported in Kenya and Malawi, and undoubtedly occurs in other countries as well (Abawi and Pastor-Corrales, 1990).

Infected plants are characteristically stunted, chlorotic and may defoliate prematurely. Severe infections also lead to reduced seed size and number of pods per plant. The symptoms are initially characterized by longitudinal, narrow, bright-red streaks on hypocotyl and taproot surfaces; infected areas become reddish brown, lack definite margins, remain superficial, and may exhibit longitudinal fissures (Schwartz and pastor-Corales, 1989).

Fusarium root rot pathogens survive between crops as thick-walled chlamydospores in the soil and may also survive by colonizing non-host plant root surfaces without causing disease. Therefore, chlamydospores and colonized non-host plant roots are the two major sources of initial inoculum in fields (Schwartz and pastor-Corales, 1989). When host plants begin to grow, the root exudates trigger chlamydospore germination and the production of hyphae of the pathogen. The hyphae either directly penetrate the dry bean tissue or enter through openings usually caused by the growth of roots in the soil or the cracks at the point of emergence of lateral roots (Harveson *et al.*, 2005).

Like most soil-borne pathogens *Fusarium solani* has a monocyclic life cycle and is dispersed mainly through the movement of soil or infected tissue by water, wind, machinery, as well as humans and animals. Contaminated seed has also been shown to be a mode of dispersal for this pathogen. Fusarium root rot severity is exacerbated by any condition that causes stress on the growth of bean plants in the soil or encourages inoculum build up. Therefore, cropping history, plant spacing, and stress factors such as drought, soil compaction, or flooding (causing oxygen deprivation) and parasitic pathogenic micro-organisms such insects and nematodes affecting the roots make the disease severe (Leslie *et al.*, 2006).

Fusarium wilt/yellows

Fusarium wilt on dry bean is caused by the pathogen *Fusarium oxysporum* (Schlecht.) f. sp. *phaseoli* Kendrick & Snyder. This disease is common and most prevalent in areas where high temperatures and drought persists for part of the growing season. It has been reported in many countries of Latin America and Africa, especially in Kenya, Malawi, and South Africa (Abawi and Pastor-Corrales, 1990).

Typical foliar symptoms are yellowing and wilting of older leaves which then proceeds to younger leaves if the disease progresses. Severely affected plants may wilt permanently. Vascular discoloration of roots and hypocotyl tissues is the primary diagnostic symptom of the disease on dry bean. Just like Fusarium root rot, Fusarium wilt is also favored by high temperature, stress (>86°F) and soil compaction and any condition that puts stress on the plant root development.

The pathogen survives in the soil as thick-walled chlamydospores which would then germinate and initiate disease when susceptible hosts are planted. The pathogen usually attacks near the root tips and penetration may occur through wounds and/or natural openings on the roots and stems (Schwartz and Pastor-Corrales, 1989). Once inside the plant the pathogen colonizes the vascular bundle and grows into the xylem where it proliferates and produces both macro- and microspores. Chlamydospores are also produced in infected tissue. The spores typically produce the red-brown diagnostic color in the vascular system. Dissemination or dispersal of the pathogen occurs by movement of infected or colonized tissue and soil by water, wind, farm equipment or animals.

Rhizoctonia root and crown rot of dry bean

Rhizoctonia solani Kühn is a soil-borne fungus that causes disease on many economically important crop plants worldwide. Strains of the fungus are traditionally grouped into genetically isolated anastomosis groups (AGs) based primarily on hyphal anastomosis reactions, and are further subdivided into intraspecific groups (ISGs) (Bolton *et al.*, 2010).

Rhizoctonia solani, was originally described on potato by Julius Kühn in 1858. It is a basidiomycete fungus that does not produce asexual spores and only occasionally will the fungus produce sexual spores (Ogoshi *et al.*,1991). In nature, *R. solani* reproduces asexually and exists primarily as vegetative mycelia and/or sclerotia. *Rhizoctonia solani* is a species complex with a wide host range and wide subspecies variability (Godoy-Lutz *et al*., 2008). At least 15 AGs exist within the species complex (Bolton *et al.*, 2010) which causes major losses in host crops such as cotton, sugar beet, potatoes and dry bean. In dry bean, *R. solani* is responsible for damping off, root and crown blight/rot in its anamorph stage but also causes web blight in its teleomorph stage under favorable environmental conditions (Godoy-Lutz *et al*., 2008). Sikora et al (2004) reported that *R. solani* is one of the most economically important root and hypocotyl diseases in the world.

Symptoms of the disease begins as small, circular or linear sunken lesions with reddish-brown borders on the hypocotyl and roots of young bean plants. Cankers may enlarge with age and may retard normal plant growth by encircling hypocotyls. Severe infections cause stunting and premature death. Infection by the fungus happens when susceptible plants grow and trigger activation of the fungus surviving as mycelia in plant debris or sclerotia in the top layers of the soil. Individual hyphae will penetrate the plant through natural openings and wounds or through the production of infection pegs on the plant cuticle (Schwartz and Pastor-Corrales, 1989. Once plants are infected, the pathogen can proceed from plant to plant down rows. Occasionally the pathogen will enter and destroy the pith, resulting in a brick-red discoloration inside the stem at the soil line which is

characteristic of *R. solani* infections. Greyish white mycelia can also be found in the pith of dead plants (Ogoshi., 1987)

Once a field becomes infested with *R. solani*, it will remain infested indefinitely. Inoculum concentration will increase if susceptible crops, including bean, potato, and sugerbeat are continually cropped. Some weedy plants are also susceptible hosts and increase inoculum in the soils. Disease in young seedlings is favored by high to moderate levels of soil moisture and cool soils. Damage is often restricted to seedlings, but also can affect older plants if stressed by temperature extremes combined with irrigation water. As with the other root rot pathogens, soil compaction and other root stressing events can increase disease severity (Schwarrtz *et al.*, 2005).

Charcoal rot/Ashy stem blight

Macrophomina phaseolina (Tassi) Goidanich (1947) causes seedling blight, root rot and stem rot of more than 500 cultivated and wild plant species (Srivastava *et al.*, 2001) including dry bean (You *et al.*, 2011). This pathogen is a problem in North and South America including the Dominican Republic and Puerto Rico (Campo-Arana, R. and R.Echavez-Badel. 2001; You *et al.*, 2011). In Africa, it has been reported in Sudan, Tanzania, Zambia, Kenya and Ethiopia (Abawi, Pastor-Corrales, 1990).

Charcoal rot/ashy stem blight is favored by dry conditions and water stress although the disease has also been found under humid tropical conditions. The *M. phaseolina* hyphae initially invade the cortical tissue of plants, followed by sclerotia formation, causing stem rot (Islam *et al.*, 2012) . Gray-black mycelia and sclerotia are produced and the infected area exhibits disease symptoms. Symptoms of the disease in the field when heavily

infested are leaf chlorosis, defoliation and wilting. Initial symptoms on the plants start as dark irregular lesions of different sizes on the cotyledons. The black sunken lesions then expand to stem tissue and move upward and downward as the plant grows until they reach the growing point and kill the plant or until the stem becomes weakened and breaks (Schwartz and Pastor-Corrales, 1989). Infected plants have a pale, ash-colored, dry rot on the stem. Adult plant stem tissues show the growth of numerous microsclerotia and pycnidia which look like small black dots especially in the dead areas. In adult plants, wilting and blockage of the vascular system occurs with production of black or grey microsclerotia (Abawi and Pastor-Corrales, 1990). The pathogen survives in and on plant debris and loosely in the soil as microsclerotia and is dispersed through movement of infected plant debris, soil and seed. (Khan, 2007, Otsyula *et al.*, 2003).

Pythium root rot.

Bean root rot, caused by *Pythium* spp. is one of the most destructive diseases affecting dry bean in east and central Africa where they are grown in intensive agricultural production systems. In such areas, complete yield loss usually occurs when susceptible varieties are planted and the environmental conditions are favorable for pathogen development (Abawi and Pastor-Corrales, 1990).

Pythium root rot symptoms may appear as seed rot, damping-off, root rot, foliar blight or pod rot. Pre- and post-emergence damping-off happens when seedlings are killed shortly after planting or immediately after germination, respectively. Seedlings that escape damping-off but are still heavily infected show extreme stunting, chlorosis and wilting (Abawi and Pastor-Corrales, 1990; Schwartz and Pastor-Corrales, 1989). Infected roots are severely reduced in mass, discolored and show severe rotting and decay. Initial

infection symptoms appear as elongated, water-soaked areas on root and lower stem tissues. Infected tissues become soft, brownish, and somewhat sunken and eventually collapse causing plant wilt and death (Otsyula *et al.*, 2003). *Pythium* spp. belong to a group of fungi-like organisms called Oomycetes and belong to the kingdom chromalveolata (Marano *et al*., 2012; Simpson *et al.*, 2004) with a unique evolutionary line distant from true fungi. *Pythium* spp. form oospores that are thick-walled and survive for long periods in the soil and in plant debris. This is usually the initial inoculum for outbreaks. Like other root rot pathogens, *Pythium* spp. are disseminated through movement of soil and plant debris by water in irrigation systems or machinery (Schwarrtz *et al.*, 2005).

Morphological and molecular identification of fungi

Morphological Identification

Identification of an organism is key to its classification and taxonomy. In the past, fungi and other organisms have been identified based on their morphological characteristics. The Saccardo system was primarily based on morphology of sporulation structures as they are known in nature as well as the morphology and pigmentation of conidia and conidiophores (Barnett and Hunter, 1972). The morphological features used to identify fungi may vary from organism to organism but largely depend on their characteristics in culture. For example, in order to have a good culture to use for identifications, certain media has to be used for sporulation and specific diurnal temperature and lighting regimes need to be followed, especially for the *Fusarium* species (Leslie *et al.*, 2006). *Fusarium* isolates are grown on carnation leaf agar (CNL) for the study of size and shape

parameters of their spores and spore bearing structures. (Burgess *et al.*, 1991). CLA promotes the growth of more uniform spores that can then be measured and also compared to standard values for identification. The most distinct characteristic of *Fusarium* species is the banana shape of their spores. The spores are borne on conidiophores as a bunch loosely or tightly held together. For production of chlamydospores, a soil broth media is favored over other media (Bennett and Davis, 2013, Leslie *et al.*, 2006). Identification of *Fusarium* species by colony pigment color is done on PDA (Leslie *et al.*, 2006, Cordova-Albores *et al.*, 2016). Although *Fusarium* species have distinct colors on PDA, care has to be taken when using this parameter for identification. A change in lighting regime, temperature, or multiple transfers can affect the color of an isolate (Leslie *et al.*, 2006). Therefore, colony color is mostly used for preliminary classifications of the *Fusarium* isolates (Cordova-Albores *et al.*, 2016). Other fungi species associated with RCR have different morphological characteristics by which they are identified. *Rhizoctonia solani* does not produce spores, however it produces micro/macrosclerotia as its resting or survival structures. Sclerotia are made from compaction of specialized hyphae called monoloid cells. *R. solani* species are usually morphologically distinguished by the color of their mycelial mat, and the color, size and abundance of sclerotia. On PDA, *R. solani* forms buff to brown mycelial mats. The color of sclerotia varies from brown to dark brown and the abundance of sclerotia depends on the AG group (Lakshman *et al.*, 2016). *Macrophomina phaseolina* isolates on PDA are characteristically grey to black in color. They produce sclerotia from specialized highly compressed hyphae cells called monoloids just like *R. solani.* Some isolates produce pycnidia on specific media. The color of the mycelial mat, and the size and color of

sclerotia are also used to morphologically identify *M. phaseolina.*(Beas-Fernández *et al.*, 2006, Aboshosha *et al.*, 2007). *Pythium* species are typically morphologically identified by features such as the presence of sexual reproductive structures (homothallic or heterothallic), the character of oogonia (smooth or ornamental), oospores (plerotic or aplerotic) and antheridia, and the type of sporangial morphology (spherical, filamentous or lobulated) (Nzungize *et al.*, 2011, Matsumoto *et al.*, 1999).

However, new techniques of DNA analysis have now been developed for quicker and more specific identification of fungi.

Molecular identification

In the past two decades, several techniques have been developed for molecular identification and quantification of organisms (Paplomatas, 2004). The choice of a technique to use usually depends on the research question that one is trying to answer (Pereira *et al.*, 2008). For most researchers where the question is detecting the presence or absence of a specific organism in a sample, conventional Polymerase Chain Reaction (PCR) is used with specific oligonucleotide probes to detect the organism of interest (White *et al.*, 1990) . The application of PCR is also widely used in diagnostic labs and clinics in both plant and medical sciences (Beckmann, 1988, Reischl and Lohmann, 1997, Cooley, 1992). PCR based identification have also been widely used in the identification of RCR pathogens (Aboshosha *et al.*, 2007, Mwang'ombe *et al.*, 2008, Zitnick-Anderson and Nelson Jr, 2015, Lakshman *et al.*, 2016). Sanger sequencing is another common molecular method that is usually employed when a deeper analysis of DNA is required (Pereira *et al.*, 2008). Sanger sequencing of DNA enables analysis of DNA for several projects such as identification of homologous genes across species or identification of

mutations or merely identification of individual species for determination of relationships among organisms. For fungi, universal primers such as the those targeting the ITS region of rRNA are used (White *et al.*, 1990). Sanger sequencing has been an important tool also in the study of pathogen populations and prevalence (Sutton *et al.*, 2006, Bardgett, 2010, Jana *et al.*, 2005) and the advancement of the molecular species concept. Identification of species in Sanger sequencing is achieved by performing a BLAST search of the sequence obtained in vast GenBank sequence database. A step from Sanger sequencing is Next Generation Sequencing (NGS). Two of the most common platforms used for NGS are Roche 454 and Illumina. 454 sequencing technology involves pyrosequencing in high-density picoliter reactors, and Illumina involves sequencing by synthesis of single-molecule arrays with reversible terminators (Morozova and Marra, 2008, Schuster, 2007). This technology has many applications in both medical and plant sciences from ecological studies of forest soils, agriculture fields to the study of microbiota in human or animal guts (Suhr *et al.*, 2016, Wang *et al.*, 2012, İnceoğlu *et al.*, 2011). Although much study has been done with NGS in ecology and environmental studies on classification and quantification of organisms, studies of identification of predominant pathogenic fungi/oomycete species are still few. NGS is thus a very promising technology that can be used in this field (Amend *et al.*, 2010).

DNA storage and transportation (FTA® cards)

For accurate identification of pathogens using molecular techniques requires quality DNA. Therefore, how the DNA is extracted, transported and stored is very important. For most pathogens infecting plants, their necrotrophic stage usually results in formation of lesions on the plants. This can be seen in the form of spots on leaves or the stem, necrotic

sunken lesions or cankers on roots stem fruit or pods. The advancing margin of the lesion or the interface of the healthy and diseased tissue is usually where the pathogen is actively surviving (Jousset *et al.*, 2011, van West *et al.*, 2003, Paplomatas, 2004, Li-Jun Ma *et al.*, 2013). Preservation of this interface on intact tissue is one way the DNA of the pathogen can be preserved and transported for extraction and analysis later. Most isolation of fungi are recovered from the interface of diseased and healthy tissue (Li-Jun Ma *et al.*, 2013). However, handling plant tissue in this manner could introduce pathogens to new areas and also may be cumbersome due to quarantine requirements where special permits are required. Flinders Technology Associates (FTA) cards have been developed where an organism's DNA can be imbedded in a matrix, stored, and recovered when needed. This technology was initially developed for use in animal and human medical sciences has also found growing applications in plant sciences and plant pathology (Liang *et al.*, 2014, Muthukrishnan *et al.*, 2008, Borman *et al.*, 2006, Ndunguru *et al.*, 2005). The method of storage and transportation of DNA of FTA® cards offer an alternative to plant tissue. In plant pathology most of the pioneering studies on FTA® cards as DNA storage and transportation media for molecular work has been on virus pathogens (Ndunguru *et al.*, 2005).

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CHAPTER 1

Comparison of Pathogenicity Testing Methods

Abstract

Root and crown rot disease (RCR) is a major constraint of dry bean production in Africa and the Americas. The main pathogens that have been associated with RCR of dry bean are *Fusarium solani, Fusarium oxysporum*, *Pythium ultimum, Rhizoctonia solani* and *Macrophomina phaseolina*, among others. Determining pathogenicity of each of the potential fungal and oomycete isolates of the root and crown rot complex requires the use of specific methods. Current methods involve performing multiple individual isolate tests which are time consuming and laborious. A single method could reduce the time and resources needed to test for pathogenicity. Detached leaf, stem, cup and straw tests were compared on their ability to detect pathogenicity of four major RCR dry bean pathogen groups. Significant differences were found among the four pathogenicity testing methods evaluated ($P<0.001$ at 0.05 significance). The straw test identified all four genera as pathogenic. The 100% disease incidence and highest mean disease damage score of 5.8 in the straw test for all the four genera tested support that the straw test as a single method can separate pathogenic from non-pathogenic fungi and oomycetes associated with RCR of dry bean.

1. Introduction

Pathogenicity refers to the ability of an organism to cause disease. The most important soil-borne disease of dry bean (*Phaseolus vulgaris* L.) is a disease complex referred to as Root and Crown Rot (RCR). The primary soil-borne pathogens associated with symptoms of RCR are *Fusarium solani*, *Fusarium oxysporum*, *Pythium ultimum*, and *Rhizoctonia solani* (Scandiani *et al.*, 2011, Kerr, 1963, Abawi and Pastor-Corrales, 1990, Clare *et al.*, 2010).

To identify the pathogens causing root rots, rhizosphere organisms have to be isolated from the infected tissue, cultured, and Koch's postulates completed. Isolation of the soil-borne pathogens from infected tissue or from soil may results in a mixture of fungi that are pathogenic and non-pathogenic. The pathogenic fungi infect plant tissue and cause lesions while most of the non-pathogenic fungi can live as saprophytes on the plant, in the soil or in root/crown tissue after pathogens degrade the tissue. To identify the pathogens causing RCR, the isolated organisms have to be tested for pathogenicity.

Disease resistance screening methods for fungal and oomycete root rots have been developed, but there is no published information on the best method to use for pathogenicity determination of fungal/oomycetes associated with RCR of dry beans (Bilgi *et al*, 2008; Kull *et al*, 2003). Published screening methods for disease resistance are mostly species or genus specific. Furthermore, these disease resistance screening methods cannot be applied to a broad range of fungal/oomycete pathogens from different genera that may or may not produce spores. For this reason, most comparisons of pathogenicity testing methods have been concentrated within specific genera. Some

pathogenicity testing methods have been adapted from one pathogen to another but since most of the tests are developed for screening for disease resistance, they are time consuming and may require specialized environment and or equipment (Chaudhary *et al.*, 2006; Scandiani *et al*, 2011; Pratt *et al.*, 1998). The main objectives of this study are: (1) to determine a quick and simple method of differentiating fungal/oomycete pathogens from saprophytes involved in the RCR complex of dry bean, (2) to determine whether the method found in objective 1 could also separate differences/variability in aggressiveness or virulence among isolates of the same pathogen.

1. Materials and Methods

1.1 Experiment 1.

1.1.1 Selection of pathogenicity testing methods

Three methods used to measure aggressiveness of a fungal nectrotroph and one used to screen dry bean root rot were tested on *Fusarium solani*, *Rhizoctonia*, *solani*, *Pythium* spp. and *Macrophomina phaseolina*. The four methods were the stem test (Kull *et al.*, 2003), the straw test (Otto-Hanson *et al.*, 2009, Zhao *et al.*, 2004), the detached leaf test (Pettitt *et al.*, 2011) and the cup/modified inoculum layering test (Schneider and Kelly, 2000, Peña *et al.*, 2013). For each of the methods, preliminary testing was done for at least one of the pathogens to determine the level of inoculum needed. For the cup test, vermmiculite was selected as the planting media because of the ease of removal from plant roots when evaluating damage.

1.1.2 Isolate Genera

All the isolates used were originally derived from infected dry bean plant root/crown tissue (Table 1.1). *Macrophomina phaseolina* was collected from USDA-ARS bean

drought tolerance nurseries in Isabela, Puerto Rico**.** These isolates were selected because they represent the four major groups of pathogens which have been associated with RCR of dry beans in Africa and the USA.

2.2.3 Inoculum preparation

Macrophomina phaseolina (Fig. 1.1A)

To restart the isolate, the diseased and healthy interface of infected plant tissue was cut into pieces 3 - 5 mm^2. The pieces were then surface sterilized by placing in 10% v/v Clorox/water for 15 - 30 sec, then in 70% alcohol for 15 - 30 s, and finally in distilled water for 1 to 2 min. The tissue pieces were then dried on filter paper and plated on WA. After 3 - 4 days, a 3 mm plug from the advancing margin of mycelia was transferred to a PDA plate and incubated at 23 ± 1^0C for 4 d under continuous light and then increased on 6 PDA plates.

Fusarium solani (Fig. 1.1b)

The isolate was restarted from inoculated Whatman filter paper by plating pieces of the filter paper on water agar and incubating at 23 ± 1^0C for 3-5 d to induce growth of the fungus. The isolate was then transferred to 6 PDA plates for mycelial increase.

Rhizoctonia solani (Fig. 1.1c)

To activate the *Rhizoctonia solani* isolates from storage on sugar beet seeds, three seeds were plated on water ager (WA) (Difco, Detroit, MI, USA) and incubated at 23 ± 1^0C for 3-5 d to induce growth of the fungus then a 6 mm plug was taken from the growing margin of the mycelia and transferred to a potato dextrose agar (PDA) (Difco, Detroit,

MI, USA) plate. The isolate was incubated at 23±1^{0}C under continuous light for 4 d and increased on 6 PDA plates.

Pythium ultimum (Fig. 1.1d)

The isolate was started from WA storage to fresh WA and incubated for 3 – 4 d at 23±1^{0}C. It was then transferred to PDA, incubated for 3 – 4 d, then increased on 6 PDA plates.

1.2.4 Planting materials

The dry bean root rot susceptible variety Pinto PINTO 114 was grown in a greenhouse at 25 - 27^{0}C under a 14 h light and 10 h dark cycle. For the stem test, PINTO 114 was planted in 6 x 4cm plastic cells in pasteurized soil without fertilizer or nutrient supplements and grown for 10 days to growth stage V1 with fully developed primary leaves (Fig. 1.2). For the straw test, the plants were grown for 14 days to reach growth stage V3 with fully expanded first trifoliate leaves (Fig. 1.2). For detached leaf test, PINTO 114 was planted in 9 cm clay pots and grown for 3 weeks when expanded trifoliate leaves were harvested for the experiment. For the cup test PINTO 114 was planted in coarse vermiculite in 266 ml transparent cups. All the plants in the experiment were watered daily.

1.2.5 Inoculation

Six clones of each isolate were grown on PDA for 6 d. Edges of advancing mycelia were marked at the bottom and top of each plate of the isolates at 2, 4, and 6 d as mycelial ages

1, 2 and 3. For each isolate, 3 mm plugs were sampled at each marked age and used as inoculum in the pathogenicity testing methods.

1.2.6 Pathogenicity testing methods

Stem Test

The stem test was a modification of the cut stem test used in screening for resistance of dry bean and soybean to *Sclerotinia sclerotiorum* (Otto-Hanson *et al*., 2009, Smith, 2004) and also the stem inoculation tests used for *Phytophthora.* The modifications were, (1) growth stage at which inoculation was done and (2) the part of the plant that was inoculated. In the cut stem test, the plant is at V3 (Fig. 1.2) and the stem above the primary leaves is completely excised. In our study, inoculation was done at the V1 stage (Fig. 1.2) at the base of the primary leaves. Uniform plants at 10 d were selected and arbitrarily assigned to each of the four isolates at each of the three inoculum ages. The plants were inoculated with the isolates by picking a 3 mm plug of the isolate with a sterilized toothpick and placing it (mycelia side down) at the base of the petiole and stem of fully opened primary leaves. The plug was held in place with sterilized petroleum jelly (Glint cosmetic Pvt. Ltd). The plants were then put into a mist chamber for 2 d at humidity ≥80% and temperature 23 ± 3^{0}C to optimize conditions for infection. Finally, plants were placed on a greenhouse bench for 24 - 48hrs. Lesion length in cm was measured for analysis. A similar method has been used to detect pathogenicity for *Sclerotinia sclerotorum* on *Brassica napus,* dry bean and soybean (Zhao *et al* 2004).

Detached leaf Test

The detached leaf test was performed as described by Kull *et al.* 2003. Secondary leaves of 3-week-old Pinto 114 plants were harvested in the greenhouse, wrapped in wet paper

towels, then placed in sealed plastic bags and transported to the laboratory. Four glass petri plates were inverted and placed in aluminum roasting pans 48 x 33 x 5 inch lined with paper towels. The petioles of the trifoliolate leaves were inserted in 7.6 cm polypropylene Aquapic orchid tubes (Syndicate Sales, Inc.) filled with distilled water and the central trifoliolate was placed on top of the inverted petri plates with the abaxial surface of the leaf facing up. The leaves were then inoculated with the isolates using a sterile toothpick to place the 3 mm plug such that the part with mycelia growth was in direct contact with the center trifoliolate. To each pan, 300 ml of distilled water was added and the pan was covered with plastic wrap to create a humid chamber. The pans were incubated on a counter top at 23 ± 3^{0}C. Lesions on the leaves were photographed at 24 h and 48 h with a ruler as a measurement reference. The photos were used to determine the lesion area in cm2 with image-J software (imagej.net). A score reflecting lesion size was calculated and recorded for each of the isolates.

Cup Test

The cup test was a modification of the inoculum layer test described by Bilgi *et al*, 2008. Transparent cut crystal plastic cups (Humtamaki de soto USA) modified to have drainage holes in the bottom were filled to two-thirds with previously autoclaved vermiculite. Each cup was arbitrarily assigned to each of the four isolates at the three different inoculum ages. Pinto 114 seeds were then planted in the cups, one seed per cup. Each seed was then inoculated with a 3 mm mycelia agar disc from the 3 different mycelial ages and then covered with a layer of vermiculite. The cups were put in a greenhouse on trays and watered daily. After 10 days plants were removed from the cups,

the roots were washed with water and evaluated using a CIAT score for root rot damage (Schneider and Kelly, 2000).

Straw Test

The straw test used was a modification of the one described by Otto-Hanson *et al.* (2011). Clear plastic drinking straws were cut into 2.5 cm long pieces and each piece was sealed on one end by heating a forceps and crimping. At the V3 growth stage (14-16 d old plants), the first trifoliate was excised at a length of 6 cm. Inoculum from the plates was picked up with the straw such that the part of the plug with mycelial growth was facing outward. The straw was then placed over the cut trifoliolate petiole and pushed in until the plug reached the sealed end of the straw. The inoculated plants were moved to a mist chamber at humidity $\geq$ 80% and temperature 23$\pm$3^{0}C for 48 h, after which plants were placed on a greenhouse bench. Lesion length for each plant was measured at 24 h and 48 h after removal from the mist chamber. To convert the lesion length to a damage score, the length of the lesion was expressed as a percentage of the total length of the petiole (6 cm) for each plant. Thus uniform units are able to be used for the comparisons among the different testing methods.

Duration of test methods.

The duration of each pathogenicity test method was measured as the number of days it took to complete from planting to the final disease scoring.

1.2 Experiment 2.

1.2.1 Evaluating the straw test's ability to detect variability in pathogenicity of different *Fusarium* spp. isolates from western Nebraska

1.2.2 Planting material and inoculum preparation.

The dry bean root rot susceptible variety Pinto PINTO 114 was grown in a greenhouse at 25-27^{0}C under a 14 h light and 10 h dark cycle for 14 d. Twenty-five *Fusarium* spp. Isolates obtained from dry bean samples from Western Nebraska (Appendix 1) and stored as dry cultures were restarted on WA and incubated at 23±3^{0}C for 3 - 4 days. The isolates where then transferred onto individual PDA plates and incubated at 23±3^{0}C for 7 d. The straw test was then used to test pathogenicity as described above. A completely randomized design (CRD) with four replicates was used. The isolates were the dependent variable and the lesion length the response variable.

1.3 Statistical Analysis

1.3.1 Experiment 1

Experimental Design

The experiment compared four different genera/species reaction to four pathogenicity testing methods compounded by 3 mycelial ages on the detection of pathogenicity. The lesion sizes from the stem, straw and detached leaf test and disease score from the cup test for each of the genera at the different mycelial ages were considered as dependent variables. The experiment was analyzed with an ANOVA for CRD split plot where genus (test pathogen) was the whole plot factor, genus (test pathogen) clones (plates) as whole plot units and methods and mycelial age as split plot factors. To statistically compare the

pathogenicity methods, the response variable from each different method was standardized into a damage score, and a damage scale of 1 to 9 was developed to match the CIAT 1 to 9 scale: 1= 0%, 2= 1 - 10%, 3= 11 - 20%, 4= 26 - 40%, 5= 41 – 60%, 6= 61 – 75%, 7= 76 – 90%, 8= 91 –99%, 9= 100%.

$$\text{Damage score} = \frac{\text{lesion length X 100}}{\text{petiole or stem length}}$$

for the straw and stem test and

$$\text{Damage score} = \frac{\text{lesion area x 100}}{\text{leaf area}}$$

for the detached leaf test.

The data was analyzed by the PROC GLM procedure using SAS software (SAS Institute Inc., 2014).

1.3.2 Experiment 2.

Descriptive summary statistics of the mean and the standard deviations of the lesion length of each *Fusarium* isolate were calculated. To see variations in the pathogenicity of the different *Fusarium* isolates and their mean lesion length, ANOVA was conducted and means separated using the Tukey Standardized Range test in SAS.

1.3 Results.

1.3.1 Experiment 1: Pathogenicity Tests

Fusarium solani, Rhizoctonia solani, Macrophomina phaseolina and *Pythium ultimum* were scored a scale of 1 to 9 for the disease severity each pathogen caused on the Pinto 114 plants in the four pathogenicity testing methods. Significant differences ($0.05 > p > 0.001$) were observed among genera (test pathogens) and methods (Table 1.2). There were highly significant differences among the genera indicating that the pathogens had differing levels of aggressiveness. There were also highly significant differences among

the test methds by genera. The significant difference (0.1 p> 0.01) in the genus by mycelial age interaction indicates that with some isolates the age of the inoculum can have an effect on the disease score.

Genera

Pythium ultimum, Rhizoctonia solani and *Macrophomina phaseolina* caused damage and were detected by all the tests while *Fusarium solani* was only detected by the straw test. However, although detected by all the pathogenicity test methods, *Pythium ultimum* and *Fusarium solani* had significantly higher disease expression in the straw test, *Macrophomina phaseolina* had significantly higher disease expression in the cup test and *Rhizoctonia solani* had a significantly lower disease expression in the detached leaf test compared to the other 3 tests (Table 1.3). This observation indicates that isolates had unique responses to the test methods.

Performance of test methods in detecting RCR pathogens

The straw test detected pathogenicity of all (100%) the isolates tested and had the highest mean disease score. There was no significant difference in the mean disease score between *Pythium ultimum, Fusarium solani* and *Macrophomina phaseolina* in the test, however, *Rhizoctonia solani* was significantly lower at 95% significance level (Table 1.3). This indicates that the straw test is able to detect pathogenicity of all the four primary RCR Pathogens tested but is less sensitive to *R.solani* (Fig 1.3).

The stem test, DLT and cup test all detected pathogenicity in 75% of the isolates (Fig 1.3).

In the stem test, *R. solani* had the highest mean disease score which was significantly different from *M. phaseolina* and *Pythium ultimum* (Table 1.3) at the 95% significance level. In the cup test, *M. phaseolina* had the highest disease score, although not significantly different from *R.solani* at the 95% significance level. Although *P. ultimum* was detected by the cup test, its mean disease score was not significantly different from 1 (i.e. no visible damage) at the 95% significance level (Table 1.3). In the detached leaf test, *P. ultimum* had the highest disease score and was significantly different from *M. phaseolina* which, although detected, had a mean disease score that was not significantly different from 1 (i.e. no visible damage) at the 95% significance level. (Table 1.3). The results suggest that the test methods are different in their sensitivity toward detecting and expressing pathogenicity of the isolates.

Isolate mycelia age

There was no significant difference in the LS means disease score of *Pythium ultimum, Rhizoctonia solani, and F.solani* within each genus at all 3 isolate mycelial ages (Table 1.4; Fig.1.4). This indicates that the mycelial age of these isolates within the first 6 days of growth does not influence their pathogenicity. For the *Macrophomina phaseolina* isolate tested, mycelial ages 1 and 2 were not significantly different from each other but were significantly different from mycelial age 3 (Table 1.4; Fig 1.4). The highest LS mean was at mycelial age 1 (margin of advancing mycelia). This indicates that the margin of the advancing mycelia tends to be more pathogenic and is the best source of inoculum for *Macrophomina phaseolina.*

Pathogenicity symptoms expressed by the different isolates were watersoaking to necrotic lesion (stem straw and detached leaf tests) and sunken necrotic lesions on hypocotyl and the roots (cup test), with slight variation for each isolate (Figure 1.5)

Duration of test methods

The average time for each test method was 18.5 days. The cup test was the shortest with 13 day and the detached leaf test took the longest with 28 days. (Table 1.5)

2.3.2 Experiment 2. 1 Evaluating the straw test to detect variability in pathogenicity of different *Fusarium* spp. isolates from western Nebraska

There was a significant difference in the isolates mean lesion length $p>0.0001$ at 0.05 significance level (Table 1.6). This shows that the straw test is able to detect and differentiate aggression levels of different *Fusarium* spp.

There were significant differences in mean lesion length of the isolates between *Fusarium* species isolates and within specific species groups at 0.05 significance level (Table 1.7). Of the 3 *Fusarium acuminatum* isolates, 2 were pathogenic but their mean lesion length was not significantly different from 0 or the nonpathogenic isolate (Fig 1.6, Table 1.7). *Fusarium acuminatum* isolates were very weak pathogens. Of the 12 *Fusarium solani* isolates, 10 were pathogenic but only 6 had a mean lesion length that was significantly different from 0 or the nonpathogenic isolate. Of the 10 *Fusarium oxysporum* isolates, 8 were pathogenic but only 3 were significantly different from 0 or the 2 nonpathogenic isolates. This shows that the isolates had differences in their aggressiveness levels and that the straw test is able to distinguish the levels.

1.4 Discussion

Detached leaf, stem, cup and straw tests were compared on their ability to detect pathogenicity of the four major dry bean RCR pathogen groups; *Fusarium solani, Pythium ultimum, Rhizoctonia solani* and *Macrophomina phaseolina* the straw test confirmed pathogenicity of all the four RCR pathogens. The straw test also had the highest LS mean score and is a relatively easy and inexpensive test. The straw test also detected differences in aggressiveness of different species and isolates of *Fusarium* spp. associated with RCR of dry bean. These results support use of the straw test to separate pathogenic from non-pathogenic fungi and oomycetes associated with RCR of dry bean.

The significant differences in the genus pathogenicity and aggressiveness reflected in the testing methods is expected. The fungi and oomycetes tested have different biologies and ecologies and thus it would not be surprising for them to have different aggression levels (Infantino *et al*., 2006). Also, since different morphological plant components were used in the test methods, pathogenicity measured is expected to be different as was observed. This would also support the high significant difference in the genus by method interactions observed. Due to differing methods used by organisms to attack plant hosts, e.g. a fungus like *Fusarium* compared to an oomycete like *Pythium* gave different levels of damage in the straw test.

All the RCR pathogens tested were soil-borne pathogens, and based on their biology and ecology, it was expected that the cup test would measure their pathogenicity since soil-borne pathogens naturally would attack the below ground parts of the plant, such as any opening or cracks on the hypocotyl or the roots (Bais *et al.*, 2006, Toledo-Souza *et al.*, 2012). The cup test was designed to match the biology of the pathogens and initiate

infection of the plants similar to natural infection in the field. Positive infection results have been obtained using the cup test with *Fusarium* and *Rhizoctonia* (Bilgi *et al.*, 2008, Al-Abdalall, 2010). The failure of the cup test to detect pathogenicity of *Fusarium* could be because *Fusarium* did not establish a high enough population early enough in the cup to trigger infection, due to low inoculum concentration . Another reason could be that the time the plants were exposed to the inoculum was too short. Additionally, this could explain why the cup test barely detected pathogenicity in *Pythium*. Further, *Pythium* as an oomycete infects plants through the production of zoospores (Nzungize *et al.*, 2011, Sutton *et al.*, 2006). Using a plug of mycelia as inoculum in the cup, along with the specified time period for the test, likely did not allow for enough zoospore production. However, the high disease score for *Macrophomina* and *Rhizoctonia* in the cup test can be attributed to optimum conditions for the two isolates in the cup test. They both have relatively high mycelial growth rates and could have become established quickly to initiate infection and cause serious damage. A lot of dead seedlings, (score damage 9) were observed for *Macrophomina* in the cup tests. *Rhizoctonia* primarily affects seedling of dry bean causing damping-off. Plants in the cup test are normally scored at the V1 stage when they are still seedlings (Abawi and Pastor-Corrales, 1990, Peña *et al.*, 2013). In the cup test, plants are therefore more susceptible to *Rhizoctonia* and this might be why it was more sensitive to the *Rhizoctonia* isolate.

The stem test also did not detect pathogenicity of the *Fusarium* isolate. However it has been reported to detect pathogenicity in screening for resistance of plants to *Phytopthora*, (Smith, 2004), *Pythium* (Linde *et al.*, 1994) and *Sclerotinia* (Kull *et al.*, 2003), species which are also soil-borne pathogens of dry bean. Since *Fusarium* is an opportunistic

pathogen , it needs an opening or wound to cause disease. Inoculating the base of the unifoliate without making any incision or creating a wounding effect could have resulted in the failure of the method to detect pathogenicity of *Fusarium*. On the other hand, *Pythium, Rhizoctonia* and *Macrophomina* possibly would have to have been more aggressive to cause a disease reaction on the stem in the test even without an entry wound (Pratt *et al.*, 1998).

The detached leaf test (DLT) detected pathogenicity of *Rhizoctonia*, *Pythium* and *Macrophomina.* Similar results have been shown for *Rhizoctonia* and *Pythium* using the detached leaf test (Sutton *et al.*, 2006; Franke and Brenneman, 2001; Pettitt *et al.*, 2011; Otto-Hanson *et al.*, 2009) where shoot inoculation and DLT were evaluated for screening peanuts for resistance to *Rhizoctonia solani* limb rot and in evaluating methods of comparing disease resistance screening. Both the DLT and the straw test had a relatively low standard deviation, which can be attributed to the more controlled environment provided by the pans used for DLT and the microclimate created inside the straws used in the straw test. In the DLT, the mean disease score for *Macrophomina* was not significantly different from 1 (no noticeable damage) possibly due to the high humidity in the pan which may have affected disease development since *Macrophomina* is a dry environment pathogen (Kishore Babu *et al.*, 2007; Abawi and Pastor-Corrales, 1990; Sharma *et al.*, 2005). Although the ease of performing each test was not quantified statistically, the amount of resources each test required, the ease and time taken for scoring damage, the time from inoculation to pathogenicity detection and the time each test took from planting experiment material to the final scoring was recorded and taken into consideration in determining the ease, resources and time requirement for each test.

The DLT required only two days for the detection and scoring disease damage for each pathogen, similar to other reports (Pettitt *et al.*, 2011, Otto-Hanson *et al.*, 2009). The leaves have open stomata and thus penetration of hyphae into the tissue and infection is expected to be more direct and faster than in any other inoculated part of the plant. However, because the test requires secondary leaves from the V3 stage, the plants had to be grown for at least 28 days making it the longest test in terms of time to complete it. The DTL, required more resources to set up than the other tests and software to calculate/score the disease damage. The cup test was the shortest test and required the least resources but it could not detect *Fusarium* and barely detected *Pythium* at the level of inoculum used. The stem test required 12 days to be completed. The process of inoculating the plants was laborious in fixing the mycelial inoculum plugs on the stem using petroleum jelly. Covering the plug with petroleum jelly could also have created an anaerobic condition which might have affected the performance of the isolates. The straw test took a maximum of 18 days to complete. It was the only test that detected pathogenicity of *Fusarium*, which could have been caused by wounding the petiole allowing faster entry of the inoculum, as most of the RCR pathogens in the soil use cracks in the roots to infect the plants. Considering the average time of all the experiments being 19 days, the straw test at 18 days maximum is thus a relatively quick method for detecting pathogenicity.

Species of *Fusarium* have long been reported acting as a complex in different crop diseases, and isolation of fungi from diseased tissue have been shown to result in more than one pathogenic isolate from a single lesion (Coleman, 2016; Herron *et al.*, 2015). *Fusarium acuminatum* was isolated from symptomatic dry bean tissue samples. The

straw test was able to detect *Fusarium acuminatum* as a weak pathogen probably because it is not a primary RCR pathogen of dry bean but may colonize the tissue after it has been already attacked. *Fusarium acuminatum,* however, has been reported as pathogenic, causing damping-off disease on Pigeonpea and Aleppo Pine in India and in Algeria, respectively (M. Sharma *et al.*, 2013, F. Lazreg *et al.*, 2013). The straw test differentiated pathogenic from nonpathogenic *Fusarium oxysporum* and *Fusarium solani* isolates, and the separation of the means of the lesion length caused by the pathogens by the Tukey's Studentized Range resulted in expression of variability in aggression levels of the pathogens confirming the ability of the straw test to show pathogenicity and variability of aggression within. *F.oxysporum* and *F.solani* have been reported in various areas as primary pathogens causing RCR and wilts of legumes and other crops (Abawi and Pastor-Corrales, 1990; Al-Abdalall, 2010). Variability in the aggression of these pathogens has also been reported (Chaudhary *et al.*, 2006; Kerr, 1963; Mwang'ombe *et al.*, 2008; Toledo-Souza *et al.*, 2012).

In conclusion, our study shows that the straw test can detect pathogenicity of the major fungal/oomycete root rot pathogens of dry bean. The straw test is easy to set up and able to detect pathogenicity within a relatively short period of time. The straw test can be used to sort out pathogenicity for fungal/oomycete root rot pathogens and lessens the time and expense incurred by setting up multiple pathogenicity testing methods. The straw test therefore provides a quick and easy tool for pathologists and agronomists to sort out the pathogens causing fungal root rot disease. The method also offers a quick tool for characterization of pathogen aggressiveness.

1.5 References

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Tables and Figures

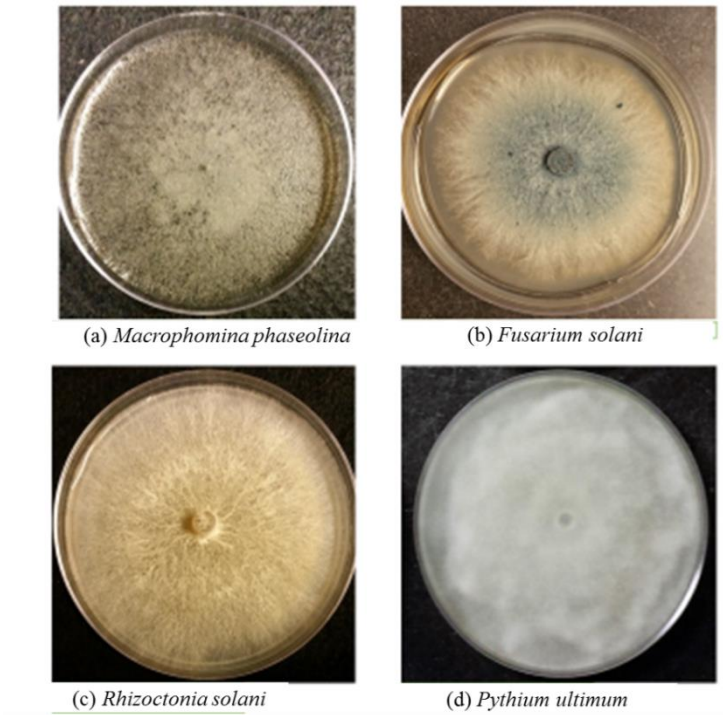


Figure 1.1 (a-d) Isolates from four different genera used in comparison of pathogenicity testing methods (a) *Rhizoctonia solani* NW (b) *Fusarium solani* f. sp. *phaseoli* 09RGBF 46 (c) *Pythium ultimum ultimum* P201 (d) *Macrophomina phaseolina*

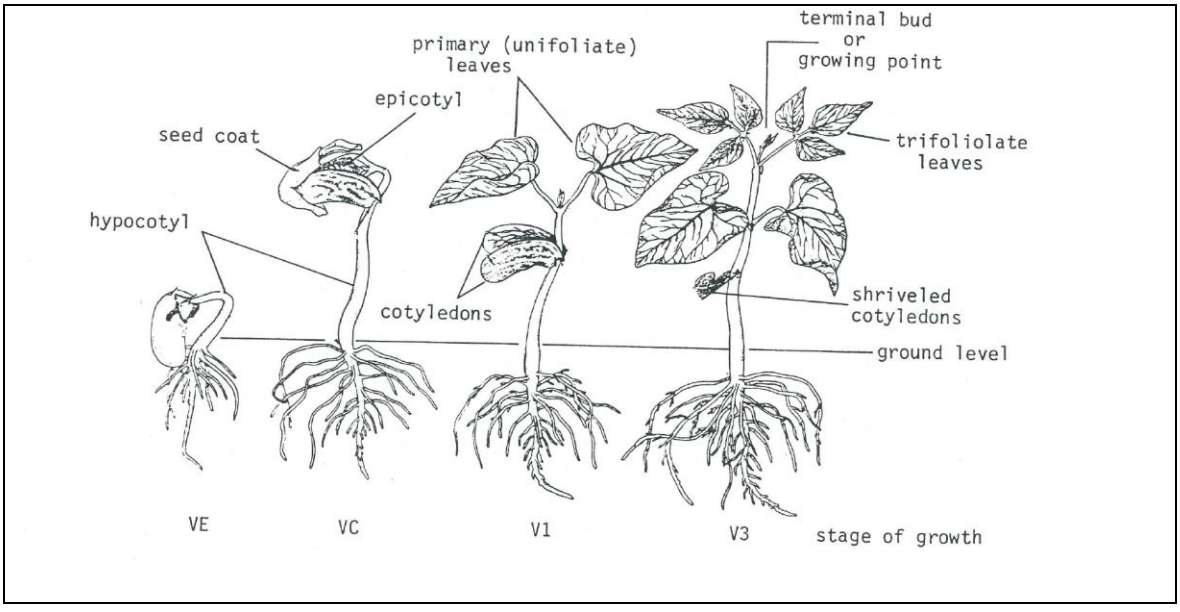
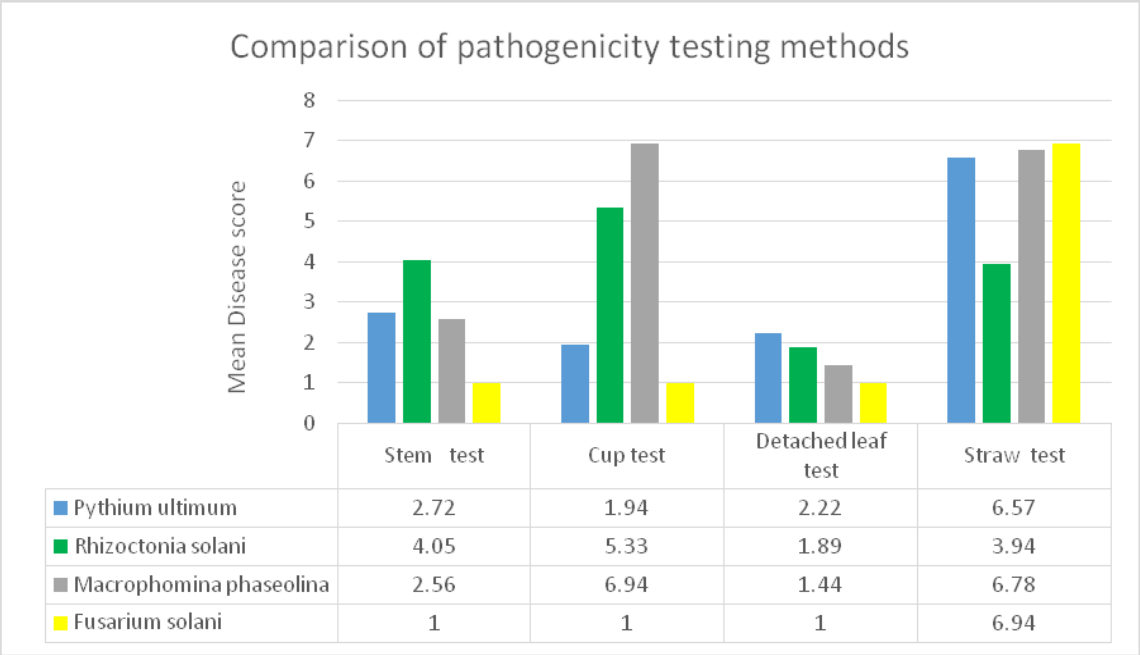
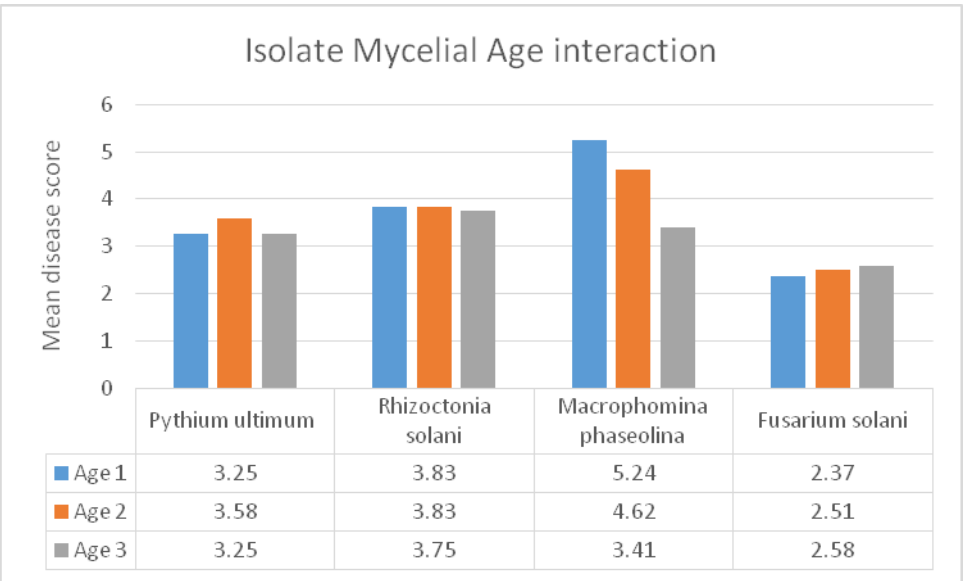


Figure 1.2 Dry bean growth stages inoculated in different pathogenicity testing methods. (Image courtesy of ADM.com)



	Stem test	Cup test	Detached leaf test	Straw test
Pythium ultimum	2.72	1.94	2.22	6.57
Rhizoctonia solani	4.05	5.33	1.89	3.94
Macrophomina phaseolina	2.56	6.94	1.44	6.78
Fusarium solani	1	1	1	6.94

Figure 1.3. Comparison of isolate performance within and between pathogenicity testing methods. *Rhizoctonia solani* and *Macrophomina phaseolina* had the highest scores in all the testing methods although differences with the other pathogens at $P<0.05$ were observed. Disease score scale 1= 0%, 2= 1 - 10%, 3= 11 - 20%, 4= 26 - 40%, 5= 41 – 60%, 6= 61 – 75%, 7= 76 – 90%, 8= 91 – 99%, 9= 100%.



	Pythium ultimum	Rhizoctonia solani	Macrophomina phaseolina	Fusarium solani
Age 1	3.25	3.83	5.24	2.37
Age 2	3.58	3.83	4.62	2.51
Age 3	3.25	3.75	3.41	2.58

Figure 1.4. Isolate by Age interaction. Performance of test isolates at 3 different culture mycelial ages. Age 1 = mycelia at 6 days (margin of advancing mycelia). Age 2= mycelia at 4 d. Age 3 =mycelia at 2 d.

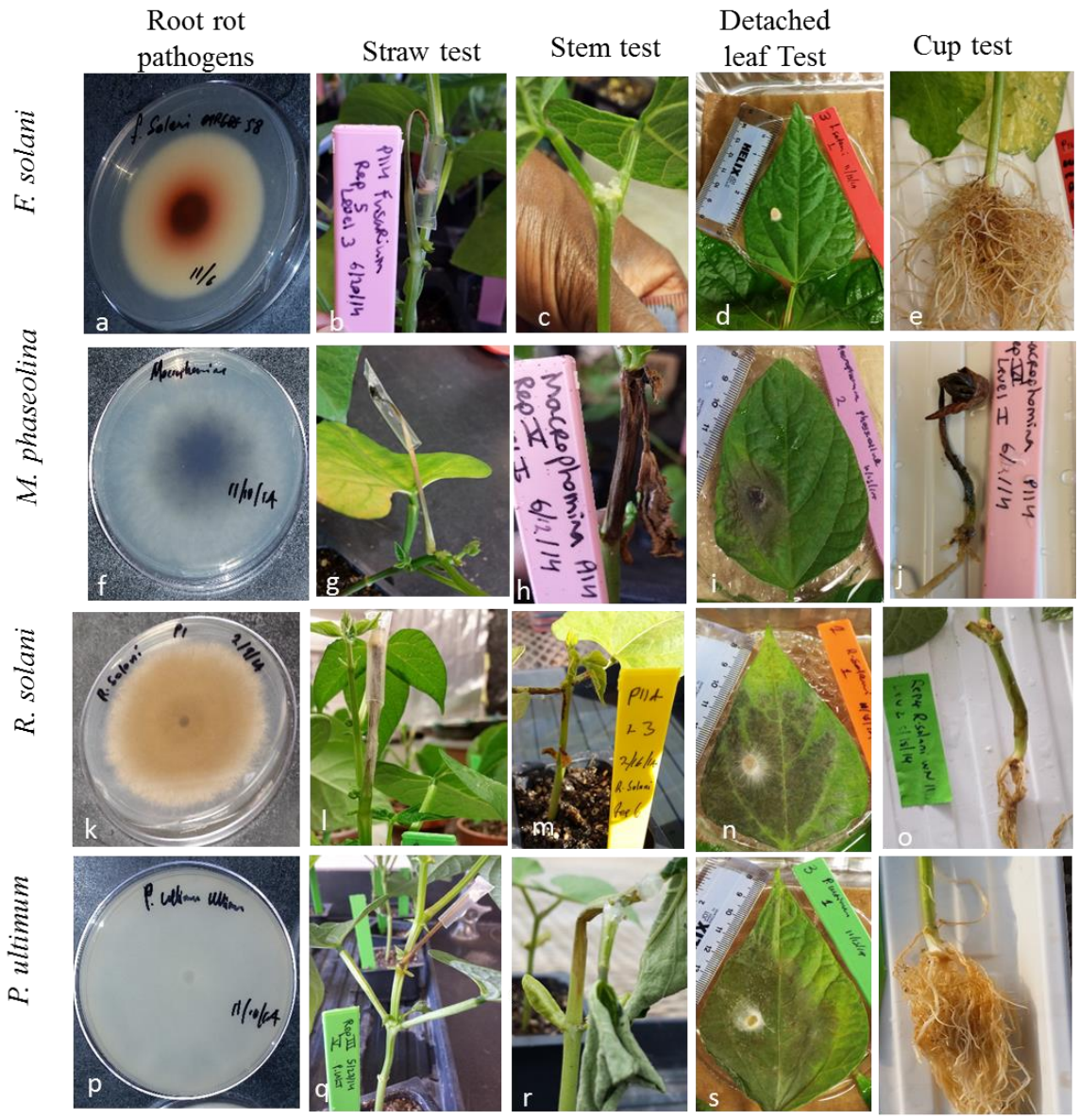


Figure 1.5 (a - t) Disease reaction caused by test isolates in the four pathogenicity testing methods. Necrosis and water soaking on petiole and stem in the straw and stem tests respectively; necrosis and water soaking on leaves in the DLT; necrotic lesions in the cup test

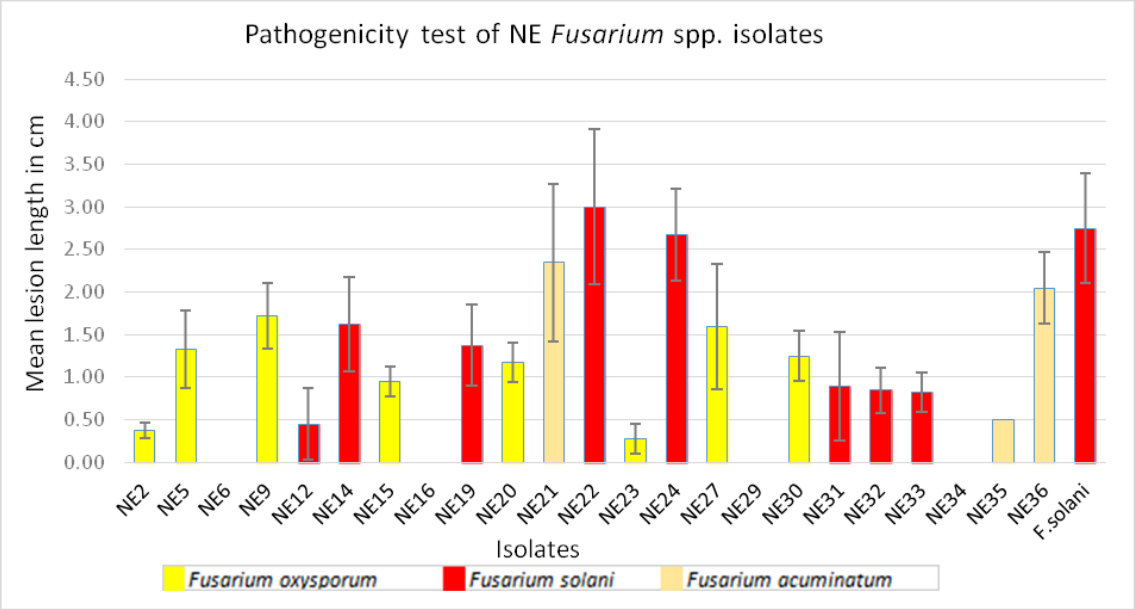


Figure 1.6. Variability in pathogenicity of NE *Fusarium* isolates between and within species groups detected by the straw test

Table 1.1 Sources of isolates used in the comparison of pathogenicity testing methods

Isolate	Source	Storage	Activation
Fusarium solani f.sp. *phaseoli*	North Dakota State University.	Dry Filter Paper	Plated on WA then Transferred to PDA
Macrophomina phaseolina	ARS-USDA dry bean fields. Puerto Rico	Infected Tissue	Isolated from tissue
Rhizoctonia solani	University Of Nebraska-Lincoln	Sugar beet seed	Plated on WA then Transferred to PDA
Pythium ultimum ultimum	University Of Nebraska-Lincoln	Water Agar (-20^0C)	Plated on WA then Transferred to PDA

Table 1.2 ANOVA for comparison of pathogenicity testing methods on standardized damage score

Source	Df	Type iii ss	Mean square	F value	pr > f
Isolate	3	144.20	48.07	20.09	<0.0001*
Sample(isolate)	20	47.87	2.40	1.31	0.177
Method	3	785.70	261.90	142.87	<0.0001*
Age	2	10.61	5.31	2.89	0.057**
Method*age	6	1.94	0.32	0.18	0.9830
Isolate*method	9	485.21	53.91	29.41	<0.0001*
Isolate*age	6	33.47	5.57	3.04	0.0070*
Isolate*method*age	18	32.29	1.80	0.98	0.485
Error	219	401.47	1.83		

*Significant at p= 0.05
**Significant at p= 0.1

Table 1.3 Least square mean disease score of isolates with four different methods

Pathogen	Stem test	Cup test	Detached leaf test	Straw test
Pythium ultimum	2.72 b	1.94	2.22 a	6.57 a*
Rhizoctonia solani	4.05 a	5.33 a	1.89 ab*	3.94
Macrophomina phaseolina	2.56 b	6.94 a*	1.44 bc	6.78 a*
Fusarium solani	1	1	1 c	6.94 a*

LS Means in a column with the same letters are not significantly different at p= 0.05 Confidence level
*LS Means significantly different in a row at p= 0.05 Confidence level
Disease score scale 1= 0%, 2= 1 - 10%, 3= 11 - 25%, 4= 26 - 40%, 5= 41 – 60%, 6= 61 – 75%, 7= 76 – 90%, 8= 91 – 99%, 9= 100%.

Table 1.4 LS mean disease score of isolates at three different mycelial ages.

Pathogen	**Age 1**	**Age 2**	**Age 3**
Pythium ultimum	3.25	3.58	3.25
Rhizoctonia solani	3.83	3.83	3.75
Macrophomina phaseolina	5.24 a*	4.62*	3.41
Fusarium solani	2.37	2.51 a	2.58

Means in a column with different letters are significantly different at p= 0.1 Confidence level

* Means significantly different in a row at p =0.1 Confidence level

Age 1 = mycelia at 6 days (margin of advancing mycelia). Age 2= mycelia at 4 d. Age 3 =mycelia at 2 d.

Disease score scale 1= 0%, 2= 1 - 10%, 3= 11 - 25%, 4= 26 - 40%, 5= 41 – 60%, 6= 61 – 75%, 7= 76 – 90%, 8= 91 – 99%, 9= 100%

Table 1.5 Time requirement in days from inoculation to disease scoring for each of the pathogenicity testing methods across all four test pathogens

Pathogenicity testing method	**Days to inoculation**	**Days to disease score**	**Total time for test**
Stem test	10	4	14
Cup test	0	13	13
Detached leaf test	28	2	30
Straw test	14	4	18
Average	15.5	5	18.75

Table 1.6. ANOVA of pathogenicity of *Fusarium* isolates from western Nebraska

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Isolates	24	76.48	3.18667	14.63	<.0001*
Error	75	16.34	0.21787		
Corrected Total	99	92.82			

*Significant differences in the isolates mean lesion length at a= 0.05, p<0.0001

Table 1.7 Tukey's Standardized Range (HSD) Test for isolate lesion length

Mean lesion length (cm)	N	Pathogen	Identity	Mean / tukey grouping	
3	4	NE23	*Fusarium oxysporum*	3	a
2.75	4	Reference isolate	*Fusarium solani*	2.75	ab
2.675	4	NE27	*Fusarium oxysporum*	2.675	ab
2.35	4	NE22	*Fusarium solani*	2.35	abc
2.05	4	NE37	*Fusarium solani*	2.05	abcd
1.725	4	NE9	*Fusarium oxysporum*	1.725	bced
1.625	4	NE14	*Fusarium solani*	1.625	bcedf
1.6	4	NE29	*Fusarium solani* strain	1.6	bcedf
1.375	4	NE20	*Fusarium oxysporum*	1.375	cedf
1.325	4	NE5	*Fusarium oxysporum*	1.325	cedf
1.25	4	NE31	*Fusarium solani* isolate	1.25	cedfg
1.175	4	NE21	*Fusarium acuminatum*	1.175	cedfg
0.95	4	NE15	*Fusarium oxysporum*	0.95	defg
0.9	4	NE32	*Fusarium solani*	0.9	defg
0.85	4	NE33	*Fusarium solani*	0.85	defg
0.825	4	NE34	*Fusarium oxysporum*	0.825	defg
0.8	4	NE16	*Fusarium solani*	0.8	defg
0.5	4	NE36	*Fusarium acuminatum*	0.5	efg
0.45	4	NE24	*Fusarium solani*	0.45	fg
0.45	4	NE12	*Fusarium solani*	0.45	fg
0.375	4	NE2	*Fusarium oxysporum*	0.375	fg
0	4	NE35	*Fusarium acuminatum*	0	g
0	4	NE6	*Fusarium oxysporum*	0	g
0	4	NE30	*Fusarium oxysporum*	0	g
0	4	NE19	*Fusarium solani* f. sp. *phaseoli*	0	g

*Means with the same letters are not significantly different. LSD = 1.2537, alpha = 0.05

Chapter 2

Molecular and culture based methods to ascertain predominant causal agents of root and crown rot of dry beans in Zambia

Abstract

Root and crown rot (RCR) of dry bean (*Phaseolus vulgaris*) has been attributed to various fungal and oomycete species, and in the past, the RCR disease complex had been considered to be among the minor diseases affecting dry bean. However, RCR has become a limiting factor to bean production in developing countries like Zambia, where the use of uncertified seed, lack of crop rotation, and lack of bean varieties with resistance to RCR pathogens has exacerbated the problem. For this study, symptomatic dry bean RCR plant samples were systematically sampled from the Andean Diversity panel and Nebraska select breeding lines grown at research stations and farmer fields in four major bean producing districts in Zambia between 2013 and 2015. A total of 56 tissue samples and 129 DNA embedded FTA® cards were collected. From the tissue samples, 203 fungal isolates were recovered from the interface of healthy and diseased tissue, and three methods of identification were then used. Cultured isolates were identified and characterized using morphological features such as colony color/texture and spore size/morphology, and taxonomically identified by Sanger-based ITS-5.8S and EF rDNA sequences. Molecular analysis of DNA extracted from FTA cards and plant tissue was done by pyrosequencing using 454 and Illumina platforms on the SSU of the rRNA gene and PCR amplification with genus/species specific primers. *Fusarium* spp. had the highest recovery rate of > 80 % (of which 90% were pathogenic) from tissue in the classical culturing method, a detection rate of > 70% in PCR-based identification and the highest relative abundance > 90% in pyrosequencing. In some samples, *Macrophomina phaseolina* and species of *Pythium* and *Rhizoctonia* were detected mostly by PCR primers not as single species but co-occurring with *Fusarium* spp. within the

same plant. Correlation of culture-based and molecular identification methods using Pearsons's, Spearman's and Polyserial rho and Kindles Tau were significant at $p<0.001$ at 0.05 with the highest correlation between betwee 0.89 and 0.9. Inter method agreement was moderate to high between FTA card and tissue DNA in transporting and preserving DNA with highest observed agreement over 80% and kappa (k) 0.6 to 0.8, $p < 0.005$ in 2014 and 2015 samples. Most isolates of *Fusarium* and all isolates of *R. solani*, and *Pythium* spp obtained from cultures were pathogenic. In spite of its limitations, only the culture method provides pathogenic isolates for use use in screening breeding lines for RCR resistance. All the three methods used in identifying the primary RCR pathogen of dry bean in Zambia identified the *Fusarium* species complex. Within this *Fusarium* species complex, pyrosequencing and culturing methods identified *F. oxysporum* to be most abundant and thus is the most likely target for initial screening for resistance in Zambia.

Identification of a primary pathogen associated with RCR of dry bean in Zambia provides evidence to direct intial breeding for RCR resistance to breeding for resistance to *Fusarium oxysporum*.

2.0 Introduction

Identification of disease causal agents is key to development of control and management strategies. Before the advent of DNA analysis, most taxonomy and identification of organisms was done based on morphological characteristics. The morphological characteristics of fungal pathogens such as type, shape and color of sexual or asexual spore forms have long been used for their taxonomy and classification (Jayasinghe and Fernando, 1998). However, with advances in technology and the availability of inexpensive molecular tools, most taxonomy is now being done with molecular tools. Nonetheless classical taxonomy still plays an important role in the identification of fungi, although steps leading to the identification of species can be very complicated, laborious, subjective, and can lead to incorrect identification (Siddiquee *et al.,* 2010). In some laboratories or plant disease diagnostic clinics, especially in developing countries, morphological identification may be the only option available for diagnosis. Koch's postulates are also used to verify that the recovered isolates are the disease causal agents (Agrios, 2005).

Different fungi and oomycetes have different growth media, temperature and light quality requirements for the production of consistent characteristics that can be used for morphological identification. Carnation Leaf-piece Agar (CLA), Spezieller Nährstoffarmer Agar (SNA), and Potato Dextrose Agar (PDA) are the standard media used in the identification of *Fusarium s*pecies.(Leslie *et al.*, 2006). *Macrophomina* species are highly variable in microsclerotia size and the ability to produce pycnidia. Microsclerotia morphology is a key taxonomic characteristic for the identification of this fungus (Almomani *et al.*, 2013; Beas-Fernández *et al.*, 2006). Pigmentation of colony,

mycelial type and rate of growth on PDA at 27^{o}C in the dark are also distinguishing morphology features that help in the identification of *Macrophomina phaseolina*. A morphological characteristic for identification of *Rhizoctonia solani* in culture are the right angles that emerging hyphae make as they branch from the main hyphae. (Ogoshi, 1987). Colony morphology and growth characters are also used in characterization of *Rhizoctonia solani,* including type of mycelia, the presence, absence and type of sclerotia, colony diameter, color, texture and width of hyphae. Sclerotia characteristics include number, size, arrangement and color. (Sharma *et al.*, 2005; Guleria *et al.*, 2007). *Pythium* species do not readily produce sexual structures on common general growth media. *Pythium* is not a fungus and is phylogenetically more related to plants than it is to fungi, but it has distinct characteristics in culture including the production of sporangia and zoospores on corn meal agar (CMA), and growth rate on potato carrot agar (PCA). (Kageyama *et al.*, 1998). Also *Pythium* colony growth is characteristically white on PDA.

While morphological identification is basic to characterizing organisms, molecular techniques have been developed which appear to be more accurate and faster than morphological techniques (Buckingham, 2011). The molecular techniques use DNA analysis of a given pathogen or host for identification. Molecular systematics have become a good alternative and compliment to classical taxonomy (Buckingham, 2011). For fungi and some fungal-like organisms, the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) is used in phylogenetic studies of closely related species (White *et al.*, 1990). Of the transcribed rDNA regions, the non-coding internal transcribed spacers, described as ITS1 and ITS2 are found on each side of the 5.8S rRNA gene. They encompass the 3’ end of the 18S gene, the ITS1 spacer region, the 5.8S gene,

the ITS2 spacer region, and the 5' end of organisms (Henry *et al.*, 2000, Siddiquee *et al.*, 2010) (Fig. 2.1).

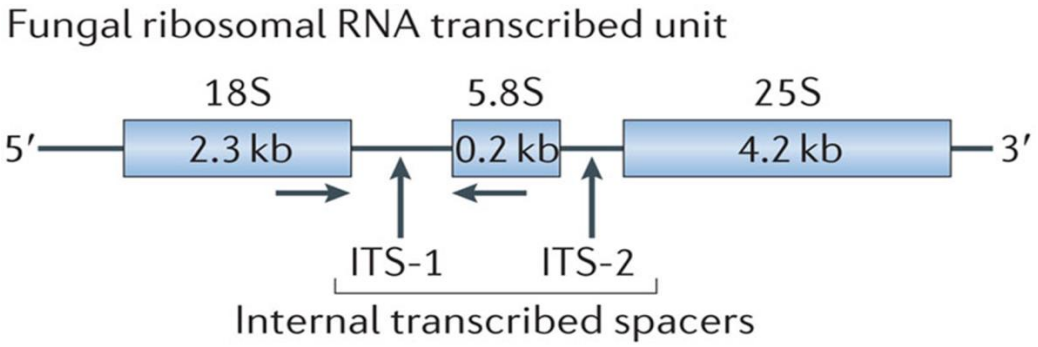


Fig 2.1. Internal Transcriber Spacer (ITS) region used for identification of fungi. (Courtesy of Nature Reviews and Immunology).

In fungi, intron-rich protein coding genes are used for species-level phylogenetics. Geiser *et al.* (2000) reported that these genes in many *Fusarium* spp. evolve at a rate greater than the species, and the fact that many *Fusarium* spp. within the Gibberella clade possess non-orthologous copies of the ITS2 presents disadvantages in the use of ITS primers in the identification of *Fusarium*, as it can lead to incorrect phylogenetic inferences (O'Donnell and Cigelnik, 1997; O'Donnell *et al.*, 1998a). Amplification of *Fusarium* DNA using general ITS primers produces gel electrophoresis bands that are between 300 – 400bp, and this is used in the identification of the genus using conventional PCR. However, the translation elongation factor 1-*alpha* (TEF) gene, which encodes an essential part of the protein translation machinery, has high phylogenetic utility. Ef1 and Ef2 primers targeting the1- *alpha* gene amplify an ~700 bp region of TEF, flanking three introns that total over half of the amplicon's length in all known *Fusarium* spp. (Geiser *et*

al., 2004). This gene appears to be consistently single-copy in *Fusarium*, and it shows a high level of sequence polymorphism among closely related species, even in comparison to the intron-rich portions of protein-coding genes such as calmodulin, beta-tubulin and histone H3 (Geiser *et al.*, 2004). For these reasons, TEF has become the marker of choice as a single-locus identification tool in *Fusarium* (Leslie *et al.*, 2006). Some species-specific PCR primers have been developed, but in most cases they have yet to be more widely tested and their reliability for analyses of strains from various crops and/or geographic locations is unproven (Rahjoo *et al.*, 2008). Most *Fusarium* species associated with wilts and root rots, especially on dry bean, are *Fusarium solani* or *Fusarium oxysporum* (Schwartz and Galvez, 1980).

Macrophomina phaseolina is a basidiomycete and has a great diversity of host plants, with genetic variation having been observed within the species (Jana *et al.*, 2005). Until recently, molecular identification was performed by using oligonucleotide-specific primers or probes targeting the ITS region. Molecular methods involving the use of PCR have been described to resolve genetic variation among the *M. phaseolina* isolates (Kishore Babu *et al.*, 2007) The molecular methods used for the differentiation of *M. phaseolina* populations have primarily been restriction fragment length polymorphism (RFLP) of rDNA ITS regions, random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) (Jana *et al.*, 2005). The ITS region has been demonstrated to selectively detect several agriculturally important fungi. Based on this discovery, Kishore *et al.* (2005) analyzed sequences of *M. phaseolina* from isolates and from GenBank, and designed *M. phaseolina* specific primer probe (MpKH1) from the conserved region, adjacent to the 5.8 S gene. Using the forward (MpKFI) and reverse

(MpKRI) primers, *M. phaseolina* can be identified by its characteristic bands of between 300 to 400 bp (Kishore Babu *et al.,* 2007). *Rhizoctonia solani* is a species complex with a wide and sometimes overlapping range of pathogenicity. Unlike other fungi, *R. solani* has been grouped and classified into anastomosis groups (AGs) based on affinities for hyphal fusion with members of designated anastomosis groups (Ogoshi, 1987). These AG groups have been used for identification and characterization of *Rhizoctonia solani*. However, with the discovery of isolates that are capable of anastomosis with more than one AG group and the loss of self-anastomosis in others, the use of AG groups may be misleading (Sharma *et al.,* 2005). This limitation can be overcome by the use of molecular markers which not only separate the isolates to individual AG groups but can also identify the genetic variations (Guleria *et al.,* 2007). *Rhizoctonia solani* species-specific primers have been developed on the basis of the differences in the ITS1 and ITS2 regions among several AGs, resulting in specific gel electrophoresis banding patterns. Primer set RS1/RS4 gives a distinctive banding patters to all *Rhizoctonia* species at 475 to 550 bp (Camporota *et al*., 2000).

Pythium species are diverse and also cause different diseases in different plant species (Martin and Loper, 1999). Genetic variability is present and morphological identification of *Pythium* species using the required specific growth conditions may be too laborious. Molecular techniques offer an easier way to identify and characterize *Pythium* spp. These techniques include restriction fragment length polymorphism (RFLP) analyses of the internal transcribed spacer (ITS) regions of rDNA, and the cytochrome c oxidase subunit II (COX II) gene (Kageyama *et al.,* 2005). The cytochrome oxidase subunit II (COX II) gene is a housekeeping gene and is thought to accumulate mutations through evolution,

indicating that this gene might be useful for determining phylogenic relationships. Moreover, because the gene is encoded in the mitochondria, it may clarify phylogenic relationships among the species in relation to different genetic backgrounds. Martin *et al.* (2000) showed that COX II sequence alignments are generally well conserved within a species but divergent among species. Amplification of the COX II gene with FM58 and FM66 as described by Martin (2000) give a distinct banding range of *Pythium* species between 544bp to 689b .

Molecular identification and genetic characterization requires the analysis of DNA of an organism. Several ways of extracting and preserving DNA have been developed and used over the years. The specific protocols for DNA extraction and handling depends on the organism and the type of analysis that needs to be made. Whatman™ FTA provides a remarkably easy way to collect and store nucleic acid samples for analysis, through FTA® cards on which virtually any type of biological sample can be applied, and nucleic acids are instantly captured and stabilized. In this method, samples are pressed or spotted onto a card and the nucleic acids within the tissue are bound to its matrix, so it can be archived until further use (Roy and Nassuth, 2005). Various chemicals and enzymes impregnated in the FTA card matrix which inhibit enzymes, microbes and chemicals that may degrade the DNA or RNA in the sample. The vast majority of the chemicals and enzymes stabilizing DNA in the matrix include chelators, denaturants and free-radical traps (Smith and Burgoyne, 2004).

Most of the work involving the use of FTA® cards has been done on viruses in both the medical and agricultural fields. Comparisons have been made on the quality as well as the accuracy of the use of FTA cards in sampling and preserving virus DNA by analyzing

the DNA for the presence of a specific virus in samples preserved in conventional ways from the infected specimen and samples preserved directly on FTA cards. In this research, we will determine whether viable DNA of dry bean RCR pathogens can be collected on FTA® cards and used in identification of the primary RCR pathogens by comparing DNA from plant tissue and from DNA-imbedded FTA® cards. The use of both molecular and culturing techniques will be compared in the determination of the predominant causal agent of RCR on dry bean in Zambia. The main objective of this research is to use morphological-cultural and molecular techniques to identify the primary pathogen causing dry bean RCR in Zambia. The specific objectives are: (1) to compare classical culture and molecular methods in identification of RCR pathogens of dry bean, and (2) to evaluate the use of FTA® cards in sampling, preservation and transportation of DNA for dry bean RCR pathogen identification.

2.1 Materials and methods

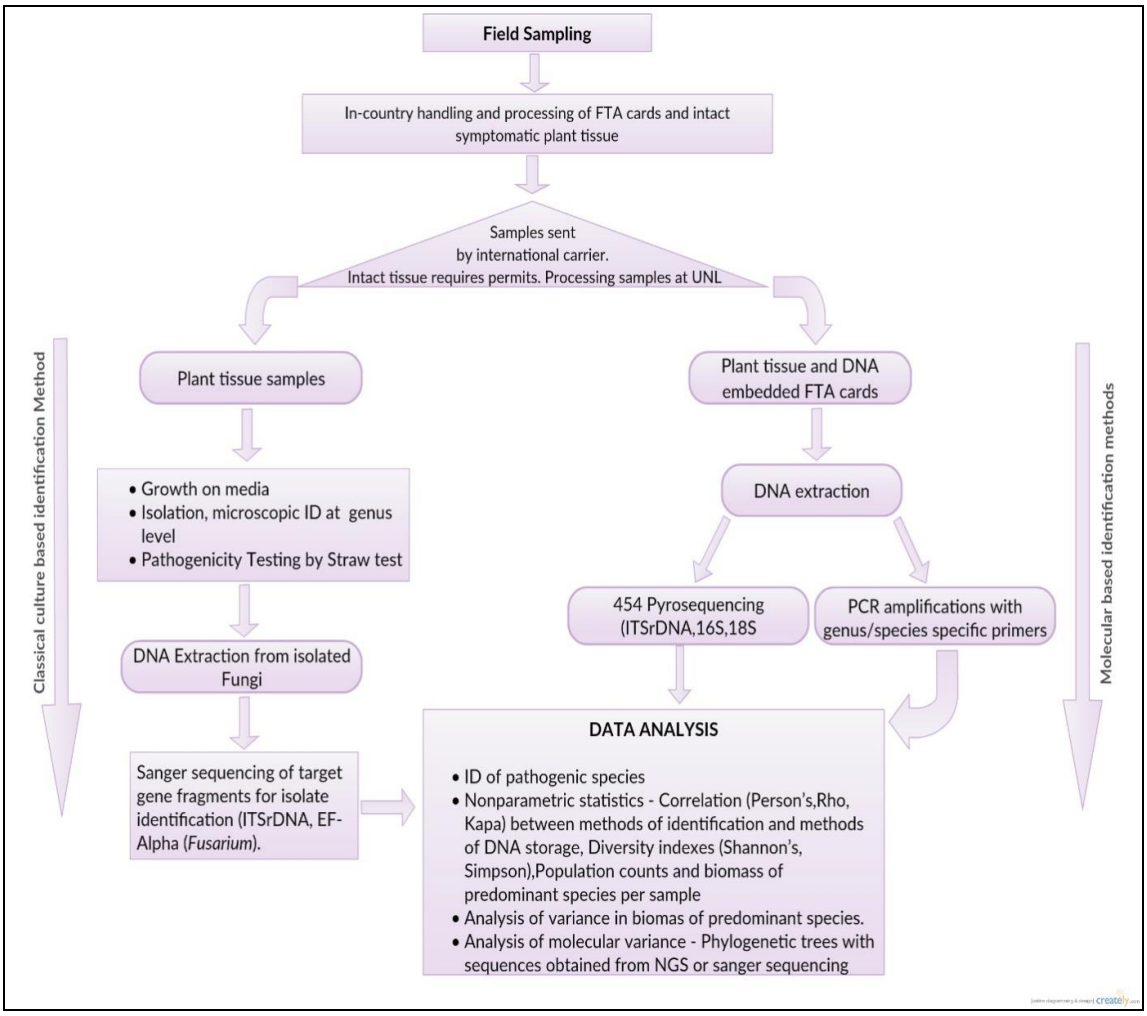


Figure 2.2. Schematic representation of the methodology for sampling and processing of samples to data analysis in this study

2.1.1 Field Sampling

Symptomatic plants were sampled systematically from the Andean Diversity Panel (ADP) (Sign et al.,1999; Cichy *et al.*, 2015) Nebraska (NE) RCR screening nurseries and farmer fields in Northern, Muchinga Central and Lusaka Provinces in Zambia from 2013 to 2015 (Fig 2.3). These areas were selected because they are in predominant bean

producing areas. The physical as well as environmental conditions of the locations and the samples collected are in Table 2.1.

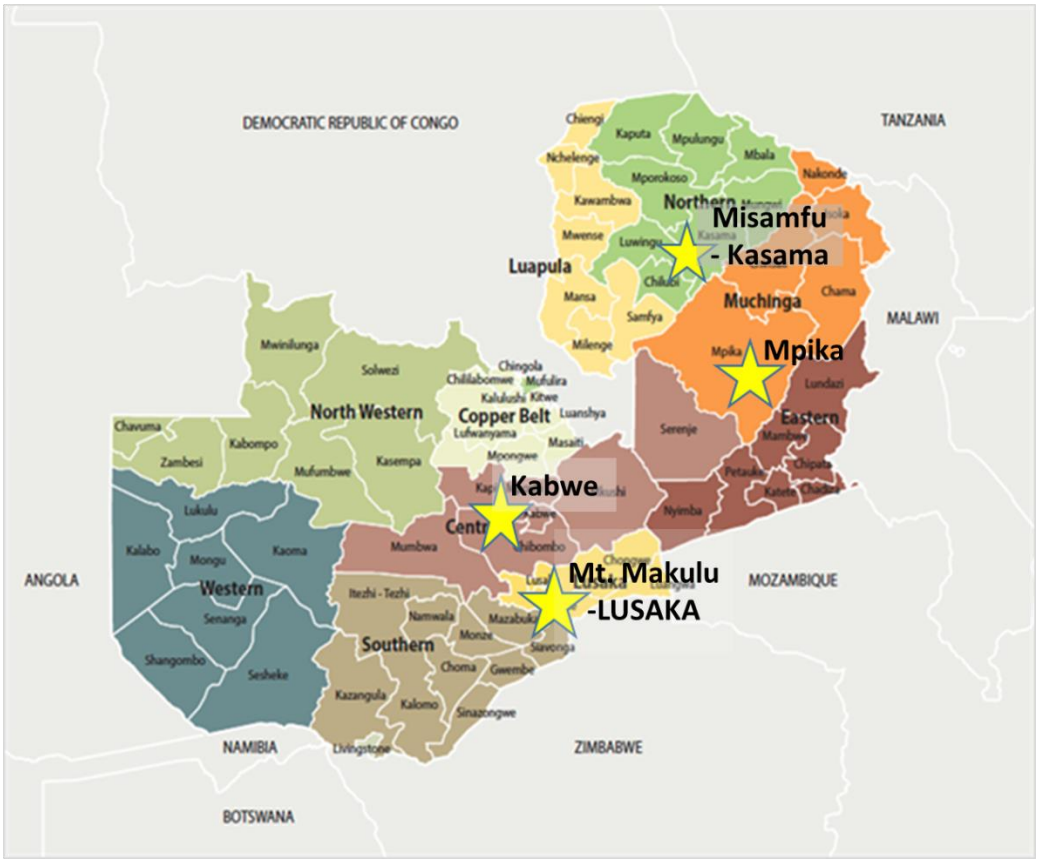


Fig 2.3. Map of Zambia showing locations where the ADP /NE dry bean nurseries and farmers fields were sampled from 2013 - 2015.

Field experiments were conducted in two locations in 2013, 2014 and 2015. In 2013, two experiments were established at Mt. Makulu (Lusaka) and Misamfu (Kasama). The experiments at Mt.Makulu and Misamfu were set up as randomized complete block designs. Each Block had 15 single plot rows 1.5 m long. Each plot was planted with 12 seeds of a single variety, with a spacing of 10 cm within rows and 60 cm between rows. The ADP/NE nurseries were evaluated primarily for root rot diseases, but foliar diseases including angular leaf spot (ALS), anthracnose (ANTH), bean rust (RST), bean common mosaic Virus (BMV), common bacterial blight (CBB) were also noted. A total of 60 (28

indeterminate and 32 determinate) best performing lines against RCR and other foliar biotic and abiotic stresses were selected and advanced to the 2014 nursery at Misamfu, with an extra 6 local lines (2 indeterminate and 4 determinate) added to complete the design. The 2014 nursery in Misamfu was planted in a Randomized Complete Block Design (RCBD) with 2-row plots of 3m length, replicated 3 times. Root rot scores were given on a scale of 1 to 3 with 1= absent, 2= present and 3= severe. Sixteen best performing lines, including local checks, were selected and advanced to 2015 nurseries in Kabwe and Misamfu. The nurseries in 2015 were planted as a split split plot in a RCBD) with 2-row plots of 3m length, replicated 3 times. The treatments were: fertilizer and insecticide; fertilizer and non-insecticide; non-fertilizer and insecticide; non-fertilizer and non-insecticide. RCR were evaluated as before and a total of 6 different lines were selected and advanced to 2016 nurseries.

Additional samples were collected from seed multiplication fields and seed multiplication lines. The samples from the seed multiplication location in Misamfu were treated as controls, since no RCR was detected during the growth of the beans. The plants sampled as controls were healthy plants with no symptoms of RCR and foliar diseases.

2.1.2 In-country processing of FTA® cards and diseased plant tissue

Sampled plants were trimmed to include only root, crown, or lower stem tissue where infected and healthy tissue meet (Fig. 2.4).

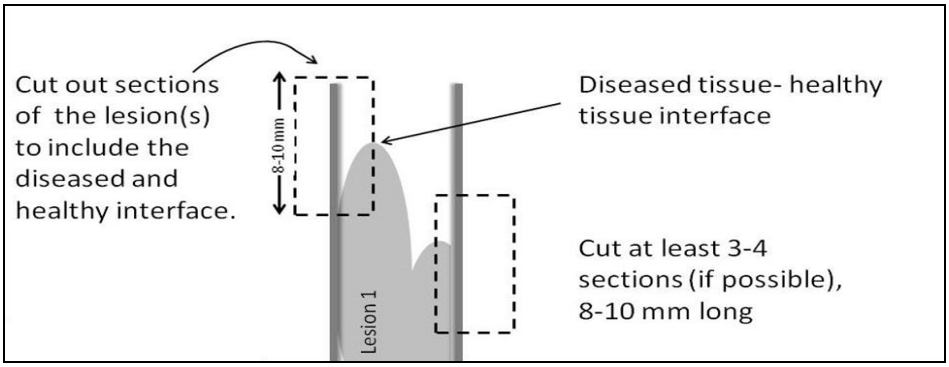


Fig 2.4 Schematic representation of the interface between healthy and diseased part of tissue on a lesion used in spotting FTA® card, tissue DNA extraction and isolations.

Pictures of the samples were taken and a number was given to each sample for identification. The samples were then packed into 2 oz. Whirl-pak™ bags (Nasco Science, Janesville, WI) and stored on ice in a cooler box for transport to the lab and further processing. At the lab, 4 to 5 sections approximately 3-5 mm^2 of the lesion(s) on the tissue that included the diseased and healthy interface were excised with a razor blade, crushed with a mortar and pestle, and ground into a homogenate with added deionized water. Approximately 125ul of the homogenate was then spotted in each circle matrix on an FTA® card (Fig. 2.5) using a wide-bore pipette.

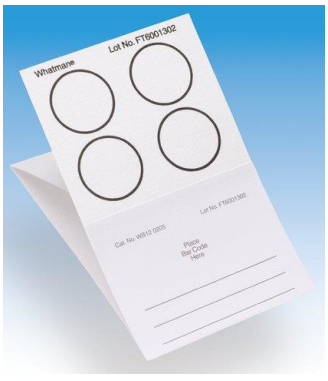


Fig 2.5. Four-sample classic Whatman™ FTA card with four sample areas per card, used in collection of DNA directly from plant tissue. (Fisher Scientific. www.fishersci.ca)
(Picture courtesy of gendna.net)

The FTA® cards imbedded with plant tissue homogenates were left at room temperature to dry for at least 2 hours. The sample number, variety, location and details of the sample were recorded on the FTA® cards. The remaining plant material was saved in small coin envelopes and tagged with the same information as on the FTA® cards. The matched FTA® card and tissue samples in coin envelopes were shipped to the laboratory at the University of Nebraska-Lincoln where they were stored in desiccators with anhydrous calcium sulfate in the bottom (W.A. Hammond Drierite Co., LTD Xenia, OH, USA) before isolations and DNA extractions. The total number of bean lines sampled from 2013 to 2015 (Appendix 2).

2.3.0 Classical culture-based identification

2.3.1 Fungal/oomycete isolations from plant tissue and morphological identification

Fungi and oomycetes were isolated from 2014 and 2015 tissue samples as described for *Macrophomina phaseolina* isolation in chapter 1. Individual pure cultures were grown on single PDA plates in duplicates for morphology identification and characterization, pathogenicity testing and DNA extraction for taxonomic classification. The total number of isolates recovered from each sample, and the different types of isolates observed from a single sample were recorded. The frequency of isolation of the different fungi was determined after taxonomic identification. Frequency tables were generated using the proc freq command in SAS software.

2.3.2 Morphological identification and characterization

Preliminary colony examination for spore producing isolates was done when cultures were on WA for separation of spore- and non-spore-producing isolates. Morphology at

micro- and macroscopic level of each isolate was observed in cultures grown on WA, PDA and CLA.

Colony color

Pure cultures of each isolate were obtained through hyphal tip or single spore and were grown on PDA under continuous light for 4 to 7 days at 24^0C $\pm 2^0$C in the lab. The colony color was examined on the top and bottom and compared to a color chart.

Growth rate

To determine the growth rates of the isolates, pure cultures of each isolate were grown on PDA in three replications under continuous darkness for 3 days at 25 ^{0}C and radial growth of the colony was measured at two perpendicular diameters on the bottom of each plate. Agar plug diameters were subtracted from every measurement. The two colony diameters for each plate were averaged, and a mean growth rate was calculated from the three replicate plates.

Spore shape and size

Fusarium-like spore producing isolates were single spored on Carnation Leaf Agar (CLA) media, and plates placed in an incubator at 25^0C with a 12 hour light and darkness cycle for two weeks to promote the production of uniform spores. The size and shape of the spore was observed and recorded using a compound microscope mounted with a Motic camera at 40x magnification. Isolates that did not produce spores were observed and the nature and distribution of sclerotia, if present, was recorded.

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2.3.3 Pathogenicity testing of fungal/oomycete isolates

To fulfil Koch's postulates, all the recovered isolates from tissue were tested for pathogenicity using the straw test and bean line PINTO 114 as described in chapter 1. The length of the lesions on the petiolate was recorded and used as a criteria to comfirm pathogenicity. The pathogenic isolates were re-isolated from the infected tissue and compared to the original isolates to complete Koch's postulates.

2.3.4 DNA extraction from FTA cards, plant tissue and isolate cultures.

Culture isolates

Total genomic DNA was extracted from mycelia pure cultures using a PowerPlant® ProDNA isolation kit (MoBio Laboroatories, Inc.). The protocol was modified to enhance DNA extraction from fungi. For each sample, 5-10 3mm plugs were collected from the advancing margin of the cultures, and the mycelia was scraped from the top of each plug and put into 2 ml PowerPlant® Bead tubes to which 450 µl of the provided PD1 solution and 50 µl of solution PD2. The bead tubes were then heated in a dry bath at 65^0C for 10 minutes. After heating, 3 µl of RNase was added to the Power Plant® Bead Tube and secured horizontally to a MO BIO Vortex Adapter (MO BIO Catalog# 13000-V1-24) and vortexed at maximum speed for 10 minutes. The bead tubes were then centrifuged at 13,000 x g for 2 minutes and the supernatant transferred to a clean 2 ml collection tube. Solution PD3 was then added at 175 µl to the collection tube and vortexed for 5 seconds to mix. The mixture was then incubated at 4°C for 5 minutes after which the tubes were centrifuged for 2 minutes at 13,000 x g. Avoiding the pellet, 600 µl of supernatant was transferred to a clean 2 ml collection tube and 600 µl of solution PD4

and 600 µl of solution PD6 were added and vortexed for 5 seconds to mix. Approximately 600 µl of this mixture was then loaded onto the spin filter and centrifuged at 10,000 x g for 30 seconds. The flow through was discarded and the spin filter placed back into the collection tube. This was repeated 3 times until all of the lysate had passed through the spin filter. Solution PD5 was then added at 500 µl to the spin filter column and centrifuged for 30 seconds at 10,000 x g. The flow through was discarded, the spin filter placed back into the same collection tube and 500 µl of solution PD6 was then added to the spin filter column and centrifuged for 30 seconds at 10,000 x g. The flow through was discarded and the spin filter placed back into the same collection tube. The spin filter tube was then centrifuged twice at maximum speed for 2 minutes to remove any excess of solution PD6. The collection tube was discarded and the spin filter column placed into a new collection tube. Solution PD7 (10 mM Tris, pH 8.0) was added at 100 µl to the center of the white filter membrane in the spin filter and incubated for 2 minutes at room temperature. This was then centrifuged for 30 seconds at 10,000 x g. The flow through was then re-loaded in the column and centrifuged once more for 30 seconds at 10,000 x g. The spin filter was discarded and DNA collected in the collection tube was stored at -20^{0}C.

Plant Tissue

To identify the causal agents of RCR of dry bean, sections of the interface between lesions and healthy tissue were removed from infected plants, ground in liquid nitrogen, stored in 1.5ul centrifuge tubes, and the DNA extracted using PowerPlant Pro DNA isolation kit and protocol (MO BIO laboratories Inc.) with a few modifications. At least 10 grams of ground tissue was aliquoted into 2 ml PowerPlant® Bead tubes to which 410

µl of solution PD1 and 40µl of Phenolic separation solution was added. The beads were then heated up to 65^0C for 10 minutes after which 50 µl of solution PD2 was added. 3 µl of RNase was then added to the PowerPlant® Bead Tube and secured horizontally to a MO BIO vortex adapter (MO BIO Catalog# 13000-V1-24) and vortexed at maximum speed for 20 minutes. The bead tubes were then centrifuged at 13,000 x g for 2 minutes and the supernatant transferred to a clean 2 ml collection tube. The DNA isolation process was then continued as described above for culture isolates.

FTA cards

To elute DNA embedded on FTA® Cards for purification, 1 cm^2 within a spotted circle on the FTA® card was excised using sterile forceps and scissors. The section was placed into a 2 ml centrifuge collecting tube and 200-300µl of TE (10Mm Trsi-HCL, 1 mM EDTA, pH=8) elution buffer was added to the tube. The tube was vortexed for 30 seconds and incubated at 4°C for an hour. The hydrated 1 cm^2 strip was squeezed with sterile forceps to release as much of the nucleic acids as possible into the elution buffer in the tube and discarded. To clean the DNA sample for further downstream analysis, PowerClean Pro DNA Cleanup kit (MoBio Laboratories, Inc.) was used. In a new collection tube, 150µl of eluted DNA was pippeted and 70µl of Power Clean® DNA Solution 1 was added. The mixture was gently inverted 3-5 times and 20 µl of clear dissolved Power Clean® DNA Solution 2 was added and inverted 3-5 times to mix. To this, 85 µl of PowerClean® DNA Solution 3 was then added and inverted 3-5 times to mix. This mixture was then incubated at 4°C for 5 minutes and then centrifuged at 10,000 x g for 1 minute at room temperature. The entire volume of supernatant of the mixture was transferred to a clean 2 ml collection tube leaving the pellet in the tube. Next, 70 µl

PowerClean® DNA Solution 4 was added and the supernatant invert 3-5 times to mix, incubated at 4°C for 5 minutes and centrifuged at 10,000 x g for 1 minute. The supernatant was transferred into a clean 2 ml collection tube to which 800 µl of PowerClean® DNA Solution 5 was added. The mixture was then vortexed for 5 seconds and 600 µl loaded into a spin filter and centrifuged at 10,000 x g for 1 minute. The flow through was discarded and the rest of the supernatum added to the spin filter and centrifuged at 10,000 x g for 1. PowerClean® DNA Solution 6 was added at 500 µl to the spin filter and centrifuged at 10,000 x g for 30 seconds. The flow through was discarded and 650µl of 100% ethanol added to the spin column and centrifuge at 10,000 x g for 30 seconds. The flow through was discarded and the spin filter dry centrifuged twice at maximum speed (13,000 x g) for 2 minutes to eliminate any residual alcohol. The spin filter was then carefully placed in a new 2 ml collection tube. Then 100 µl of PowerClean® DNA Solution 7 was added to the center of the white filter membrane and centrifuged at 10,000 x g for 30 seconds. The spin filter was then discard and the DNA collected in the 2 ml collection tube and stored at -20° until use. The procedure was done at room temperature except for the specified incubation temperatures.

DNA based identification methods

2.3.4 Detection of fungi and oomycetes in DNA from FTA® card and plant tissue samples

2.3.4.1 PCR based methods

Amplification of DNA from FTA card and plant tissue with genus/species specific primers

To determine the pathogens associated with dry bean RCR symptoms, the extracted DNA was amplified by conventional PCR with species and genus specific primers designed to identify the four major RCR pathogens of dry bean. Each sample was amplified with ITSFu1F/ITSFu1R to identify *Fusarium* species (Abd-Elsalam *et al*., 2003), RS1/4 to identify *Rhizoctonia solani* (Camporata *et al.*, 2000), MpkF1/MpkR1 for *Macrophomina phaseolina* (Babu *et al.,* 2007) and FM66/58 COX II for *Pythium* species (Martin, 2000). PCR amplification reactions were performed by adding 1µl of genomic DNA solution to 24 µl reaction mixture: 9.5 µl PCR graded sterile ddH2O, 12.5 µl Econotaq PLUS GREEN 2X Master mix, 1µl of 0.2Mm/µl reverse and forward of each primer set to a final volume of 25µl. Amplifications were performed in PTC-100 thermal cycler (Bio-Rad laboratories, Hercules, CA USA). The PCR temperature reaction regimes were set specifically for each group of organisms (Table 2.2). PCR products were electrophoresed in 1.5% Ultra-pure® and Quick dissolve agarose (Invitrogen, Carlsbad, CA, USA) gel in 0.5X Tris-borate EDTA buffer at 100v for 1h, using ethidium bromide stain and visualized in a ChemiDoc EQ System with the Quantity One software (Bio-Rad Laboroatories, CA). A 100bp ladder was used as a marker (Fig. 2.9)

2.3.5 DNA Sanger sequencing to identify culture isolates from plant tissue samples

PCR Amplification and conditions using general fungal primers ITS4 and ITS5

Reaction mixtures and thermocycler program were done according to White *et al.* (1990). The PCR amplification reactions were performed by adding 1µl of genomic DNA solution to 24 µl reaction mixture containing 9.5 µl PCR grade sterile ddH2O, 12.5 µl Econotaq PLUS GREEN 2X Master mix, 1µl of 0.2Mm/µl of ITS4 /ITS5 reverse and

forward primers to a final volume of 25µl. Amplifications were performed in PTC-100 thermal cycler (Bio-Rad laboratories, Hercules, California, USA).

To identify isolates into taxonomic groups, each isolate DNA was amplified with universal primers ITS4/ITS5 and for *Fusarium* species, the elongation factor primers EF1/EF2 were also used. Amplifications were performed as described above for fungi detection. However, in order to increase the yield of the amplicons, the volumes used were multiplied by a factor of 4. Amplicons were electrophoresed as mentioned elsewhere and those amplicons that showed a single band were purified directly using the Ultra-Clean PCR Clean-up Kit and protocol (MO BIO Laboratories, Inc.). Amplicons that showed multiple bands were run again and the gel excised and cleaned using the IBI Gel Extraction (IBI Scientific, IA, USA) sequencing protocol as specified by the manufacturer. Cleaned amplicons were sent for Sanger sequencing at ACGT, Inc. (http://www.acgtinc.com). Sequenced DNA were returned as electrophenograms which were opened with Chomast. V2.0 to generate FASTA files. Sequences were then blast searched in NCBI GenBank and *Fusarium* ID databases for their closest match. The closest matches were used to confirm the exact taxonomic group of each isolate.

2.3.6 High–throughput sequencing of DNA from FTA cards and plant tissue samples

2.3.6.1 Roche 454 Pyrosequencing

Barcoded amplicon sequencing was performed as described by Callaway *et al*., 2010; Dowd *et al*., 2008 and Williams *et al*., 2010 with modifications, using 16S and 18S universal Eubacterial primers, and ITS1/4 Universal fungal primers. A single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used in

the following temperature and cycle regime: 94ºC for 3 minutes, 28 cycles of 94ºC for 30 seconds; 53ºC for 40 seconds and 72ºC for 1 minute; with a final elongation step at 72ºC for 5 minutes . All the PCR amplicon products from different samples were then mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing Roche 454 FLX titanium instruments following manufacturer's guidelines.

Sequence data was processed using a proprietary analysis pipeline (www.mrdnalab.com , MR DNA, Shallowater, TX). Sequences were depleted of barcodes and primers, then short sequences (< 200bp), sequences with ambiguous base calls, and sequences with homopolymer runs exceeding 6bp were removed. Noisy sequences were removed and operational taxonomic units were then defined clustering at 3% divergence (97% similarity) followed by removal of singleton sequences and chimeras. Final Operational Taxonomic Units (OTUs) were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI.

2.3.6.2 Illumina Miseq

Universal Eukaryote primers Euk7F as well as ITS1/4 Universal fungal barcode primers were used in a 30 cycle PCR (5 cycle used on PCR products) using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After amplification, PCR products were visualized in a 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple (approximately 100) samples were pooled together in equal proportions based on their molecular weight and

DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then the pooled and purified PCR product was used to prepare a DNA library by following Illumina TruSeq DNA library preparation protocol. Sequences were joined, depleted of barcodes, sequences less than 150bp, and sequences with ambiguous base calls. Sequences were denoised, OTUs generated and chimeras removed. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI.

2.4 Phylogenetic relationships of isolates from plant tissue and OTUs generated from high-throughput sequencing

To determine the phylogenetic relationships and variabilities of isolates within species, we generated multiple sequence alignments using Clustal Omega 1.2.2 (Sievers *et al*., 2011) (www.ebi.ac.uk) of OTUs from FTA® cards, tissue and culture, and assembled neighbor-joining trees rendered using iTOL (Letumik and Bork, 2016).

2.5 Data analysis

2.5.0 Culture based isolate identification

2.5.1. Morphological identification and characterization

The presence or absence, shape and size of spores on WA and CLA, and color and growth rates of colonies grown on PDA were used separate isolates at genus level with a Fusarium key (Leslie, 2006).

The number of colonies of fungi recovered from each sample was recorded and groups of different species that were identified were expressed using descriptive statistics as

frequency of recovery and also as percentage of abundance. Each recovered isolate taxonomically identified was considered an OTU.

The frequency of isolation was calculated as the average recovery of an isolate in the samples. For each isolate this was calculated as OTUs of an isolate recovered from samples divided by the total number of OTUs

***IF* (Isolation Frequency) = number of OTUs of a species/total number of recovered OTUs**

2.5.2 Pathogenicity testing

To determine differences in the aggressiveness of the pathogenic isolates, the mean lesion length in cm and standard deviations were calculated and means separated by the Tukey Standardized Range test using the SAS software (SAS Institute, Inc., Cary, NC).

2.6.0 Molecular-based identification

2.6.1 Conventional PCR

Detection Frequency

The frequency of detection of each species/genus in DNA from both FTA cards and plant tissue was calculated using the binary data of presence (1) or absence (0) of each species for each sample. The presence or absence data were analyzed using SAS PROC FREQ procedure. Frequency tables were generated which gave the most frequently detected pathogens, and these were compared within and between years. Vein diagrams (http://creately.com/Draw-Venn-Diagrams-Online, 2008-2016 Cinergix Pty. Ltd) were used to show single and multiple detegtions of pathogen groups in the samples.

2.6.2 High–throughput sequencing of DNA from FTA cards and plant tissue samples

Relative Abundance

Data from both Roche 454 and Illumina were analyzed as OTU counts (number of OTUs) and as biomass (reads of sequences). This was used to determine qualitatively and quantitatively the most abundant species in the samples in the different locations and also the most prevalent species over the different years. Abundance was analyzed as relative abundance of reads of species known to be associated with RCR. Analysis of variance of reads of species known to be associated with RCR was done in SAS and means of species separated by the Tukey Standardized Range test, to determine the most important (abundant) group of pathogens.

2.6.3 Comparison of methods for preserving and transporting DNA- FTA® cards vs. plant tissue

Comparisons between methods of preserving and transporting DNA of RCR pathogens was done by analyzing DNA from matched samples of the DNA embedded FTA® cards and plant tissue. The analysis was done by calculating the correlation of four major RCR pathogens between matched samples of FTA® cards and plant tissue. The analysis was done with parametric correlations Pearson's (r) and non-parametric rank ordinal correlations; Spearman's (rs), and Kendall's Tau (rho) using the Paleontological Statistics software package (PAST) (Hammer *et al*., 2001) and SAS. The rate of agreement between the two methods of DNA preservation, transportation and RCR sampling was calculated for both sequencing and PCR data using Kappa Statistic (Viera

& Garrett, 2005; McHugh, 2012). Pyrosequencing abundance data was first transformed into binary presence (1) and absence (0) before calculating the Kappa statistic. Kappa statistic were calculated in excel as well as statistical software SPSS "IBM SPSS Version 19.0"

Kappa statistic = [P(o)-P(e)] / [1-P(e)], where, P(o) is the observed agreement between methods and P(e) is the expected agreement between methods.

2.6.4 Comparison of methods for identification of RCR pathogens

Comparisons between the classical culture, molecular PCR and high-throughput sequencing based methods was done by calculating the correlation between the methods and the rate of agreement. The analysis was done using the Paleontological Statistics software package (PAST) rank ordinal non-parametric statistics; Spearman's (rs), and Polyserial (rho). Rate of agreement of the methods was calculated by first transforming abundance to presence (1)/absence (0) and then calculating the Kappa statistic.

2.6.5 Diversity index

Diversity of fungi/oomycetes associated with RCR symptomatic bean plants among locations and years were calculated using the Shannon diversity indices calculated as $H=\_\_Si\ 1(ni/N)\mathrm{In}(ni/N)$ (Shannon, 1963) and Simpson diversity indices, calculated as $(1\_D)\_Si\ 1(ni/N)2$ (Simpson, 1949) to take into consideration the species richness and dominance. For the analysis with pyrosequencing data, only OTUs with sequence reads above 20 were considered. The indices were calculated with the PAST v.3.12 program.

2.6.6 Phylogenetic relationships

Multiple sequencing alignments were generated with Clustal Omega (1.2.2.) software, and trees rendered using iTOL of DNA sequences from FTA® cards and plant tissue, per location/year. Venn diagrams ((http://creately.com/Draw-Venn-Diagrams-Online, 2008-2016 Cinergix Pty. Ltd)) were used to display shared and unique OTUs.

2.7 Results

2.7.1 Morphological Identification and Characterization

A total of 204 isolates were recovered from symptomatic dry bean plant tissue sampled between 2014 and 2015, with 128 isolates recovered from samples collected at Misamfu in 2014 and 2015, 67 isolates from samples collected at Kabwe in 2015 and 9 samples from Mpika (Appendix 3). The 204 isolates from Misamfu and Kabwe were morphologically separated into 5 groups based on colony morphology on PDA and micro-and macroscopic characteristics on WA and CLA (Fig. 2.6 A – E). Group A isolates were consistent with *Fusarium* spp. with typical crescent shaped spores of different sizes and varying colors and growth rates on PDA (Fig 2.6 A, Table 2.3), Group B isolates were consistent with *Rhizoctonia* spp. (Fig. 2.6 B) and Group C isolates were consistent with *Pythium* spp. (Fig. 2.6 C). Group D isolates were consistent with *Macrophomina phaseolina* (Fig. 2.6 D) and group E isolates were a group of unidentified fungi (Fig. 2.6 E).

Group A isolates consisting of species of *Fusarium* had the highest isolation frequency of 43% and 30 % at Misamfu in 2014 and 2015 respectively, and 93% and 100 % in Kabwe and Mpika repectively in 2015 (Fig. 2.7; Table 2.4). This indicates that the *Fusarium*

species, specifically *F. oxysporum*, are the most important species associated with RCR of dry bean.

2.7.2 Pathogenicity Testing

Of the isolates recovered from tissue samples from Misamfu in 2014 (Appendix 4), 38 were pathogenic of which 60 % were *Fusarium* spp. Of the isolates recovered from 2015 samples from Misamfu, Kabwe and Mpika, 41, 56 and 9 respectively were pathogenic of which 41%, 98% and 100% were *Fusarium* spp. respectively (Table 2.4). Eight isolates were recovered from healthy (control) dry bean tissue and 6 were pathogenic.

2.7.3 Taxonomic classification of culture isolates based on Sanger sequencing

Sanger Sequencing taxonomically classified isolates into 32 different species. The largest group of isolates was the *Fusarium* spp. group (55 %) followed by *Rhizoctonia solani* (31 %) *Macrophomina* and *Pythium* were 7 % and 6 % respectively (Fig. 2.8A). Within the pathogenic *Fusarium* species, *Fusarium oxysporum* was the largest group constituting 55% of the isolates, followed by *Fusarium equiseti* with 33 %, *Fusarium solani* 7 %, and *Fusarium proliferatum* and *Fusarium incarnatum* 1% each (Fig. 2.8B). *Fusarium oxysporum* and *Fusarium equiseti* had the highest isolation frequency, indicating that these two species are more prevalent.

2.7.4 Species/genus detection frequency in PCR analysis

Fusarium spp, *Rhizoctonia solani*, *Macrophomina phaseolina* and *Pythium* spp. amplified with genus/species specific primers banded at respective lengths.(Fig. 2.9). Based on PCR amplification, *Fusarium* spp. had the highest detection frequency across the years, except in FTA® cards from Misamfu in 2013 and tissue samples from Kabwe in 2015, in which *Macrophomina phaseolina* detection frequency was higher (Fig 2.10.)

Fusarium spp. were detected in 100% of the samples on FTA® cards and plant tissue from Misamfu in 2015 and on FTA® cards from Kabwe in 2015. On average, the detection frequency of *Fusarium* spp. was 85 % in plant tissue and 71 % on FTA® cards at Misamfu between 2014 and 2015. The high detection frequency of *Fusarium* species relative to the other groups indicate that *Fusarium* species were the most prevalent group of pathogens associated with RCR in the areas sampled. More than one taxa was detected in most of the samples collected on both FTA® cards and plant tissue (Table 2.5, Fig. 2.11). *Fusariun* spp had the highest number of single and multiple occurances (combination) with other species groups in the samples. This indicates that the samples may have been infected by more than one pathogen and *Fusarium* species were the primary group in all the samples.

2.7.5 High–throughput sequencing of DNA from FTA® cards and plant tissue samples

Relative Abundance

Pyrosequencing of DNA from FTA® cards and plant tissue samples showed a diverse group of fungi (Fig 2.12 A-B). There were significant differences (0.05, $P<0.001$) in the number of reads of the RCR pathogens detected in the DNA by pyrosequencing in all the samples from 2013 – 2015 (Table 2.6). The *Fusarium* species were the most abundant taxa in all DNA analyzed. Within the *Fusarium* group, *Fusarium oxysporum* was the most abundant in all the years (Fig 2.13). The mean number of reads of *Fusarium oxysporum* was significantly higher and different ($p = 0.05$) from the other *Fusarium* species and fungi associated with RCR (Table 2.7). This indicates that among the *Fusarium* species, *Fusarium oxysporum* is most important.

2.7.6 Comparison of methods of preserving and transporting DNA and RCR identification

There were significant correlations between FTA® cards and plant tissue samples at 0.05 and 0.01 levels, in all the years (Table 2.8, Appendix 5) as well as high inter-method agreements between FTA® cards and plant tissue with high observed agreement averaging at 70% and a relatively high Kapa 0.6. (Table 2.8). This indicates that FTA® cards can be used as a substitute to plant tissue in sampling and transporting DNA in identification of RCR pathogens of dry beans. Significant correlations between methods of RCR identification (Culture based vs PCR vs Pyrosequencing) indicate that these methods are similar in identification of RCR and using either method would lead to the same conclusion.

2.7.8 Phylogenetic relationships and diversity indices

Diversity indices

There were differences in the diversity of species at the locations sampled in the different years. Kabwe had the highest Simpson 1-D and Shannon H was 0.93 and 2.7 and 0.95 and 2.6 in FTA® card and plant tissue samples respectively (Fig 2.14). This indicates that Kabwe may have a more diverse population of fungi in the soil than Misamfu and Lusaka. There was also an increase in the diversity at Misamfu between 2014 and 2015 (Fig. 2.14).

Phylogenetic Relationships

There were common and specific OTUs of all the primary pathogens associated with RCR between Misamfu and Kabwe (Fig 2.17). Also specific and common OTUs were found between tissue and FTA® cards within and between locations. This indicates that

some of the pathogens may be the same strains present at the different locations simultaneously.

2.8 Discussion

Identification of pathogens causing disease on plants is an integral part of development of control strategies. Both morphological and molecular techniques can be employed to this end. In most diagnostic clinical labs, the use of quick and efficient methods in the diagnosis of plant diseases is favored. Most of the isolates that were recovered from symptomatic bean tissue were able to be classified into different groups depending on their morphological features. The largest of these groups had isolates which had features consistent with *Fusarium* species.

Most of the isolates associated with RCR in Zambia were found to belong to the *Fusarium* species. Most isolates in this group produced banana or canoe shaped spores that were septate. Isolates of *Rhizoctonia solani, Pythium* spp. and *Macrophomina phaseolina* also were identified. In some samples, isolates belonging to different genera were recovered from the same sample. This is in agreement with what other researchers have found, confirming the principle of synergistic action of root rot pathogens (Abawi and Pastor-Corrales, 1990; Bolton *et al.*, 2010). *Rhizoctonia solani* and *Pythium* spp. were never isolated alone from plant samples but were always in association with *Fusarium* spp. This affirms the predominance and abundance of Fusarium. Over 90% of the isolates of Fusarium recovered were pathogenic. Through the pathogenicity tests we were able to prove or ascertain that the isolates we recovered were actually the ones that had caused the disease. Most of the classification that was done based on morphological features matched with the classification derived from use of fungi and oomycete specific

markers and the ITS –rDNA. However, there are isolates that could not be placed in any taxa based on morphology. Morphological identification is a tedious and time consuming activity in most cases, and the classification is only accurate to the genus level. However, obtaining isolates in this classical method of identification is very important, especially in terms of screening for disease resistance in the development of resistant varieties.

Conventional PCR is a very easy procedure used in the detection of pathogens. If the interest is not in how much of a pathogen but just whether it is present or absent, species and genus specific oligonucleotides can be used to address this. With genus and species specific primers for *Rhizoctonia solani, Fusarium* spp. and *Macrophomina phaseolina,* we were able to determine that *Fusarium* spp. were present in almost all the samples that we had analyzed using ITSFu1F/ITSFu1R primers. This means that *Fusarium* spp. had a very high detection frequency. Using the data from analyzing DNA from plant tissue over the locations in the Northern Province where samples were collected, the trend was the same as in the central parts of Zambia. Since Fusarium was detected in nearly all the samples, it is most likely to be the primary causal agent of root-crown rots in these areas. Mukankusi *et al.* (2003) found similar results when looking at root rots in western Uganda. While PCR might be a fast and easy way to detect some species of pathogens, it is possible that it can also amplify species that are not really pathogenic but might just be commensals or saprophytes. This is especially true when using primers that are not entirely specific to a pathogenic strain of an isolate. *Fusarium* is known to be a ubiquitous and it is found wherever dry bean is grown (Harveson *et al.,* 2005), thus any primer that would amplify the genus as a whole would pick up all sorts of *Fusarim* spp. This would support the observation of high detection frequencies of *Fusarium* in the

samples. DNA extraction from the bean plant tissue is done on the pieces of tissue cut at the interface of the healthy and infected part of the lesion to make sure that the organism causing disease is included (Smith and Burgoyne, 2004). However, it is a possibility that other opportunistic fungi also occupy the tissue and thus are amplified when performing PCR. Using PCR analysis we detected the presence of more than one pathogen being amplified in a sample. This is more so with species of *Rhizoctonia, Pythium and Macrophomina* and reflects what we found with isolations from tissue where different isolates from different genera were detected from one sample. In 2013, *Macrophomina phaseolina* was detected at rate of 100% (Fig..2.10) meaning it was present in all the samples that had been collected. However, *Macrophomina phaseolina* has never been reported as a major pathogen on dry bean in the southern Africa region. Therefore its high frequency in samples collected in 2013 and then the reduced frequency afterwards could be explained by environmental factors. *Macrophomina phaseolina* is a pathogen favored by hot dry weather (Khan, 2007), the hot dry spell occuring in 2013 migh have favored proliferation of *Macrophomina phaseolina* and the higher rainfall received in 2014 might have decreased its incidence as only a few tissue samples were recorded with *Macrophomina phaseolina.*

The use of pyrosequencing produced more data in terms of diversity as well as abundance of fungi found in association with RCR symptomatic plants, than simple isolation culturing and PCR. Direct counts of OTU's as well as the number of DNA sequence reads of each of the species taxonomically identified showed that *Fusarium* spp. were the most dominant and abundant not only in every sample but also at every location. Deep sequencing has more resolution and is thus able to generate more data from DNA.

We initially were not getting any OTUs of *Pythium* from pyrosequencing data although we were detecting it in PCR and also recovered it a few times in culture. Therefore, we expanded the genome region we were looking at from 16 to 18S and changed primers. Euk7 primer that covers the entire 18S region was able to detect *Pythium* species. This demonstrates that the quality of what you are looking for will also depend on the genome regions you investigate and the primers that you are using. *Pythium* is not a fungus, and in this case using ITS primers did not amplify the *Pythium* DNA, and an error indicating its absence could have been made. Nonetheless, pyrosequencing resulted in more diversity and showed new species associated with RCR, which showed strong correlations with known RCR pathogens.

Studying root rots sometimes can be very challenging due to the nature of their ecology. In 2015 we collected data from control plants that showed no symptoms of RCR but after analysis we isolated species of *Fusarium* and *Rhizoctonia* in culture that were pathogenic. This was corroborated in the analysis of DNA by PCR and pyrosequencing. The existence of pathogens as endophytes has been reported by other researchers (Rodrigues, A. A. C., & Menezes, M. 2005). This seems to suggest that there are mechanisms within the pathogen that might trigger or halt pathogenesis and it may do so when conditions are optimal.

We compared the classical isolation and culturing methods, finding strong correlations on all the methods and on the use of FTA® cards as a means of DNA collection and preservation. FTA® cards have primarily been used in medical sciences but are slowly becoming a media of choice for DNA preservation and transport. Therefore in studies

concerning root rot fungi, FTA® cards can also be a simple means of DNA collection and preservation.

Culture based methods of identification as well as molecular based methods all revealed that *Fusarium* spp. were the primary fungi associated with RCR in Zambia. The use of advanced molecular techniques such as pyrosequencing gave strong evidence of the prevelance of pathogens associated with RCR and also more information on the particular *Fusarium* spp in abandance as *Fusarium oxysporum*. Therefore DNA analysis alone can give direction as to which pathogens are important in an area and should be considered for identiying a primary pathogen that can be used in the development of RCR resistance. The significant corelation between the culture method and sequencing also means it might not always be necessary to do isolations from symptomatic samples to determine which pathogens are important. Having determined the primary pathogens causing RCR of dry bean in Zambia, it will now be imperative to screen germplasm in the greenhouse for resistanct to the pathogen and incoperate lines showing resistance in the national dry bean breeding program.

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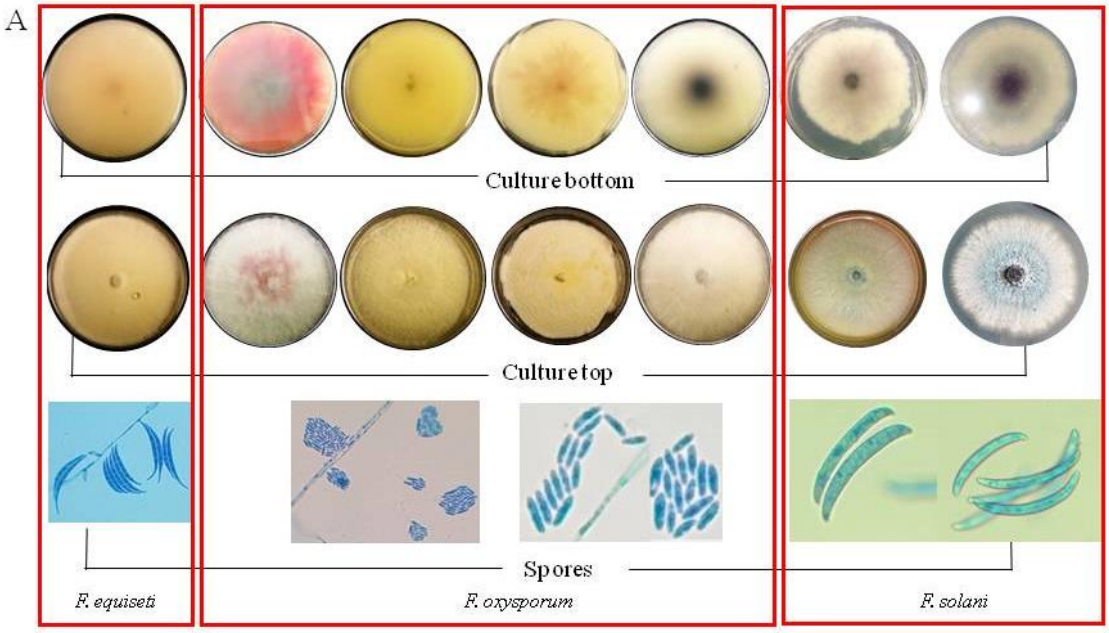
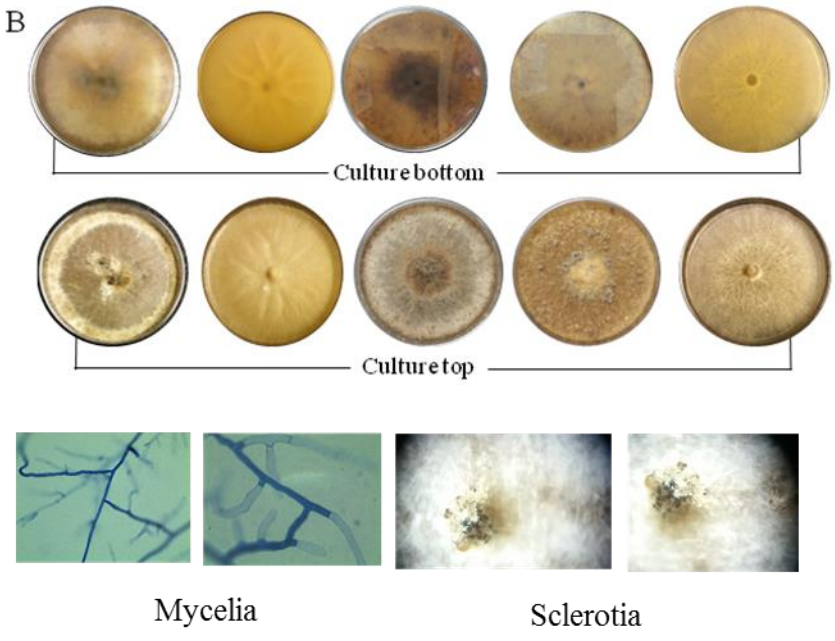
Figures and Tables.





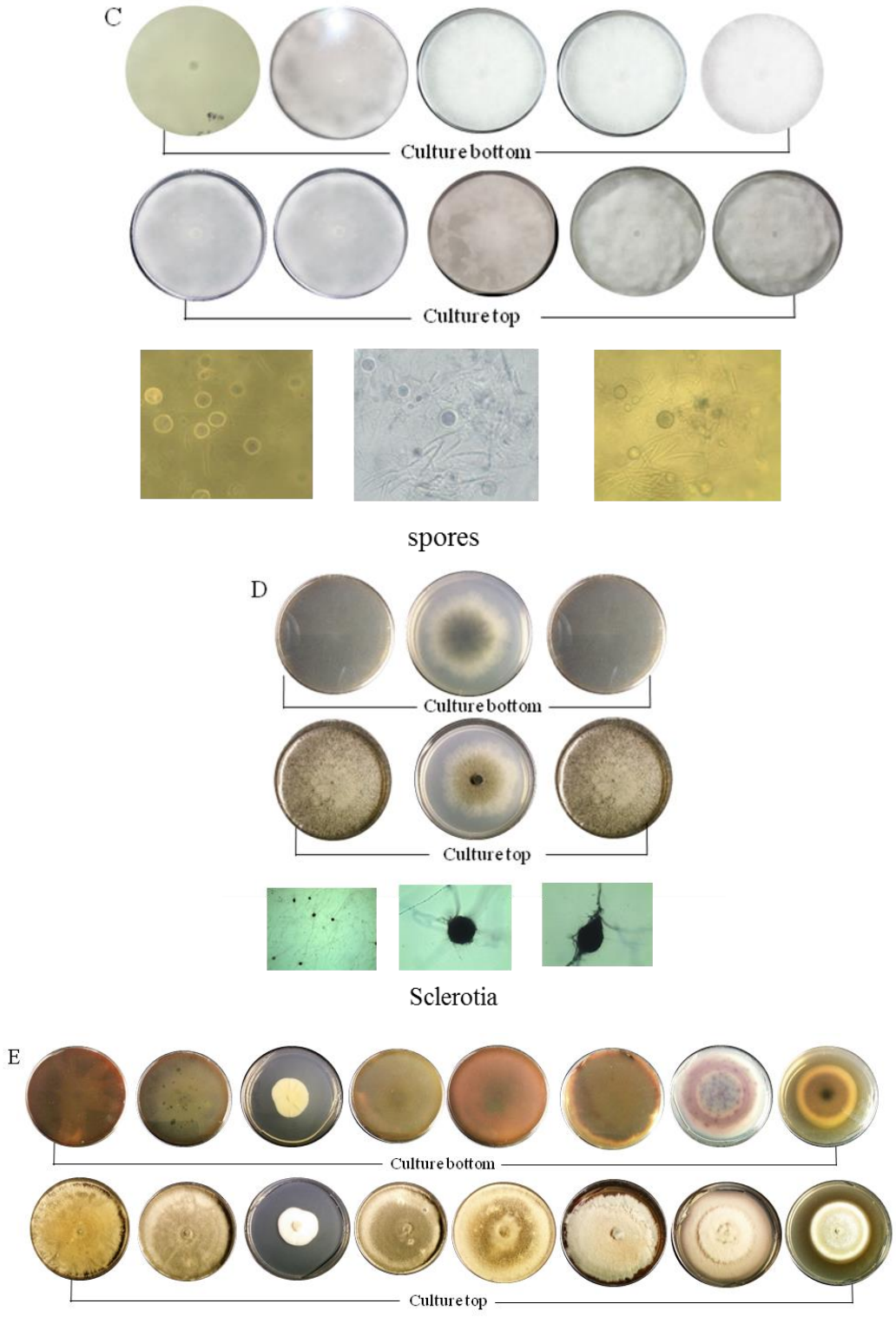


Figure 2.6 A-E Culture characteristics of RCR pathogens examined in this study. A-*Fusarium* spp., B- *Rhizoctonia solani*, C-*Pythium* spp., D- *Macrophomina phaseolina*, E-other fungi

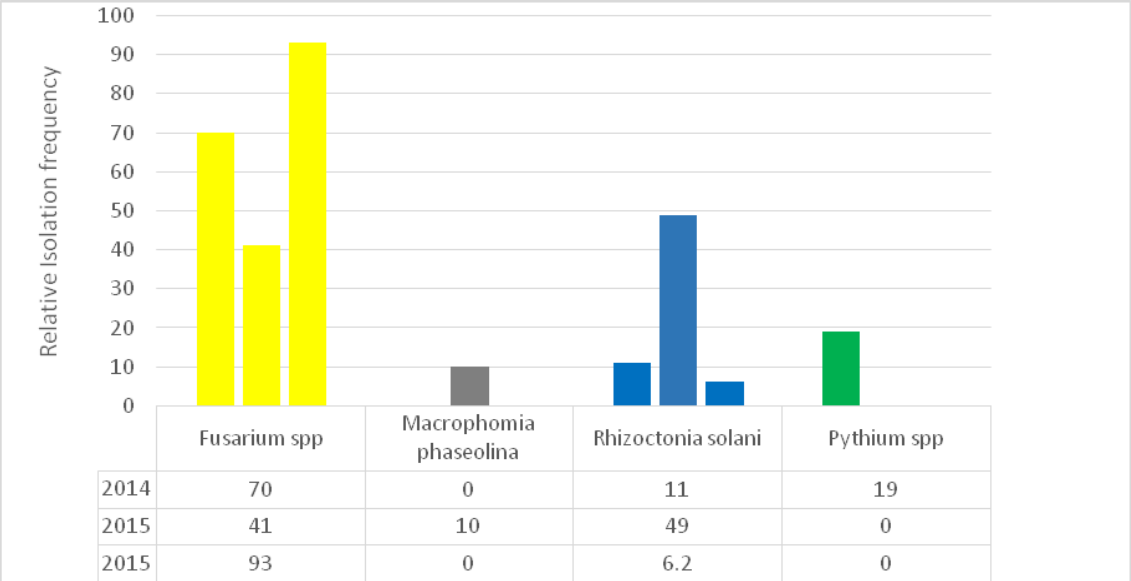
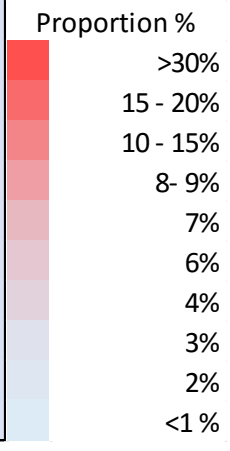
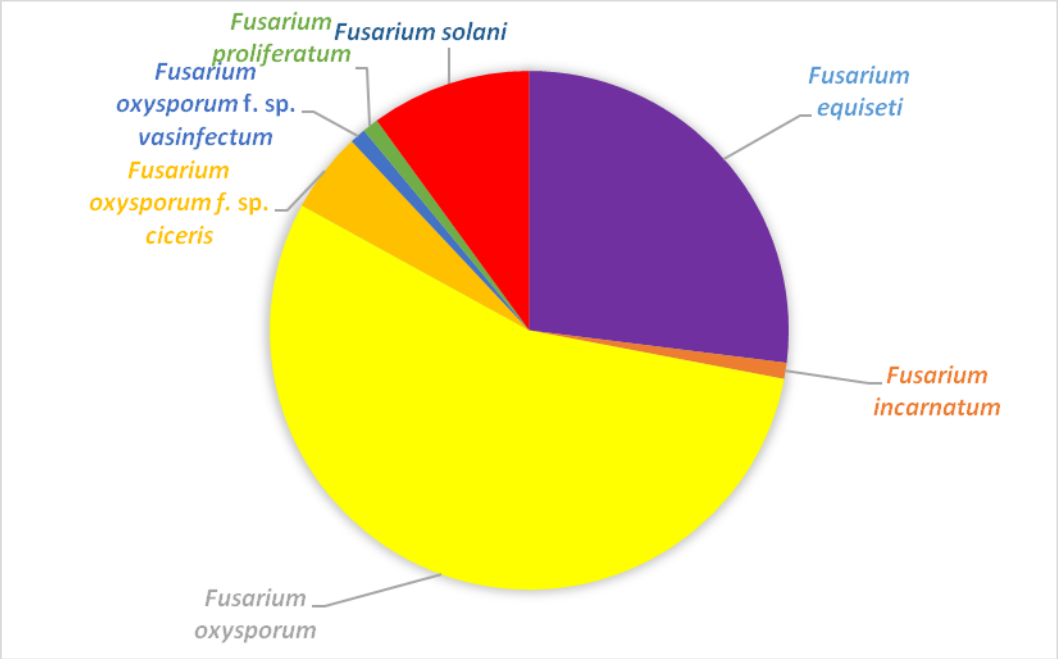


Figure 2.7. Relative isolation frequencies of pathogenic fungi recovered from symptomatic tissue samples of dry beans from Misamfu and Kabwe, Zambia from 2014 and 2015.

Species	Proportion
Fusarium oxysporum (30 %)	
Fusarium equiseti (17.6%)	
Rhizoctonia solani (10 %)	
Phoma sp. NT-2015a (6 %)	
Fusarium solani (5 %)	
Alternaria sp. 1 TMS-2011(2.5 %)	
Fusarium oxysporum f. sp. Ciceri (2.5 %)	
Macrophomina phaseolina (2.5 %)	
Phoma cf. herbarum G11(2.5 %)	
Alternaria longissima (2. %)	
Chaetomium globosum (2.0 %)	
Pythium myriotylum (2 %)	
Ceratobasidium sp. (1.0%)	
Curvularia trifolii (1%)	
Epicoccum sorghinum(1 %)	
Penicillium janthinellum (1 %)	
Alternaria alternata(0.5 %)	
Alternaria burnsii (0.5 %)	
Aspergillus sp. 085241B (0.5%)	
Chaetomium coarctatum (0.51 %)	
Chaetomium sp. NRRL 66025 (0.5 %)	
Curvularia borreriae (0.5 %)	
dothideomycete sp (0.5 %)	
Fusarium incarnatum (0.5 %)	
Fusarium oxysporum f. sp. Vasinfectum (0.5 %)	
Fusarium proliferatum(0.5 %)	
Fusarium sp. (0.5 %)	
Gibberella sp (0.5 %)	
Phoma glomerata (0.5 %)	
Phoma herbarum (0.5 %)	
Pleosporales sp. RKDO795 (0.5 %)	
Rhizoctonia bataticola (0.5 %)	
Rhizoctonia fragariae (0.5 %)	
Septoria arundinacea (0.5 %)	
Setophoma terrestris (0.5 %)	
Sistotrema brinkmannii (0.5 %)	
Talaromyces cellulolyticus CF-2612 (0.5 %)	
Thielavia sp (0.5 %)	



A



B

Figure 2.8. (A) species of fungi/oomycetes recovered from plant tissue samples of dry beans from Misamfu and Kabwe from 2014 and 2015 taxonomically identified to species by Sanger sequencing with universal primers ITS 4/5. (B) Percentages of recovered pathogenic *Fusarium* speciesin total samples examined from 2014 to 2015

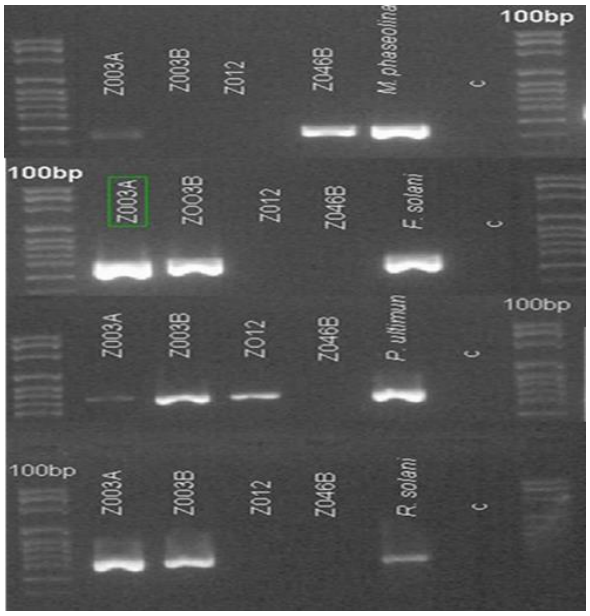


Figure 2.9 Gel electrophoresis of PCR amplification of DNA samples form RCR infected plants with genus specific primers used in this study. Columns 1-8: 1) 100 bp ladder 2-5) samples 6) positive control: *M. phaseolina, F. solani, P. ultimum, R. solani* 7) Negative control 8) 100 bp ladder. (Multiple detection of groups in sample Z003B)

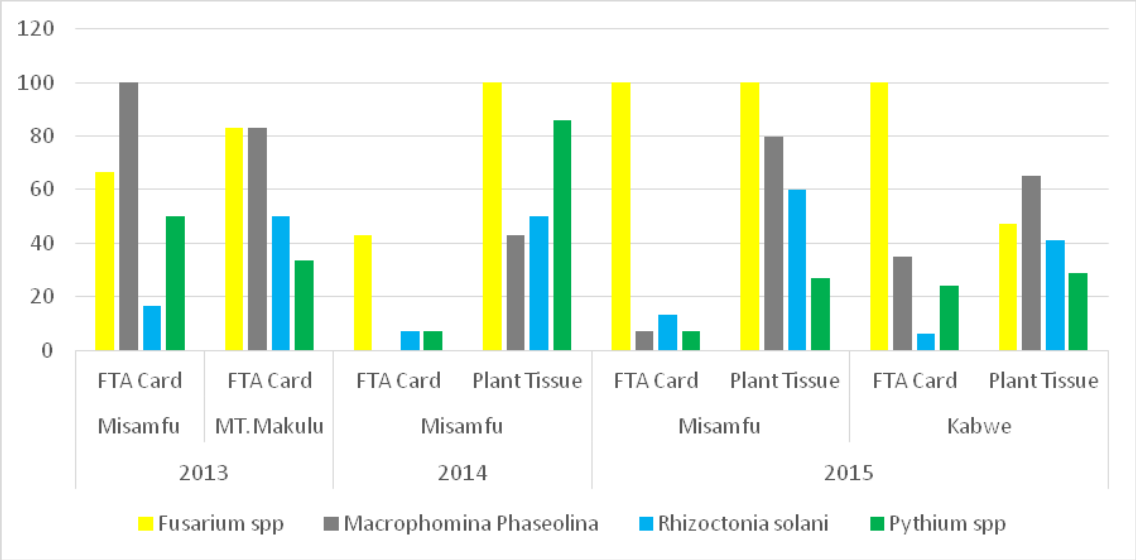


Figure 2.10 PCR analysis detection frequencies of the four major pathogen groups associated with RCR of dry bean in DNA from FTA cards and tissue samples collected between 2013 to 2015 in Misamfu, Mt. Makulu and Kabwe.

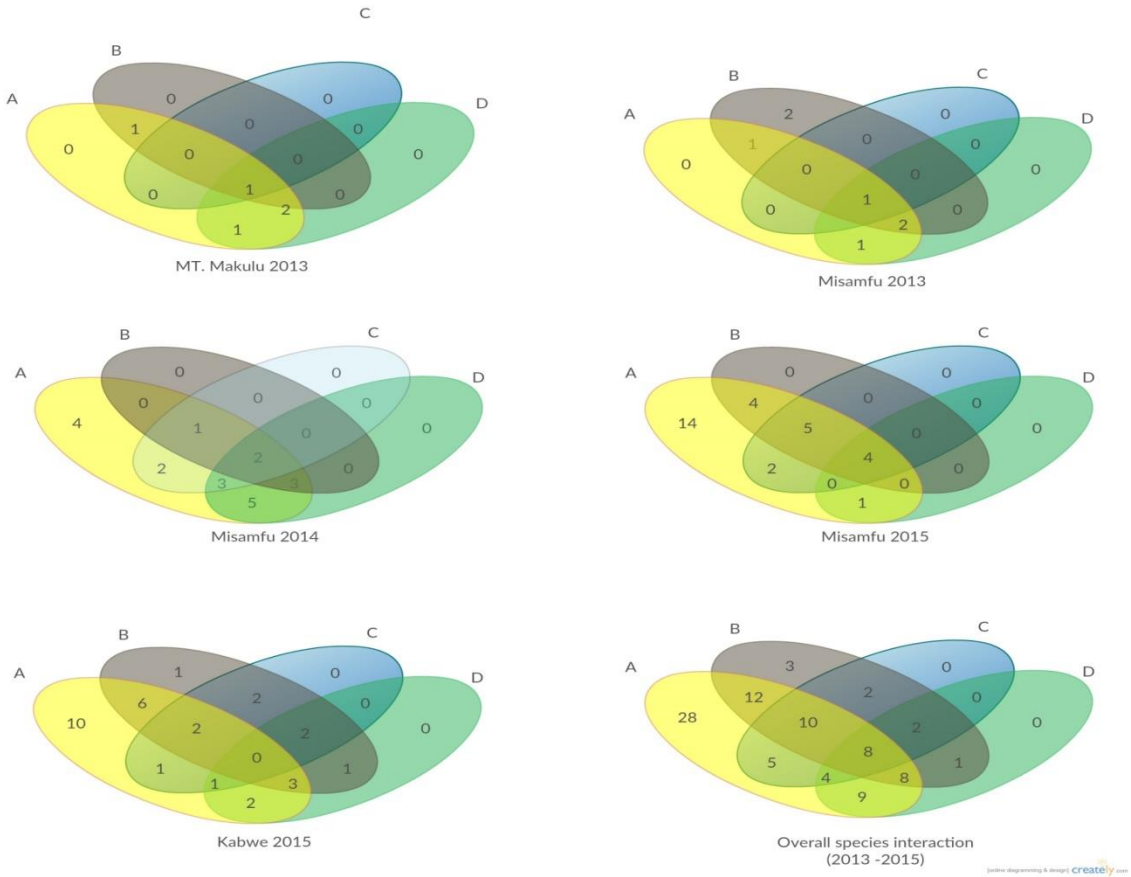


Figure. 2.11 Single and multiple species detection by amplification using genus-specific primers. DNA from samples collected at Mt. Makulu, Misamfu and Kabwe, Zambia in 2013-2015. The numbers indicate the number of times a species group was detected alone and in combination with other groups. A= *Fusarium* species,*Macrophomina phaseolina*, C= *Rhizoctonia solani*, D= *Pythium species*.

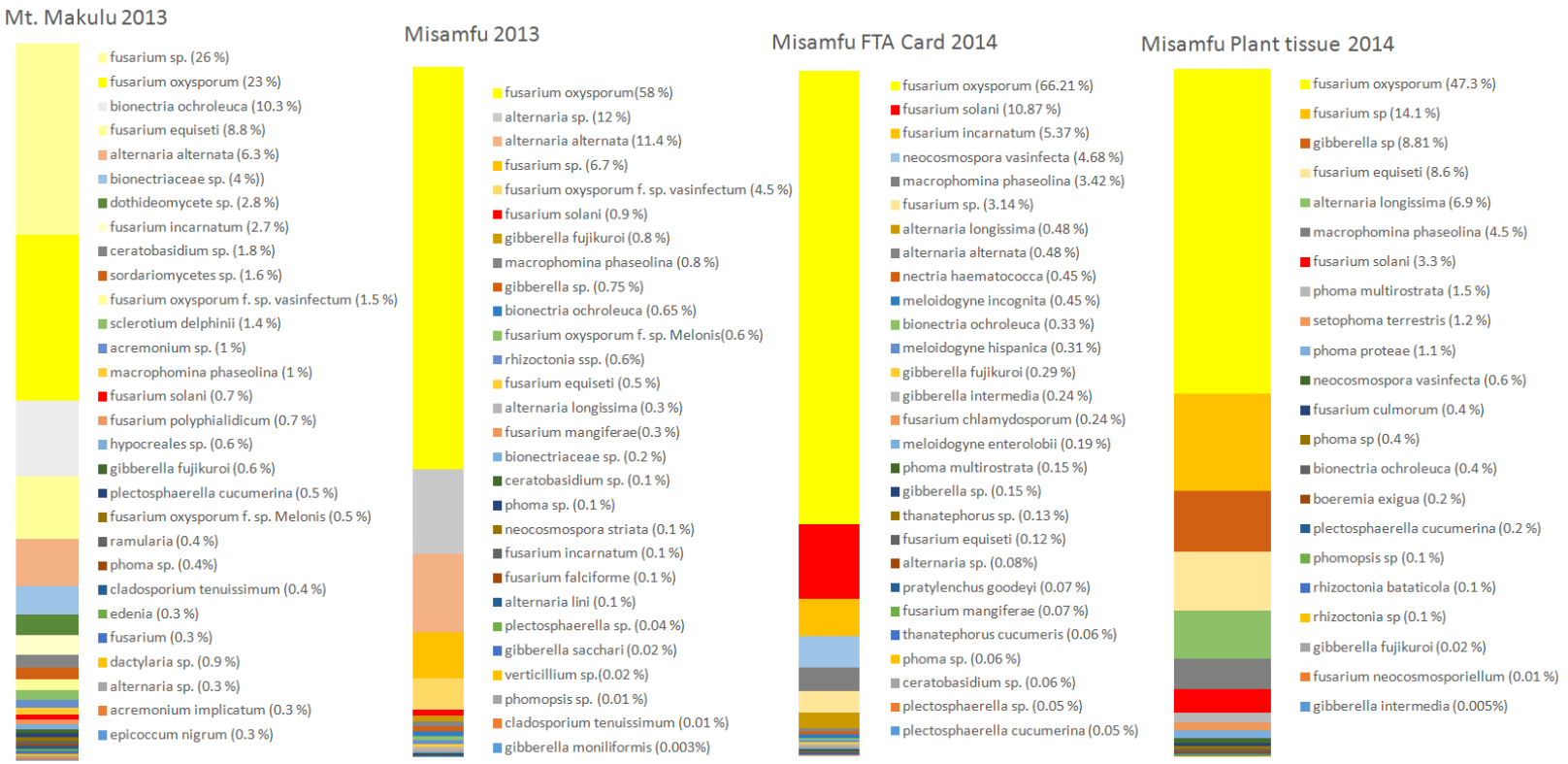

Mt. Makulu 2013
fusarium sp. (26 %)
fusarium oxysporum (23 %)
bionectria ochroleuca (10.3 %)
fusarium equiseti (8.8 %)
alternaria alternata (6.3 %)
bionectriaceae sp. (4 %))
dothideomycete sp. (2.8 %)
fusarium incarnatum (2.7 %)
ceratobasidium sp. (1.8 %)
sordariomycetes sp. (1.6 %)
fusarium oxysporum f. sp. vasinfectum (1.5 %)
sclerotium delphinii (1.4 %)
acremonium sp. (1 %)
macrophomina phaseolina (1 %)
fusarium solani (0.7 %)
fusarium polyphialidicum (0.7 %)
hypocreales sp. (0.6 %)
gibberella fujikuroi (0.6 %)
plectosphaerella cucumerina (0.5 %)
fusarium oxysporum f. sp. Melonis (0.5 %)
ramularia (0.4 %)
phoma sp. (0.4%)
cladosporium tenuissimum (0.4 %)
edenia (0.3 %)
fusarium (0.3 %)
dactylaria sp. (0.9 %)
alternaria sp. (0.3 %)
acremonium implicatum (0.3 %)
epicoccum nigrum (0.3 %)
Misamfu 2013
fusarium oxysporum(58 %)
alternaria sp. (12 %)
alternaria alternata (11.4 %)
fusarium sp. (6.7 %)
fusarium oxysporum f. sp. vasinfectum (4.5 %)
fusarium solani (0.9 %)
gibberella fujikuroi (0.8 %)
macrophomina phaseolina (0.8 %)
gibberella sp. (0.75 %)
bionectria ochroleuca (0.65 %)
fusarium oxysporum f. sp. Melonis(0.6 %)
rhizoctonia ssp. (0.6%)
fusarium equiseti (0.5 %)
alternaria longissima (0.3 %)
fusarium mangiferae(0.3 %)
bionectriaceae sp. (0.2 %)
ceratobasidium sp. (0.1 %)
phoma sp. (0.1 %)
neocosmospora striata (0.1 %)
fusarium incarnatum (0.1 %)
fusarium falciforme (0.1 %)
alternaria lini (0.1 %)
plectosphaerella sp. (0.04 %)
gibberella sacchari (0.02 %)
verticillium sp.(0.02 %)
phomopsis sp. (0.01 %)
cladosporium tenuissimum (0.01 %)
gibberella moniliformis (0.003%)
Misamfu FTA Card 2014
fusarium oxysporum (66.21 %)
fusarium solani (10.87 %)
fusarium incarnatum (5.37 %)
neocosmospora vasinfecta (4.68 %)
macrophomina phaseolina (3.42 %)
fusarium sp. (3.14 %)
alternaria longissima (0.48 %)
alternaria alternata (0.48 %)
nectria haematococca (0.45 %)
meloidogyne incognita (0.45 %)
bionectria ochroleuca (0.33 %)
meloidogyne hispanica (0.31 %)
gibberella fujikuroi (0.29 %)
gibberella intermedia (0.24 %)
fusarium chlamydosporum (0.24 %)
meloidogyne enterolobii (0.19 %)
phoma multirostrata (0.15 %)
gibberella sp. (0.15 %)
thanatephorus sp. (0.13 %)
fusarium equiseti (0.12 %)
alternaria sp. (0.08%)
pratylenchus goodeyi (0.07 %)
fusarium mangiferae (0.07 %)
thanatephorus cucumeris (0.06 %)
phoma sp. (0.06 %)
ceratobasidium sp. (0.06 %)
plectosphaerella sp. (0.05 %)
plectosphaerella cucumerina (0.05 %)
Misamfu Plant tissue 2014
fusarium oxysporum (47.3 %)
fusarium sp (14.1 %)
gibberella sp (8.81 %)
fusarium equiseti (8.6 %)
alternaria longissima (6.9 %)
macrophomina phaseolina (4.5 %)
fusarium solani (3.3 %)
phoma multirostrata (1.5 %)
setophoma terrestris (1.2 %)
phoma proteae (1.1 %)
neocosmospora vasinfecta (0.6 %)
fusarium culmorum (0.4 %)
phoma sp (0.4 %)
bionectria ochroleuca (0.4 %)
boeremia exigua (0.2 %)
plectosphaerella cucumerina (0.2 %)
phomopsis sp (0.1 %)
rhizoctonia bataticola (0.1 %)
rhizoctonia sp (0.1 %)
gibberella fujikuroi (0.02 %)
fusarium neocosmosporiellum (0.01 %)
gibberella intermedia (0.005%)

Figure 2.12 A.

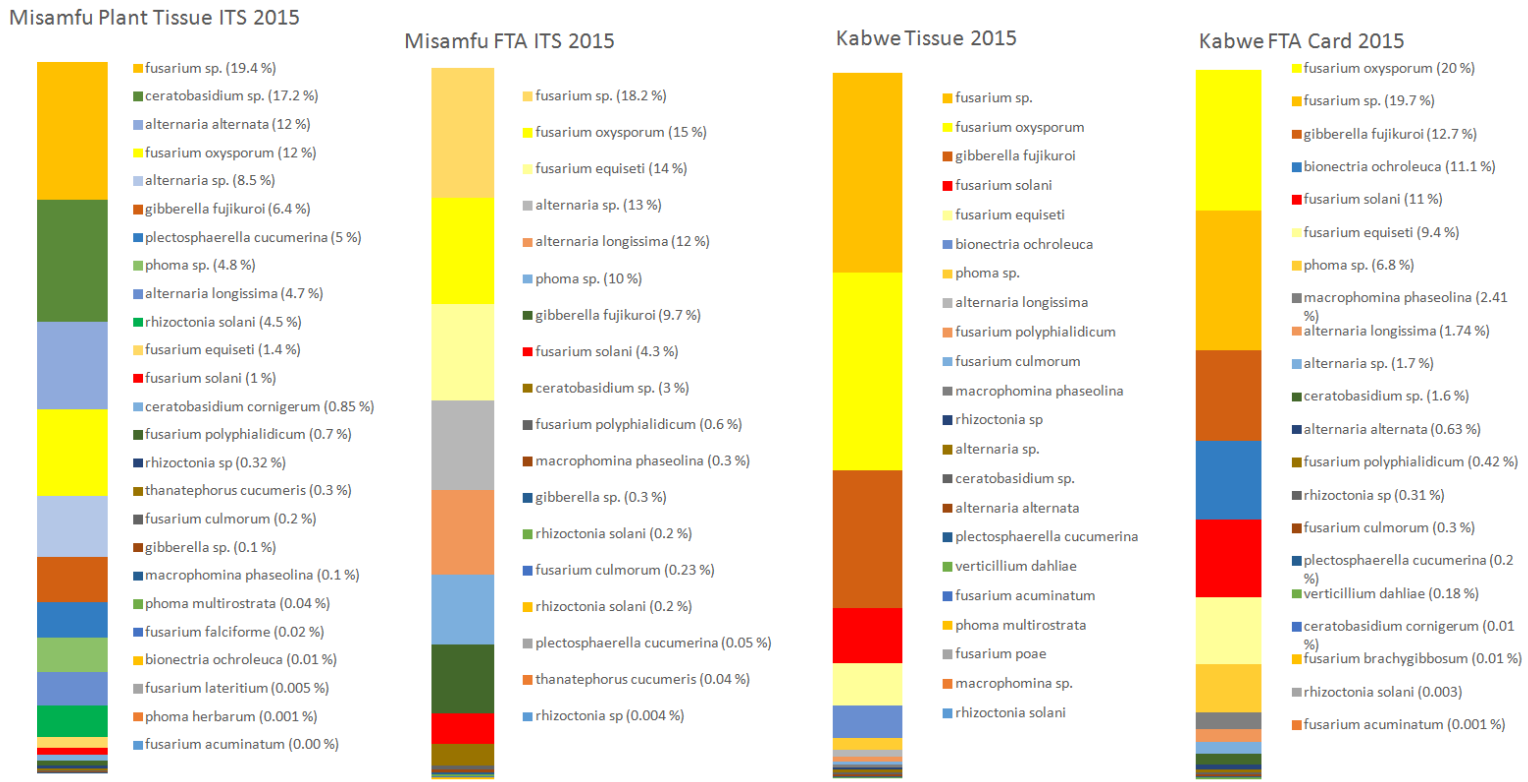


Figure 2.12 B

Figure 2.12. A – B Relative Abundance and diversity of fungi species detected in DNA from RCR symptomatic dry bean plant tissue and embedded FTA cards from Mt. Makulu Central Research Station (MMCRS), Misamfu Regional Research Center (MRRC) and Kabwe Research Station (KRS). DNA was analyzed by Rosche 454 pyrosequencing. Fungi in each column are colored according to species and are listed according to their abundance.

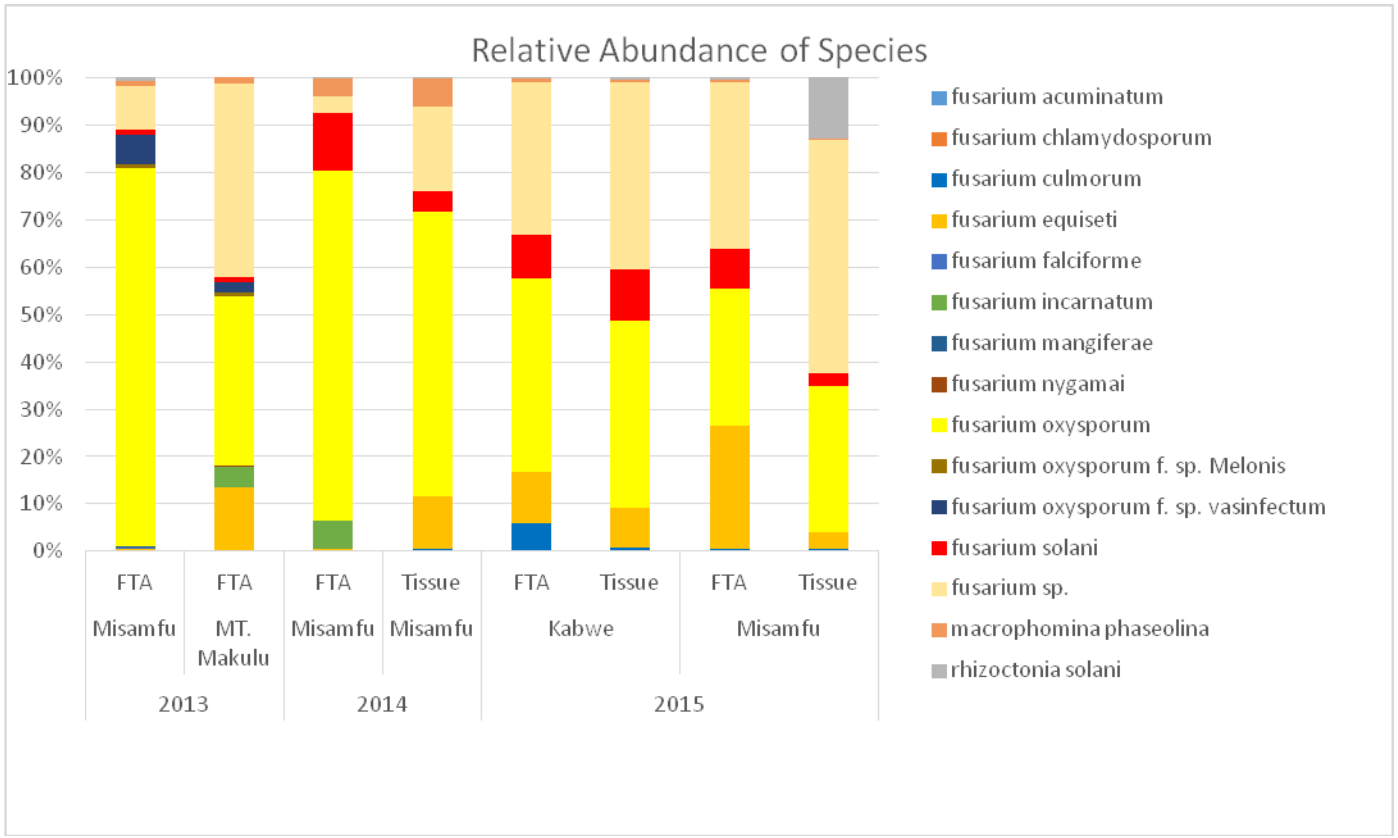


Figure 2.13 Relative abundance of species known to be associated with RCR of dry bean, *Fusarium* spp, *Rhizoctonia solani*, and *Macrophomina phaseolina*. Abundance is based on the number the total of reads of each species. Only OTUs with reads above 20 were considered. OTUs were generated from Pyrosequencing of DNA from FTA cards and tissue samples collected between 2013 and 2015 in Misamfu, Mt. Makulu and Kabwe.

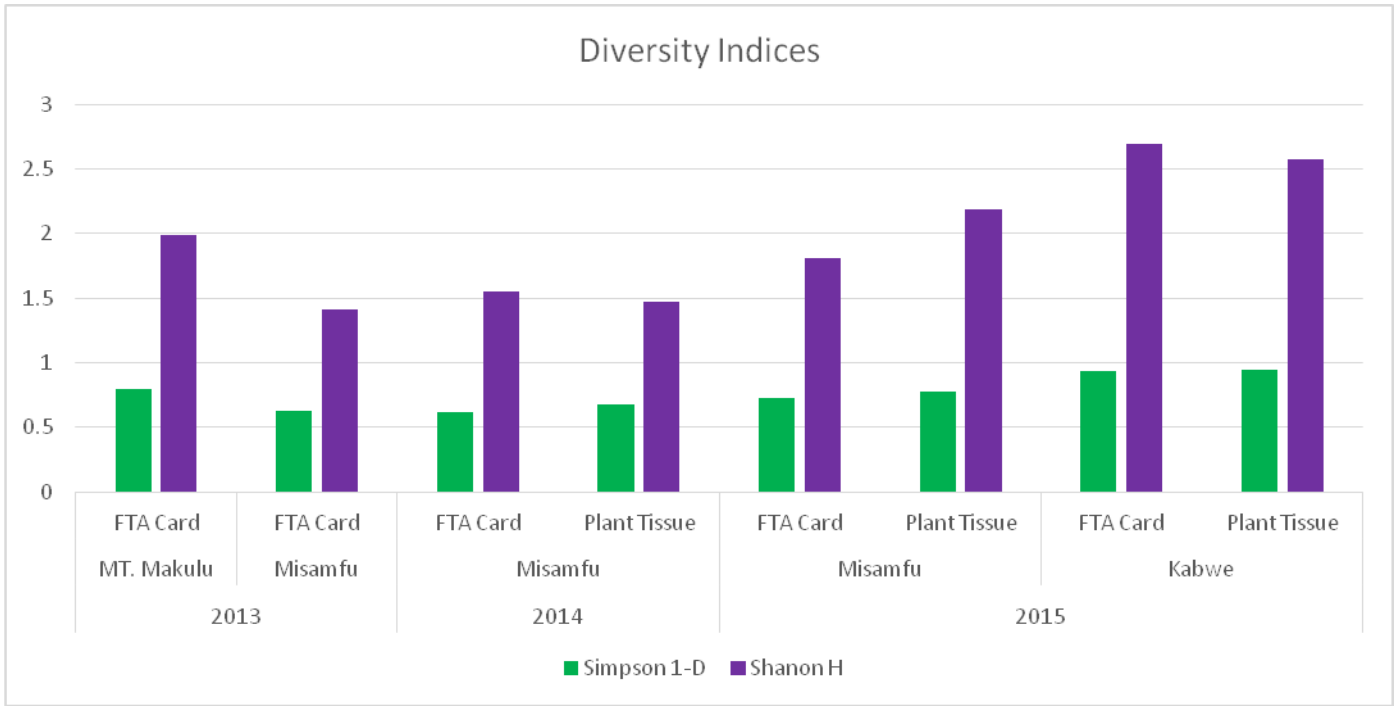


Figure 2.14 Diversity indices of species identified by Roche 454 pyrosequencing of plant tissue and DNA embedded FTA card samples collected between 2013 and 2015 from Misamfu, Mt. Makulu and Kabwe, Zambia

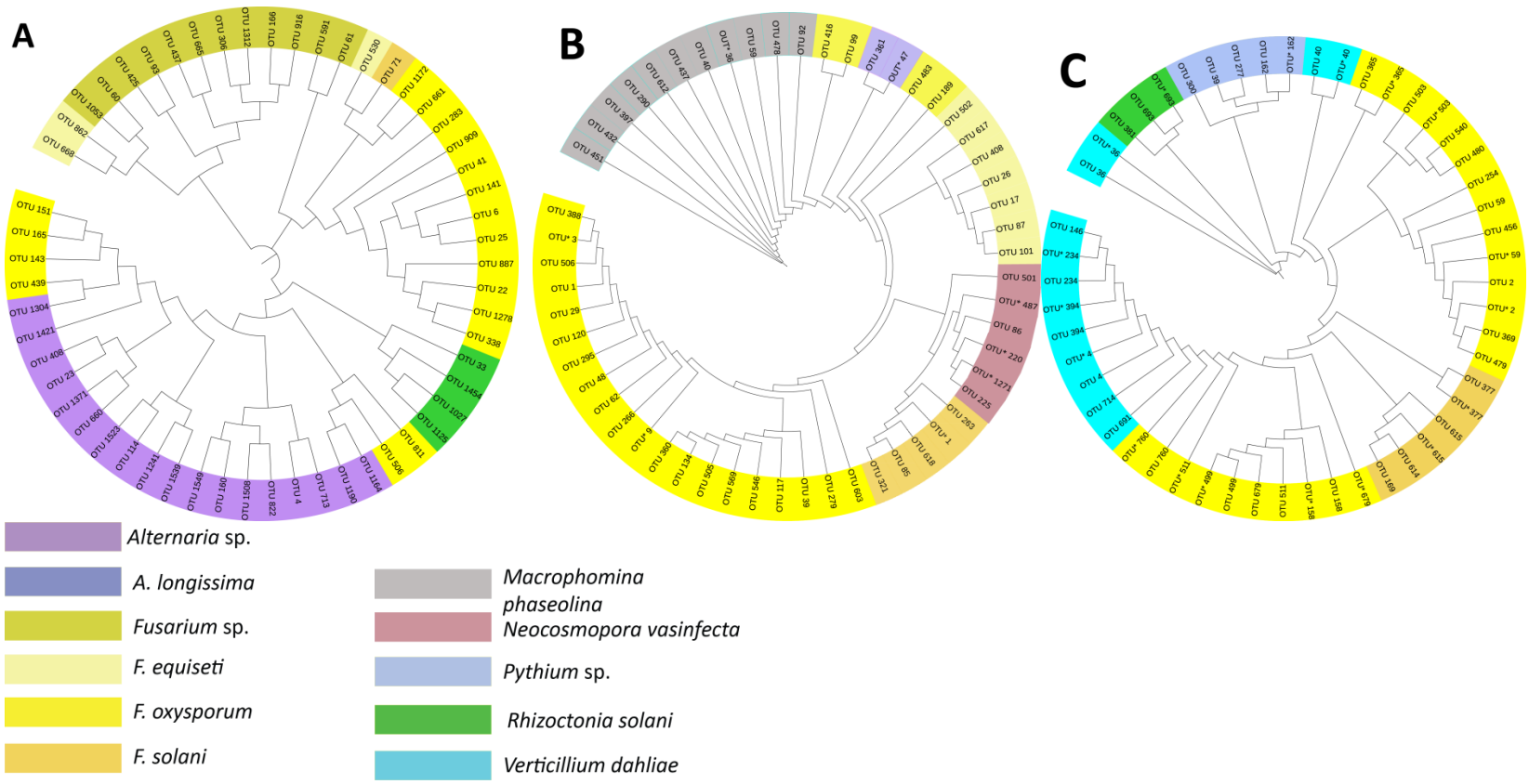


Figure 2.15 Neighbor-joining phylogenetic tree of the most relevant OTUs inferred fom the partial 16S region generated by 454 (A) and 18S generated by Illumina (B,C) pyrosequencing of bean RCR DNA extracted from FTA cards and tissue from Misamfu in 2013 (A), 2014 (B) and 2015 (C). Trees were rendered using iTOL (Letunic & Bork, 2007). Color represents fungal/oomycete group species.

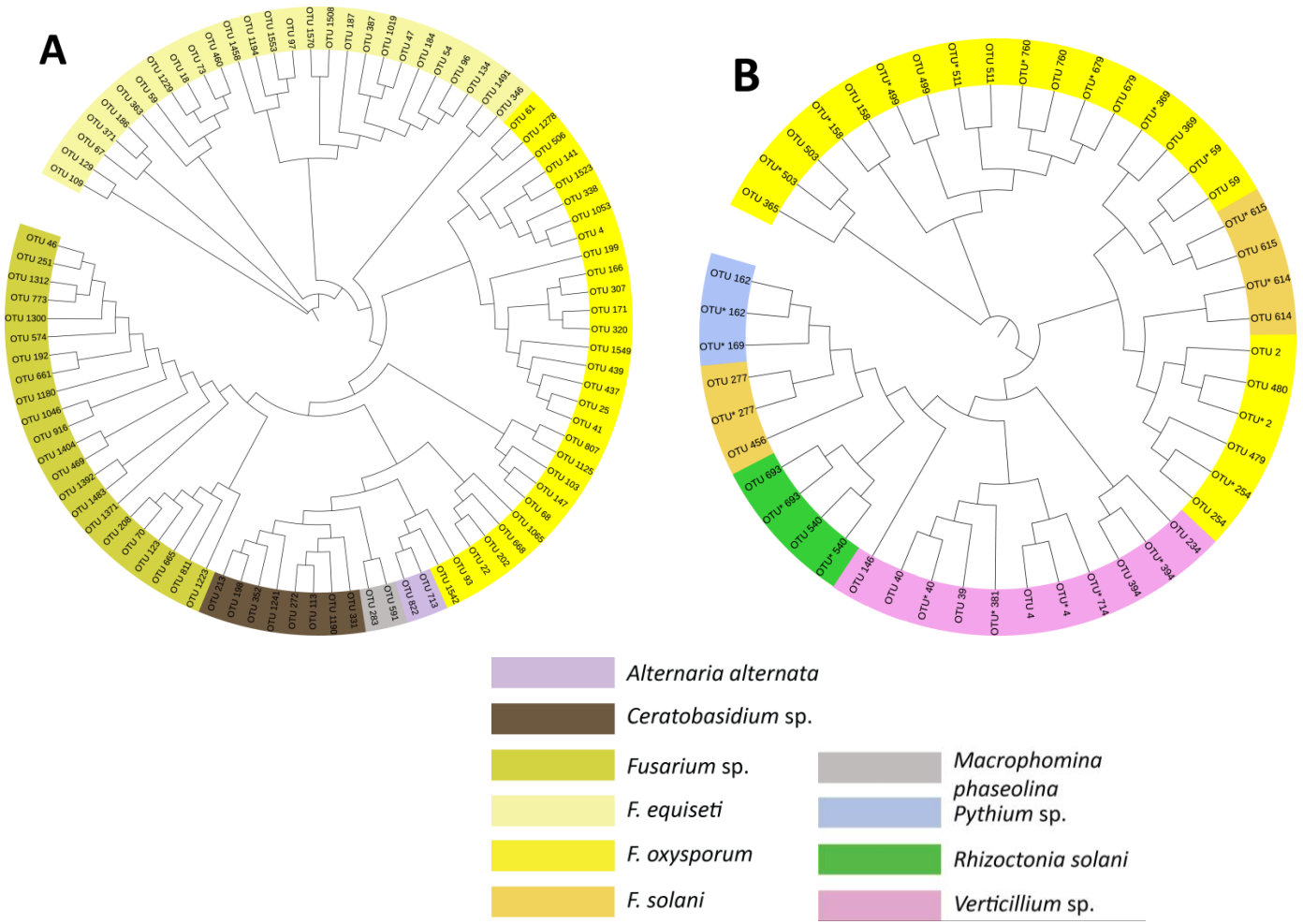


Fig. 2.16 Neighbor-joining phylogenetic tree of the most relevant OTUs inferred fom the partial 16S region generated by 454 (A) and 18S generated by Illumina (B) pyrosequencing of bean RCR DNA extracted from FTA cards and tissue from A) Mt. Makulu in 2013 and B) Kabwe in 2015. Trees were rendered using iTOL (Letunic & Bork, 2007). Color represents fungal/oomycete group species.

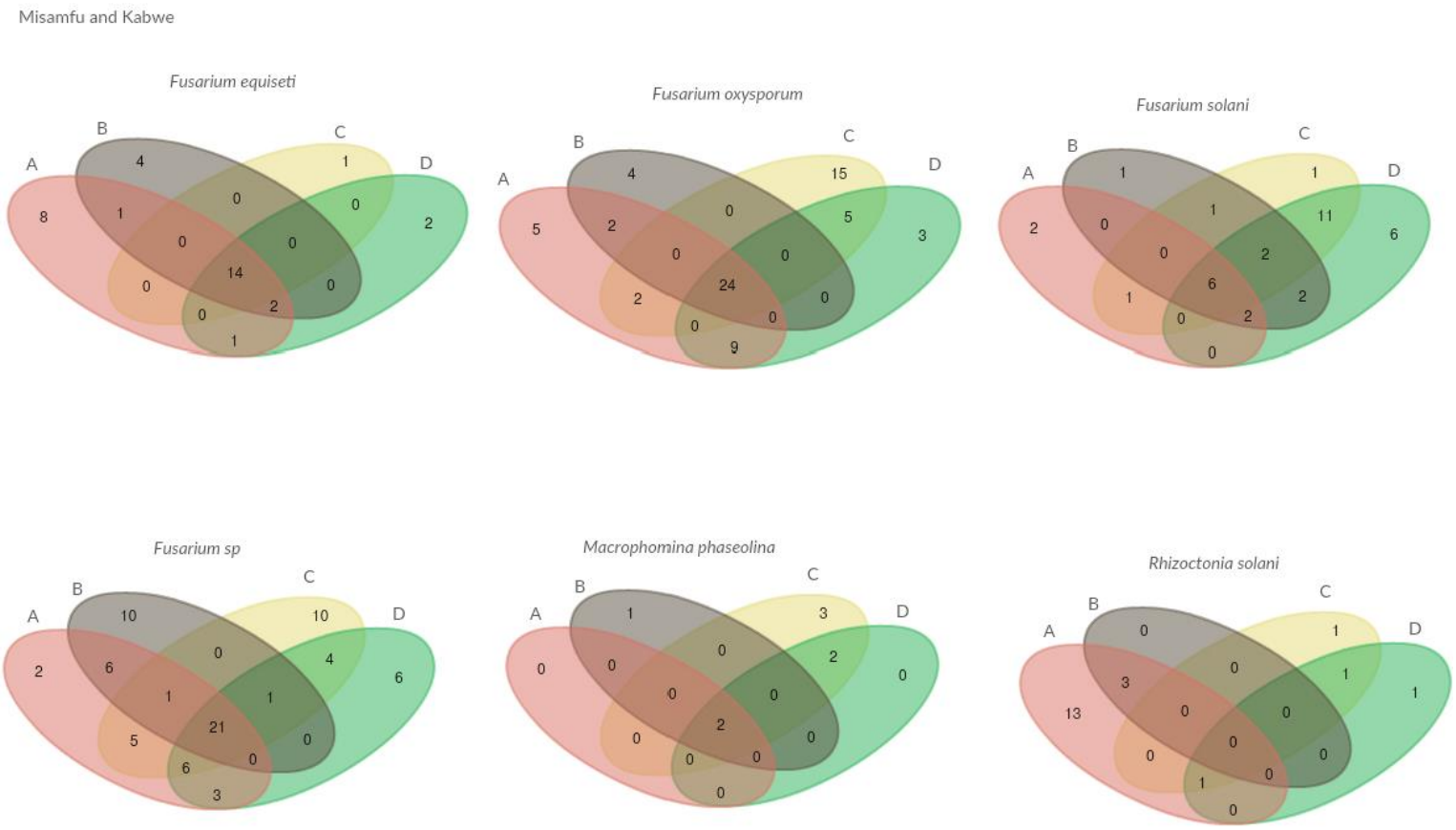


Fig. 2.17 Schematic Venn diagram representation of the number of unique and shared OTUs of RCR pathogens generated from 454 pyrosequencing of DNA from tissue and FTA card samples from Misamfu and Kabwe, Zambia. A) Misamfu tissue sample B) Misamfu FTA card C) Kabwe tissue sample, D) Kabwe FTA card

Table 2.1 ADP/NE nursery locations, soil characteristics and environmental conditions 2013-2015

Year	Physical Properties			Soils Data			Sampling					Weather Data		
												Rainfall (mm)	Mean Temperature (C)	
	Location	Coordinates	Elevation (masl)*	p.H	OC	Texture	Planting Date	Sampling Date	FTA Cards	Plant Tissue	Number of samples		Min	Max
2013	Mt. Makulu	15°33'S 28°11'E	1,998	7	1	Sandy loam	1/22/2013	5/22/2013	26	0	26	1187.3	12.65	25.6
	Misamfu	10°13'S 30°08'E	1,384	6	1.1	clay loamy	1/15/2013	5/7/2013	46	7	53	1187.3	15.23	26.89
2014	Misamfu	10°13'S 30°08'E	1,384	6	1.1	clay loamy	2/5/2014	05/25-27/2014	19	18	37	1582.2	16.58	27.95
2015	Misamfu	10°13'S 30°08'E	1,384	6	1.1	clay loamy	1/20/2015	5/5/2015	16	21	37	1119.5	15.56	27.72
	Kabwe	14°42'S 328°45'E	1,182	6	0.9	clay to loam	1/15/2015	5/13/2015	17	17	34	861.6	12.15	26

*masl-meters above sea level ,OC-soil organic carbon

Table 2.2 Oligonucleotides for polymerase chain reaction analysis of important RCR associated pathogens used in this study

Specificity	Primer Code	Primer Sequence	Gene	Amplified Product Size (bp)	PCR Programs	Reference
Pythium spp.	FM 66	TAGGATTTCAAGATCCTGC	cytochrome oxidadse 11	544 bp-689	35 cycles; 94°C for 30 sec, 52°C for 30 sec, 72°C for 60 sec	Martin, F. 2000.
	FM 58 COX II	CCACAAATTTCACTACATTGA				
Macrophomina phaesolina	MpkF1	CCGCCAGAGGACTATCAAAC	Internal Transcribed Spacer rRNA	300-400	35 cycles; 95°C for 30 sec, 56°C for 60 sec, 72°C for 120 sec	Kishore Babbu et al. 2007
	MpkR1	CGTCCGAAGCGAGGTGTATT				
Fusarium spp.	ITSFu1F	CAACTCCCAAACCCCTGTGA	Internal Transcribed Spacer rRNA	300-400	40 cycles; 94°C for 60 sec, 58°C for 60 sec, 72°C for 120 sec	Ed Elsalam KA et al 2003.
	ITSFu1R	GCGACGATTACCAGTAACGA				
	EF1	ATGGGTAAGGA(A/ G)GACAAGAC	Translation Elongation Factor (1-a)	~700	40 cycles; 94°C for 60 sec, 53°C for 60 sec, 72°C for 120 sec	O'Donnell et all., 1998c
	EF2	GGA(G/A)GTACCAGT(G/C)ATCATGTT				
Rhizoctonia spp.	R1	CCTGTGCACCTGTGAGACAG	Internal Transcribed Spacer rRNA	475-550	35 cycles; 94°C for 30 sec, 56°C for 30 sec, 72°C for 60 sec	Comprota,p et al. 1999; 2000
	R4	TGTCCAAGTCAATGGACTAT				
Phytophthora	FMPh-8bf	AAAAGAGAAGGTGTTTTTTATGGA	cytochrome oxidadse 11	457	35 cycles; 94°C for 30 sec, 53°C for 45 sec, 72°C for 120 sec	Drenth et al 2006
	FMPh-10b	GCAAAAGCACTAAAAATTAAATATAA				
Fungi General (Peronosporaceae)	ITS5	GGAAGTAAAAGTCGTAACAAGG	Internal Transcribed Spacer rRNA	550-700	35 cycles; 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec	White et all. 1990.
	ITS4	TCCTCCGCTTATTGATATGC				

Table 2.3 Morphology characteristics of representative *Fusarium* spp isolates recovered from symptomatic dry bean plant tissue.

	Color of colony		Microconidia	Macroconidia				chlamydospore	Growth Rate	Species
	Top	Bottom	Shape/septa	Apical	Basal	Size(µm)	# septa			
1	White	pink	cresent	slender	foot	30	5	yes	20	*Fusarium*
2	White	cream	Rod/1	pointed	foot	25	4	yes	25	*Fusarium*
3	White	cream	oval	blunt	Notched	54	4	yes	26	*Fusarium*
4	yelow	cream	oval	blunt	notched	55	4	yes	27	*Fusarium*
5	White	cream	oval	hooked	notched	55	4	yes	28	*Fusarium*
6	pink	Deep red	globose	hooked	pointed	30	3	no	29	*Fusarium*
7	purple streaked	cream	pyriforme	blunt	blunt	27	3	no	30	*Fusarium*
8	White	yellow	oval	pointed	notched	53	4	no	31	*Fusarium*
9	Yellow	cream	oval	pointed	Barely notched	25	4	singe	32	*Fusarium*
10	White	cream	globase	blunt	footshape	40	no	yes	33	*Fusarium*
11	White	cream	Obovoid	Hooked	foot shaped	48	no	yes	34	*Fusarium*
12	White	cream	oval	Hooked	foot shaped	46	no	pared	35	*Fusarium*
13	pink	pink	globae	Hooked	pointed	no	4	no	36	*Fusarium*
14	White	pink	globase	blunt	foot shped	no	3	no	37	*Fusarium*
15	White	cream	oval	blunt	foot	44	3	yes	38	*Fusarium*
16	Yellow	cream	oval	blunt	foot	49	3	single	39	*Fusarium*
17	blue	blue	oval	blunt	blunt	no	4	single	40	*Fusarium*
18	Cream	cream	globose	pointed	Barely notched	55	4	single smooth	41	*Fusarium*

Table 2.4 Isolation frequencies (IF) of pathogenic (P) and non-pathogenic (NP) fungi/oomycetes recovered from dry bean plant tissue collected from Misamfu, Kabwe and Mpika between 2014 and 2015

Morphology Group	Taxonomic group	2014				2015											
		Misamfu				Misamfu				Kabwe				Mpika			
		P	NP	Total isolates	*IF* (%)	P	NP	Total isolates	*IF* (%)	P	NP	Total isolates	*IF* (%)	P	NP	Total isolates	*IF* (%)
Group A	*Fusarium* spp.	22	2	24	43	17	5	22	30	55	7	62	94	9	0	9	100
Group B	*Macrophomina phaseolina*	0	0	0	0	5	0	5	7	0	0	0	0	0	0	0	0
Group C	*Rhizoctonia solani*	4	0	4	7	15	0	15	20	1	0	1	1.5	0	0	0	0
Group D	*Pythium* spp.	6	0	6	11	0	0	0	0	0	0	0	0	0	0	0	0
Group E	Other	6	15	21	38	4	28	32	43	0	3	3	4.5	0	0	0	0
P	Total	38	17	55	100	41	33	74	100	56	10	66	100	9	0	9	100
Percentage (%)		70	30	100	100	55	45	100	100	85	15	100	100	100	0	100	100

Table 2.5. Single and multiple species group detections in DNA

Species group combinations	2013		2014		2015				Total
	Mt. Makulu	Misamfu	Misamfu		Misamfu		Kabwe		
	FTA Card	FTA Card	FTA Card	Plant Tissue	FTA Card	Plant Tissue	FTA Card	Plant Tissue	
A	0	0	4	0	11	3	9	1	28
B	0	2	0	0	0	0	0	1	3
C	0	0	0	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0	0
AB	1	1	0	0	1	3	4	2	12
AC	0	0	1	1	2	0	0	1	5
AD	1	0	1	4	1	0	1	1	9
BC	0	0	0	0	0	0	0	2	2
BD	0	0	0	0	0	0	0	1	1
CD	0	0	0	0	0	0	0	0	0
ABC	2	0	0	1	0	5	0	2	10
ABD	0	2	0	3	0	0	2	1	8
ACD	0	0	0	3	0	0	1	0	4
BCD	0	0	0	0	0	0	0	2	2
ABCD	1	1	0	2	0	4	0	0	8

A-*Fusarium* spp., B-*Macrophomina phaseolina* , C-*Rhizoctonia solani* , D-*Pythium* spp.

Single and multiple species detection in dry bean DNA samples from FTA® cards and plant tissue collected between 2013 and 2015. Numbers indicate the number of times a species group was detected alone and in combination with other groups.

Table 2.6 Anova of reads of species associated with RCR generated by 454 pyrosequencing of DNA from plant and DNA embedded FTA cards from Mt. Makulu, Misamfu and Kabwe research stations between 2013 and 2015.

Year	Location	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
2013	Mt. Makulu	FTA Card species Reads	10	5688076.39	568807.639	13.09	<.0001
		Error	66	2868760.286	43466.065		
		Corrected Total	76	8556836.675			
	Misamfu	FTA Card species Reads	11	27690965.57	2517360.51	15.46	<.0001
		Error	108	17585128.8	162825.27		
		Corrected Total	119	45276094.37			
2014	Misamfu	FTA Card species Reads	4	1465603.057	366400.764	9.02	<.0001
		Error	65	2641631.643	40640.487		
		Corrected Total	69	4107234.7			
		Plant Tissue species Reads	6	3543192.35	590532.06	6.62	<.0001
		Error	91	8113498.93	89159.33		
		Corrected Total	97	11656691.28			
2015	Misamfu	Plant Tissue species Reads	7	52297507.5	7471072.5	2.46	0.0221
		Error	112	340437403.7	3039619.7		
		Corrected Total	119	392734911.2			
		FTA Card species Reads	7	14137852.37	2019693.2	3.06	0.0056
		Error	112	74005345.6	660762.01		
		Corrected Total	119	88143197.97			
	Kabwe	Plant Tissue species Reads	6	30066957.1	5011159.5	6.69	<.0001
		Error	112	83836738.2	748542.3		
		Corrected Total	118	113903695.3			
		FTA Card species Reads	10	217657805.7	21765780.6	21.08	<.0001
		Error	176	181728964.7	1032550.9		
		Corrected Total	186	399386770.4			

Significant differences in the number of reads of different pathogens at alpha 0.01 and 0.05.

Table 2.7 Tukey's Studentized Range (HSD) test for OTU Reads. Mean seperation/grouping of species associated with RCR based on reads generated by Roche 454 pyrosequencing of DNA from plant and DNA embedded FTA cards from Mt. Makulu, Misamfu and Kabwe research stations in Zambia between 2013 - 2015

Pathogen	Turkey Mean Grouping*					
	2013		2014		2015	
	Mt. Makulu	Misamfu	Misamfu		Misamfu	
	FTA Card	FTA Card	Plant Tissue	FTA Card	Plant Tissue	FTA
Fusarium oxysporum	548.3 a	1037.7 a	577.6 a	385.71 a	2498.9 a	1413.2 a
Fusarium sp	597.4 a	129.9 b	167.8 b	-	2372.9 a	992.7 ab
Fusarium equiseti	145.6 b	2.5 b	78.8 b	-	503.5 b	718.1abc
Fusarium incarnatum	66.6 b	-	-	30.07 b	-	-
Fusarium oxysporum f. sp. vasinfectum	11.1 b	251.7 b	-	-	-	-
Ceratobasidium sp.	39.3 b	-	-	-	20.7	104.2 bc
Macrophomina phaseolina	5 b	4.2 b	53.4 b	15.14 b	30.7 b	23.5 c
Fusarium solani	-	4.8 b	27 b	34.93 b	693.1 b	756.1abc
Fusarium oxysporum f. sp. Melonis	6.7 b	11.9 b	-	-	-	-
Verticillium sp.	-	-	-	-	9.2 b	-
Rhizoctonia solani	-	6.1 b	3.1 b	-	32.5 b	-
neocosmospora striata	-	-	3.3 b	17.64 b	-	-
plectosphaerella cucumerina	-	-	-	-	10.1 b	16 c

Means in a column with the same letter are not significantly different at 0.05.

***(-)** Species not present and not included in analysis

Table 2.8 Parametric and non-parametric correlation coefficients.

Year	Location	Source	Method	Pearson 's	Spearman	Kandall's	Kappa		
				r	Rho	Tau	P(0)	P(e)	Kappa
2013	Misamfu	FTA Card	Pyrosequencing Vs PCR	0.27	0.12	0.44**	0.7	0.7	NA
	MT. Makulu		Pyrosequencing Vs PCR	0.23	0.6*	0.5**	0.71	0.5	0.43**
2014	Misamfu	FTA Card	Pyrosequencing Vs PCR	0.12	0.43**	0.41**	0.55	0.42	0.23
			Pyrosequencing Vs Culture	0.21	0.46**	0.4**	0.7	0.5	0.31**
			PCR Vs Culture	0.06	0.02	0.2	0.7	0.7	NA
		Plant Tissue	Pyrosequencing Vs PCR	0.25	0.4**	0.4**	0.6	0.5	0.15
			Pyrosequencing Vs Culture	0.07	0.4**	0.3**	0.7	0.5	0.25
			PCR Vs Culture	0.3**	0.12	0.1	0.4	0.5	-0.05
		FTA Card Vs Tissue	Pyrosequencing	0.55**	0.82**	0.93**	0.8	0.5	0.6**
			PCR	0.2	0.20	0.16	0.8	0.5	0.6**
2015	Misamfu	FTA Card	Pyrosequencing Vs PCR	0.52**	0.7**	0.63**	0.85	0.6	0.6**
			Pyrosequencing Vs Culture	0.219	0.5**	0.45**	0.8	0.6	0.5**
			PCR Vs Culture	0.13	0.4**	0.38**	0.8	0.6	0.5**
		Plant Tissue	Pyrosequencing Vs PCR	0.25	0.4**	0.36**	0.6	0.5	0.3
			Pyrosequencing Vs Culture	0.28	0.5**	0.43**	0.8	0.5	0.5*
			PCR Vs Culture	0.23	0.3*	0.27	0.6	0.4	0.2
		FTA Card Vs Tissue	Pyrosequencing	0.5**	0.69**	0.57**	0.8	0.6	0.5*
			PCR	0.42*	0.41*	0.57**	0.6	0.6	0.1
	Kabwe	FTA Card	Pyrosequencing Vs PCR	0.6**	0.65**	0.61**	0.8	0.5	0.6**
			Pyrosequencing Vs Culture	0.8**	0.79**	0.73**	0.9	0.6	0.8**
			PCR Vs Culture	0.6**	0.61**	0.59**	0.8	0.6	0.6*
		Plant Tissue	Pyrosequencing Vs PCR	0.06	0.09	0.083	0.6	0.5	0.1
			Pyrosequencing Vs Culture	0.9**	0.85**	0.77**	0.6	0.5	0.1
			PCR Vs Culture	0.01	0.02	0.02	0.8	0.6	0.5*
		FTA Card Vs Tissue	Pyrosequencing	0.87**	0.81**	0.72**	0.9	0.8	0.2
			PCR	0.014	0.01	0.0141	0.5	0.5	0.01

Comparison of methods of RCR identification and extent of agreement between the use of FTA cards and direct plant issue in sampling and identification of RCR pathogens. Culculations are based on the four important pathogens associated with RCR disease of dry bean. * Significant at 0.05 level. ** Significant at 0.01 level. NA-value in denominator or numerator was 0.

Appendix 1. *Fusarium* spp. isolated from RCR symptoms on dry beans (*Phaseolus vulgaris*) grown in Western Nebraska and Wyoming and used in pathogenicity testing with the straw tes

Sample ID	Location	Sampling date	Variety	NCBI Genbank closest match	Sequence accesion Numbers for Closest match	Coverage/ Identity (%)
NE2	Lisco, NE	7/30/2013	Aries	*Fusarium oxysporum* Isolate 850	JN232136.1	98/99
NE5	Lisco, NE	7/30/2013	Aries	*(Fusarium oxysporum* Isolate 820*)*	JN232177	100/99
NE6	Lisco, NE	7/30/2013	Aries	*Fusarium oxysporum* Strain ZJ	KF278962.1	98/99
NE9	Bayard, NE	7/30/2013	Marquis	*Fusarium oxysporum* Isolate FPV-32	HG423346.1	100/99
NE12	Scottsbluff, NE	6/16/2014	Pinto	*Fusarium solani* Isolate FWC27	JQ625562.1	97/97
NE 14	Scottsbluff, NE	6/16/2014	Pinto	*Fusarium solani* Isolate RSPG 229	KC478532	98/99
NE15	Scottsbluff, NE	6/16/2014	Pinto	*Fusarium oxysporum* Isolate F19	JF439472.1	98/99
NE16	Scottsbluff, NE	6/16/2014	Pinto	*Fusarium solani* Isolate 851	JN232141	98/99
NE19	Scottsbluff, NE	6/16/2014	Pinto	*Fusarium solani* f. sp. *Phaseoli*	L36630.1	96/99
NE20	Scottsbluff, NE	6/16/2014	Pinto	*Fusarium oxysporum* Isolate FPV-32	HG423346.1	100/98
NE21	Scottsbluff, NE	6/16/2014	Pinto	*Fusarium acuminatum* strain CHS-3	KJ082098.1	96/99
NE22	Scottsbluff, NE	6/16/2014	Pinto	*Fusarium solani* Isolate 851	JN232141.1	98/99
NE23	Scottsbluff, NE	9/5/2013	Pinto	*Fusarium oxysporum* Isolate 281	JN232163.1	96/99
NE24	Scottsbluff, NE	9/5/2013	Pinto	*Fusarium solani* Isolate 832	JN232138.1	97/99
NE27	Scottsbluff, NE	9/5/2013	Pinto	*Fusarium oxysporum* Isolate FPV-32	HG423346.1	97/100
NE29	Scottsbluff, NE	7/7/2014	Kniess	*Fusarium solani* Strain D3	KJ019827.1	97/98
NE30	Scottsbluff, NE	7/7/2014	Kniess	*Fusarium oxysporum* Isolate F19	JF439472.1	98/98
NE31	Scottsbluff, NE	7/7/2014	Kniess	*Fusarium solani* Isolate FWC27	JQ625562.1	96/99
NE32	Scottsbluff, NE	7/7/2014	Kniess	*Fusarium solani* Strain xsd08086	FJ478128.1	96/99
NE33	Lingle, WY	7/7/2014	Pinto	*Fusarium solani* Strain xsd08086	FJ478128.1	96/99
NE34	Lingle, WY	7/7/2014	Pinto	*Fusarium oxysporum* Isolate F19	JF439472.1	97/99
NE35	Wyoming	7/7/2014	Pinto	*Fusarium acuminatum* Strain CHS-3	KJ082098.1	97/99
NE36	Wyoming	7/7/2014	Pinto	*Fusarium acuminatum* Strain CHS-3	KJ082098.1	97/99
NE37	Lingle, WY	7/7/2014	Pinto	*Fusarium solani* Isolate 851	JN232141.1	97/99

Appendix 2. ADP, NE and local landrace dry bean lines sampled from RCR nurseries between 2013 and 2015 at Misamfu, Mt. Makulu and Kabwe research stations, Zambia

Bean Line	Location	Year	Sample Type
ADP 117	Mt. Makulu	2013	FTA Card
ADP 472	Mt. Makulu	2013	FTA Card
NE-34-12-40	Mt. Makulu	2013	FTA Card
NE34-12- 49	Mt. Makulu	2013	FTA Card
CAPRI BC 95	Mt. Makulu	2013	FTA Card
Kalungu	Mt. Makulu	2013	FTA Card
Lyambai	Mt. Makulu	2013	FTA Card
ADP-97	Misamfu	2013	FTA Card
ADP-100	Misamfu	2013	FTA Card
ADP-113	Misamfu	2013	FTA Card
ADP-117	Misamfu	2013	FTA Card
ADP-472	Misamfu	2013	FTA Card
NE34-12-39	Misamfu	2013	FTA Card
NE34-12-40	Misamfu	2013	FTA Card
NE34-12-50	Misamfu	2013	FTA Card
Lyambai	Misamfu	2013	FTA Card
Kalungu	Misamfu	2013	FTA Card
CIMSUG02-15-1	Misamfu	2014	FTA Card /Plant tissue
CIMSUG07-ALS-2	Misamfu	2014	FTA Card /Plant tissue
CIMSUG07-ALS-3	Misamfu	2014	FTA Card /Plant tissue
CIMSUG07-ALS-5	Misamfu	2014	FTA Card /Plant tissue
Local Landrace 6	Misamfu	2014	FTA Card /Plant tissue
LocalLandrace 1	Misamfu	2014	FTA Card /Plant tissue
ADP 517	Misamfu	2014	FTA Card /Plant tissue
Local landrace 3	Misamfu	2014	FTA Card /Plant tissue
ADP 629	Misamfu	2014	FTA Card /Plant tissue
ADP 461	Misamfu	2014	FTA Card /Plant tissue
NE34-12-37	Misamfu	2014	FTA Card /Plant tissue
Local Landrace 5	Misamfu	2014	FTA Card /Plant tissue
CIM-CLIMB03-48	Misamfu	2014	FTA Card /Plant tissue
Local landrace 4	Misamfu	2014	FTA Card /Plant tissue
Carioca Kihala	Misamfu	2015	FTA Card /Plant tissue
cim climb 03-49	Misamfu	2015	FTA Card /Plant tissue
Incomparable	Misamfu	2015	FTA Card /Plant tissue
NAK2	Misamfu	2015	FTA Card /Plant tissue

Appendix 2 (Continued)

Lyambai	Misamfu	2015	FTA Card /Plant tissue
NE 34-12-47	Misamfu	2015	FTA Card /Plant tissue
NE34-12-50	Misamfu	2015	FTA Card /Plant tissue
NE34-12-45	Misamfu	2015	FTA Card /Plant tissue
cim-climb 03-48	Misamfu	2015	FTA Card /Plant tissue
Larga Comercial	Misamfu	2015	FTA Card /Plant tissue
Mblamtwe	Misamfu	2015	FTA Card /Plant tissue
cim-climb 03-48	Misamfu	2015	FTA Card /Plant tissue
G10994	Misamfu	2015	FTA Card /Plant tissue
NE34-12-45	Misamfu	2015	FTA Card /Plant tissue
Carioca Kihala	Misamfu	2015	FTA Card /Plant tissue
Mbereshi	Misamfu	2015	FTA Card /Plant tissue
NE 32-12-47	Kabwe	2015	FTA Card /Plant tissue
Incomparable	Kabwe	2015	FTA Card /Plant tissue
NE34-12-28	Kabwe	2015	FTA Card /Plant tissue
Mbulumutwe	Kabwe	2015	FTA Card /Plant tissue
NE34-12-47	Kabwe	2015	FTA Card /Plant tissue
910994	Kabwe	2015	FTA Card /Plant tissue
nak2	Kabwe	2015	FTA Card /Plant tissue
USD-K4	Kabwe	2015	FTA Card /Plant tissue
Carioca kihala	Kabwe	2015	FTA Card /Plant tissue
USD-K4	Kabwe	2015	FTA Card /Plant tissue
cim-climb03-48	Kabwe	2015	FTA Card /Plant tissue
Mbereshi	Kabwe	2015	FTA Card /Plant tissue
NE34-12-50	Kabwe	2015	FTA Card /Plant tissue
Clouseau	Kabwe	2015	FTA Card /Plant tissue
Local 2(lyambai)	Kabwe	2015	FTA Card /Plant tissue

Appendix 3. NCBI closest match of isolated fungi/oomycetes from symptomatic beans plant tissue.

Isolate Lab ID	NCBI Genebank closest match	Sequence accession number of closest match	Coverage/ identity	Primers
ZM1	*Chaetomium coarctatum*	JN209863.1	99/97	ITS 4/5
ZM2	*Dothideomycete* sp.	EU680544.1	98/93	ITS 4/5
ZM3	*Alternaria* sp.	HQ630996.1	96/99	ITS 4/5
ZM4	*Fusarium oxysporum*	KC215120.1	100/99	ITS 4/5
ZM5	*Penicillium janthinellum*	KM268710.1	90/98	ITS 4/5
ZM6	*Fusarium proliferatum*	GU723438.1	99/99	ITS 4/5
ZM7	*Fusarium equiseti*	KJ562376.1	99/99	ITS 4/5
ZM8	*Epicoccum sorghinum*	KP050561.1	97/99	ITS 4/5
ZM9	*Alternaria longissima*	KJ572139.1	97/99	ITS 4/5
ZM10	*Fusarium equiseti*	KP205542.1	98/99	ITS 4/5
ZM11	*Pythium myriotylum*	KJ162354.1	99/99	ITS 4/5
ZM12	*Fusarium equiseti*	KJ562376.1	100/99	ITS 4/5
ZM13	*Fusarium oxysporum* f. sp. *ciceris*	JN400681.1	100/98	ITS 4/5
ZM14	*Fusarium equiseti*	KJ562376.1	99/99	ITS 4/5
ZM15	*Fusarium equiseti*	KJ854378.1	97/99	ITS 4/5
ZM16	*Phoma* sp. F175	KM979945.1	83/96	ITS 4/5
ZM17	*Rhizoctonia solani*	AY684921.1	98/99	ITS 4/5
ZM18	*Fusarium oxysporum* f. sp. *ciceris*	JN400681.1	99/99	ITS 4/5
ZM19	*Rhizoctonia solani*	AY684921.1	98/99	ITS 4/5
ZM20	*Rhizoctonia solani*	AY684921.2	98/100	ITS 4/5
ZM21	*Fusarium oxysporum* f. sp. *ciceris*	KM817208.1	100/99	ITS 4/5
ZM22	*Rhizoctonia solani*	DQ102435.1	97/96	ITS 4/5
ZM23	*Rhizoctonia solani*	JF701771.1	90/95	ITS 4/5
ZM24	*Rhizoctonia solani*	JX454673.1	81/95	ITS 4/5
ZM25	*Epicoccum sorghinum*	HQ328047.1	99/99	ITS 4/5
ZM26	*Fusarium oxysporum*	JN631751.1	46/89	ITS 4/5
ZM27	*Fusarium oxysporum*	JQ886414.1	99/99	ITS 4/5
ZM28	*Fusarium oxysporum*	KP050556.1	99/100	ITS 4/5
ZM29	*Fusarium oxysporum*	KM268673.1	100/99	ITS 4/5
ZM30	*Pythium myriotylum*	KJ162354.1	100/99	ITS 4/5
ZM31	*Alternaria* sp. 1 TMS-2011	HQ630996.1	99/99	ITS 4/5
ZM32	*Alternaria* sp. 1 TMS-2011	HQ630996.1	99/99	ITS 4/5
ZM33	*Alternaria* sp. 1 TMS-2011	HQ630996.1	99/99	ITS 4/5
ZM34	*Pythium myriotylum*	KJ162354.1	99/99	ITS 4/5
ZM35	*Pleosporales* sp. RKDO795	KJ812284.1	94/90	ITS 4/5

Appendix 3. (continued)

Isolate Lab ID	NCBI Genebank closest match	Sequence accession number of closest match	Coverage/ identity	Primers
ZM36	*Fusarium equiseti*	EF611087.1	98/99	ITS 4/5
ZM37	*Penicillium janthinellum*	KM268648.1	99/98	ITS 4/5
ZM38	*Talaromyces cellulolyticus* CF-2612	AB474749.2	96/99	ITS 4/5
ZM39	*Curvularia trifolii*	JN712459.1	99/99	ITS 4/5
ZM40	*Fusarium equiseti*	KR094440.1	100/99	ITS 4/5
ZM41	*Fusarium* cf. *oxysporum* B164	KR812231.1	100/99	ITS 4/5
ZM42	*Fusarium oxysporum* f. sp. *ciceris*	JN400681.1	100/98	ITS 4/5
ZM43	*Fusarium solani*	KF918580.1	100/99	ITS 4/5
ZM44	*Fusarium equiseti*	KJ562376.1	100/99	ITS 4/5
ZM45	*Curvularia borreriae*	HE861848.1	99/99	ITS 4/5
ZM46	*Fusarium oxysporum*	KT358875.1	100/99	ITS 4/5
ZM47	*Phoma* sp. NT-2015a	KT462714.1	99/99	ITS 4/5
ZM48	*Chaetomium* sp. NRRL 66025	KM030305.1	99/99	ITS 4/5
ZM49	Fusarium oxysporum f. sp. *vasinfectum*	EU849584.1	99/99	ITS 4/5
ZM50	*Fusarium oxysporum* f. sp. *ciceris*	KM817208.1	98/99	ITS 4/5
ZM51	*Fusarium oxysporum*	KR094464.1	100/99	ITS 4/5
ZM52	*Pythium myriotylum*	KJ162354.1	97/99	ITS 4/5
ZM53	*Aspergillus* sp. 085241B	KP059102.1	99/99	ITS 4/5
ZM54	*Alternaria* sp. 1 TMS-2011	HQ630996.1	99/99	ITS 4/5
ZM55	*Setophoma terrestris*	KP789315.1	82/97	ITS 4/5
ZM56	*Macrophomina phaseolina*	KR012878.1	100/99	ITS 4/5
ZM57	*Macrophomina phaseolina*	KR012878.1	100/99	ITS 4/5
ZM58	*Macrophomina phaseolina*	EF545133.1	98/99	ITS 4/5
ZM59	*Macrophomina phaseolina*	KM519193.1	100/99	ITS 4/5
ZM60	*Macrophomina phaseolina*	KT768135.1	94/87	ITS 4/5
ZM61	*Rhizoctonia solani*	KF372651.1	92/95	ITS 4/5
ZM62	*Rhizoctonia solani*	FR734293.1	97/97	ITS 4/5
ZM63	*Rhizoctonia solani*	KF372651.1	93/97	ITS 4/5
ZM64	*Phoma* sp.	KT462714.1	100/99	ITS 4/5
ZM65	*Fusarium equiseti*	KR094440.1	98/99	ITS 4/5
ZM66	*Fusarium equiseti*	HM008677.1	99/99	ITS 4/5
ZM67	*Rhizoctonia solani*	JX294349.1	97/79	ITS 4/5
ZM68	*Rhizoctonia bataticola*	EU375549.1	82/86	ITS 4/5
ZM69	*Rhizoctonia solani*	JX294349.1	98/86	ITS 4/5
ZM70	*Fusarium solani*	KP784419.1	100/100	ITS 4/5

Appendix 3. (continued)

Isolate Lab ID	NCBI Genebank closest match	Sequence accession number of closest match	Coverage/ identity	Primers
ZM71	*Fusarium solani*	JN232141.1	98/83	ITS 4/5
ZM72	*Rhizoctonia solani*	JX294349.1	97/99	ITS 4/5
ZM73	*Rhizoctonia solani*	JF701771.1	97/98	ITS 4/5
ZM74	*Phoma* sp.	KM979787.1	98/99	ITS 4/5
ZM75	*Phoma* sp.	KP734248.1	99/99	ITS 4/5
ZM76	*Thielavia* sp.	HQ435667.1	95/94	ITS 4/5
ZM77	*Phoma* cf. *herbarum* G10	KP734248.1	100/99	ITS 4/5
ZM78	*Phoma* cf. *herbarum* G11	KM259932.1	95/99	ITS 4/5
ZM79	*Phoma* cf. *herbarum* G10	KP734248.1	99/99	ITS 4/5
ZM80	*Phoma* cf. *herbarum* G11	KP734248.1	99/99	ITS 4/5
ZM81	*Alternaria longissima*	JQ676198.1	96/99	ITS 4/5
ZM82	*Alternaria longissima*	JQ676198.1	96/99	ITS 4/5
ZM83	*Septoria arundinacea*	KF498861.1	94/99	ITS 4/5
ZM84	*Alternaria longissima*	KT835049.1	95/99	ITS 4/5
ZM85	*Fusarium oxysporum*	KJ019830.1	98/99	ITS 4/5
ZM86	*Chaetomium globosum*	KM268646.1	99/100	ITS 4/5
ZM87	*Phoma glomerata*	EU273521.1	100/99	ITS 4/5
ZM88	*Phoma* cf. *herbarum* G10	KP734248.1	100/99	ITS 4/5
ZM89	*Phoma* sp.	GU045305.1	99/99	ITS 4/5
ZM90	*Phoma* sp.	KT199712.1	100/99	ITS 4/5
ZM91	*Phoma* sp.	KM979998.1	95/99	ITS 4/5
ZM92	*Fusarium equiseti*	HM008677.1	97/99	ITS 4/5
ZM93	*Fusarium equiseti*	HM008677.1	99/99	ITS 4/5
ZM94	*Fusarium equiseti*	HM008677.1	99/99	ITS 4/5
ZM95	*Fusarium equiseti*	HM008677.1	98/99	ITS 4/5
ZM96	*Phoma* sp.	GU045305.1	99/99	ITS 4/5
ZM97	*Fusarium equiseti*	KR094440.1	98/99	ITS 4/5
ZM98	*Phoma* sp.	KM979987.1	98/99	ITS 4/5
ZM99	No significant similarity	-	-	ITS 4/5
ZM100	*Fusarium equiseti*	KR094440.1	99/99	ITS 4/5
ZM101	*Rhizoctonia solani*	KF372652.2	96/94	ITS 4/5
ZM102	*Rhizoctonia solani*	KF372652.1	96/94	ITS 4/5
ZM103	*Rhizoctonia solani*	KF372652.0	96/94	ITS 4/5
ZM104	*Rhizoctonia solani*	KF372652.1	96/94	ITS 4/5

Appendix 3. (continued)

Isolate Lab ID	NCBI Genebank closest match	Sequence accession number of closest match	Coverage/ identity	Primers
ZM105	*Rhizoctonia fragariae*	DQ979011.1	71/82	ITS 4/5
ZM106	*Rhizoctonia solani*	KF372651.1	92/95	ITS 4/5
ZM107	*Rhizoctonia solani*	KF372651.1	92/93	ITS 4/5
ZM108	*Fusarium equiseti*	HM008677.1	99/99	ITS 4/5
ZM109	*Fusarium equiseti*	KR094440.1	97/99	ITS 4/5
ZM110	*Fusarium oxysporum*	KP132220.1	71/75	ITS 4/5
ZM111	No significant similarity	-	-	ITS 4/5
ZM112	*Chaetomium globosum*	KJ863531.1	99/99	ITS 4/5
ZM113	*Chaetomium globosum*	KT780353.1	99/99	ITS 4/5
ZM114	*Alternaria burnsii*	KR604838.1	99/99	ITS 4/5
ZM115	*Alternaria alternata*	KJ002057.1	99/99	ITS 4/5
ZM116	*Phoma* sp.	KM979976.1	99/99	ITS 4/5
ZM117	*Chaetomium globosum**	KJ863531.1	81/83	ITS 4/5
ZM118	*Fusarium oxysporum**	KJ019830.1	97/99	ITS 4/5
ZM119	*Ceratobasidium* sp.*	DQ279021.1	89/92	ITS 4/5
ZM120	*Ceratobasidium* sp.*	DQ279021.1	86/93	ITS 4/5
ZM121	No significant similarity	-	-	ITS 4/5
ZM122	*Fusarium* sp.*	FJ904916.1	96/99	ITS 4/5
ZM123	*Gibberella* sp*	KT268931.1	98/99	ITS 4/5
ZM124	*Phoma herbarum**	KT254322.1	100/99	ITS 4/5
ZM125	*Fusarium solani**	KU382502.1	99/99	ITS 4/5
ZM126	*Fusarium oxysporum*	KJ019830.1	100/99	ITS 4/5
ZM127	No significant similarity	-	-	ITS 4/5
ZM128	*Fusarium oxysporum*	KJ439203.1	100/99	ITS 4/5
ZM129	*Fusarium equiseti*	HM008677.1	99/99	ITS 4/5
ZM130	*Fusarium oxysporum*	KJ439203.1	100/99	ITS 4/5
ZM131	*Fusarium equiseti*	HM008677.1	99/99	ITS 4/5
ZM132	*Fusarium oxysporum*	KJ439203.1	100/99	ITS 4/5
ZM133	*Fusarium oxysporum*	KJ019830.1	100/99	ITS 4/5
ZM134	*Fusarium oxysporum*	KP267760.1	98/99	ITS 4/5
ZM135	*Fusarium oxysporum*	KF278962.1	99/99	ITS 4/5
ZM136	*Fusarium equiseti*	KR094440.1	99/99	ITS 4/5
ZM137	*Fusarium equiseti*	HM008677.1	99/99	ITS 4/5
ZM138	*Fusarium equiseti*	HM008677.1	100/99	ITS 4/5
ZM139	*Fusarium oxysporum*	KJ082096.1	99/96	ITS 4/5
ZM140	No significant similarity	-	-	ITS 4/5

Appendix 3. (continued)

Isolate Lab ID	NCBI Genebank closest match	Sequence accession number of closest match	Coverage/ identity	Primers
ZM141	*Fusarium oxysporum*	KJ699122.1	80/96	ITS 4/5
ZM142	*Fusarium oxysporum*	KJ439203.1	98/99	ITS 4/5
ZM143	*Fusarium oxysporum*	KC787019.1	99/100	ITS 4/5
ZM144	*Fusarium oxysporum*	KT898585.1	94/94	ITS 4/5
ZM145	*Fusarium oxysporum*	JN631751.1	79/92	ITS 4/5
ZM146	*Rhizoctonia solani*	KF372673.1	93/80	ITS 4/5
ZM147	*Fusarium oxysporum*	KJ439203.1	100/99	ITS 4/5
ZM148	*Fusarium equiseti*	KR094440.1	99/99	ITS 4/5
ZM149	*Sistotrema brinkmannii*	DQ899094.1	96/99	ITS 4/5
ZM150	*Fusarium oxysporum*	KF278962.1	99/99	ITS 4/5
ZM151	*Fusarium equiseti*	HM008677.1	99/99	ITS 4/5
ZM152	*Fusarium incarnatum*	KJ562367.1	96/99	ITS 4/5
ZM153	*Fusarium oxysporum*	EU326216.1	100/99	ITS 4/5
ZM154	*Fusarium oxysporum*	KJ439169.1	100/99	ITS 4/5
ZM155	*Fusarium oxysporum*	KJ439203.1	99/99	ITS 4/5
ZM156	*Fusarium oxysporum*	KF555228.1	94/92	ITS 4/5
ZM157	*Fusarium oxysporum*	KU056819.1	98/99	ITS 4/5
ZM158	*Fusarium oxysporum*	KT898585.1	91/99	ITS 4/5
ZM159	*Fusarium oxysporum*	KJ439203.1	100/99	ITS 4/5
ZM160	*Fusarium equiseti*	KR094440.1	99/99	ITS 4/5
ZM161	*Fusarium equiseti*	KF863780.1	99/99	ITS 4/5
ZM162	*Fusarium oxysporum*	KJ019830.1	99/99	ITS 4/5
ZM163	*Fusarium equiseti*	EU625404.1	99/99	ITS 4/5
ZM164	*Fusarium oxysporum*	KJ715962.1	97/100	ITS 4/5
ZM165	*Fusarium oxysporum*	KJ439203.1	97/100	ITS 4/5
ZM166	*Fusarium oxysporum*	KJ439203.1	98/99	ITS 4/5
ZM167	*Fusarium oxysporum*	KJ439203.1	99/99	ITS 4/5
ZM168	*Fusarium oxysporum*	KR364590.1	97/97	ITS 4/5
ZM169	*Fusarium equiseti*	JQ936180.1	99/99	ITS 4/5
ZM170	*Fusarium solani*	KU382502.1	98/99	ITS 4/5
ZM171	*Fusarium solani*	KJ528882.1	99/99	ITS 4/5
ZM172	*Fusarium solani*	JN006816.1	89/99	ITS 4/5
ZM173	*Fusarium solani*	KU382502.1	99/99	ITS 4/5
ZM174	*Curvularia trifolii*	KM979920.1	97/99	ITS 4/5
ZM175	*Phoma* sp.	JN207352.1	98/99	ITS 4/5

Appendix 3. (continued)

Isolate Lab ID	NCBI Genebank closest match	Sequence accession number of closest match	Coverage/ identity	Primers
ZM176	*Fusarium oxysporum*	KJ439203.1	100/99	ITS 4/5
ZM177	*Fusarium oxysporum*	KP050556.1	99/99	ITS 4/5
ZM178	*Fusarium oxysporum*	FR731133.1	99/100	ITS 4/5
ZM179	*Fusarium oxysporum*	KU872840.1	97/99	ITS 4/5
ZM180	*Fusarium oxysporum*	KJ528881.1	99/99	ITS 4/5
ZM181	*Fusarium equiseti*	HM008677.1	98/97	ITS 4/5
ZM182	*Fusarium equiseti*	HM008677.1	99/99	ITS 4/5
ZM183	*Fusarium equiseti*	HM008677.1	99/99	ITS 4/5
ZM184	*Fusarium oxysporum*	KJ439203.1	99/99	ITS 4/5
ZM185	*Fusarium oxysporum*	KU931543.1	99/98	ITS 4/5
ZM186	*Fusarium oxysporum*	KJ019830.1	99/99	ITS 4/5
ZM187	*Fusarium oxysporum*	KU056819.1	99/99	ITS 4/5
ZM188	*Fusarium oxysporum*	KC577178.1	97/100	ITS 4/5
ZM189	*Fusarium oxysporum*	KJ715962.1	97/99	ITS 4/5
ZM190	*Fusarium oxysporum*	HM008677.1	99/99	ITS 4/5
ZM191	*Fusarium oxysporum*	KJ439203.1	99/99	ITS 4/5
ZM192	*Fusarium oxysporum*	KJ439203.1	100/99	ITS 4/5
ZM193	*Fusarium oxysporum*	KJ082096.1	98/99	ITS 4/5
ZM194	*Fusarium equiseti*	KJ562376.1	98/99	ITS 4/5
ZM195	*Fusarium equiseti*	KR094440.1	99/99	ITS 4/5
ZM196	*Fusarium equiseti*	KJ562376.1	100/99	ITS 4/5
ZM197	*Fusarium oxysporum*	HQ649814.1	97/100	ITS 4/5
ZM198	*Fusarium oxysporum*	KU056819.1	99/99	ITS 4/5
ZM199	*Fusarium oxysporum*	KU056819.1	100/99	ITS 4/5
ZM200	*Fusarium oxysporum*	KJ019830.1	98/99	ITS 4/5
ZM201	*Fusarium solani*	JQ277276.1	97/99	ITS 4/5
ZM202	*Fusarium solani*	KJ528882.1	99/99	ITS 4/5
ZM203	*Fusarium oxysporum*	KF278962.1	99/99	ITS 4/5
ZM204	*Fusarium oxysporum*	KU872840.1	99/100	ITS 4/5

Taxonomic classification of fungal and oomycete isolates recovered from dry bean plant tissue samples. Isolate ZM1 – ZM55 and ZM56-ZM128 from Misamfu 2014 and 2015 respectfully, ZM129-ZM195 and ZM96-ZM204 from 2015 Kabwe and Mpika respectively.

Appendix 4. Pathogenicity Testing of Isolates recovered from symptomatic dry bean tissue samples collected from Misamfu, Kabwe and Mpika between 2014 and 2015

Taxonomic ID	Isolate Lab ID	Lesion Length (cm)				std
		Rep1	Rep2	Rep3	Mean	
Chaetomium coarctatum	ZM1	0	0	0	0	0.00
Dothideomycete sp.	ZM2	0	0	0	0	0.00
Alternaria sp.	ZM3	1	1	0.5	2.5	0.29
Fusarium oxysporum	ZM4	7	7	6.9	20.9	0.60
Penicillium janthinellum	ZM5	0	0	0	0	0.00
Fusarium proliferatum	ZM6	5	1.8	4.2	11	1.67
Fusarium equiseti	ZM7	7	4	5	16	1.53
Epicoccum sorghinum	ZM8	0	0	0	0	0.00
Alternaria longissima	ZM9	0	0	0	0	0.00
Fusarium equiseti	ZM10	5.5	2	3.4	10.9	1.76
Pythium myriotylum	ZM11	7.5	7.5	7.5	22.5	0.00
Fusarium equiseti	ZM12	0.5	0	1	1.5	0.50
Fusarium oxysporum f. sp. *ciceris*	ZM13	4.5	5.5	4.9	14.9	0.50
Fusarium equiseti	ZM14	4	7	5	16	1.53
Fusarium equiseti	ZM15	0.5	0	0	0.5	0.29
Phoma sp. F175	ZM16	3	2.5	2.9	8.4	0.26
Rhizoctonia solani	ZM17	2	3	2.3	7.3	0.51
Fusarium oxysporum f. sp. *ciceris*	ZM18	2	2.5	2.3	6.8	0.25
Rhizoctonia solani	ZM19	5	4	4	13	0.58
Rhizoctonia solani	ZM20	3	3.5	3	9.5	0.29
Fusarium oxysporum f. sp. *ciceris*	ZM21	7	2.5	6.3	15.8	2.49
Rhizoctonia solani. AG-F	ZM22	3	4	4.2	11.2	0.64
Rhizoctonia solani	ZM23	5.5	5.5	5.9	16.9	0.23
Rhizoctonia solani	ZM24	6	5	6	17	0.58
Epicoccum sorghinum	ZM25	0	0.5	0	0.5	0.29
Fusarium oxysporum	ZM26	5.5	3.7	4.4	13.6	0.91
Fusarium oxysporum	ZM27	2	1.5	2.2	5.7	0.36
Fusarium oxysporum	ZM28	5	5.5	5	15.5	0.29
Fusarium oxysporum	ZM29	3	3	3	9	0.00
Pythium myriotylum	ZM30	7	7	7	21	0.00
Alternaria sp.	ZM31	0	0	0	0	0.00
Alternaria sp.	ZM32	1	0	0	1	0.58
Alternaria sp.	ZM33	0	0	0	0	0.00
Pythium myriotylum	ZM34	7.5	7	7	21.5	0.29

Appendix 4 (continued)

Taxonomic ID	Isolate Lad ID	Lesion Length (cm)				Std
		Rep1	Rep2	Rep3	Mean	
Pleosporales sp.	ZM35	0	0	0	0	0.00
Fusarium equiseti	ZM36	4	2.5	3	9.5	0.76
Penicillium janthinellum	ZM37	0	0	0	0	0.00
Talaromyces cellulolyticus	ZM38	7.5	3	4.2	14.7	2.30
Curvularia trifolii	ZM39	3.5	4	4	11.5	0.29
Fusarium equiseti	ZM40	2.5	2	2	6.5	0.29
Fusarium cf. *oxysporum*	ZM41	6	4.5	5	15.5	0.76
Fusarium oxysporum f. sp. *ciceris*	ZM42	6	6.5	6	18.5	0.29
Fusarium solani	ZM43	7	3	5	15	2.00
Fusarium equiseti	ZM44	6.5	4	5	15.5	1.26
Curvularia borreriae	ZM45	5	6	5.5	16.5	0.50
Fusarium oxysporum	ZM46	7	4	5	16	1.53
Phoma sp.	ZM47	0	0	0	0	0.00
Chaetomium sp.	ZM48	0	0	0	0	0.00
Fusarium oxysporum f. sp. *vasinfectum*	ZM49	5	3	4	12	1.00
Fusarium oxysporum f. sp. *ciceris*	ZM50	7	4	4	15	1.73
Fusarium oxysporum	ZM51	7.5	5	5	17.5	1.44
Pythium myriotylum	ZM52	7	5	5	17	1.15
Aspergillus sp. 085241B	ZM53	0	0	0	0	0.00
Alternaria sp. 1 TMS-2011	ZM54	0	0	0	0	0.00
Setophoma terrestris	ZM55	0	0	0	0	0.00
Macrophomina phaseolina	ZM56	6	6.2	6.1	18.3	0.10
Macrophomina phaseolina	ZM57	6.3	6.1	6.2	18.6	0.10
Macrophomina phaseolina	ZM58	6.2	6.2	6.3	18.7	0.06
Macrophomina phaseolina	ZM59	6.1	6	6	18.1	0.06
Macrophomina phaseolina	ZM60	6.1	6.1	6.3	18.5	0.12
Rhizoctonia solani	ZM61	1.9	1.8	1.5	5.2	0.21
Rhizoctonia solani	ZM62	2	2.2	2.4	6.6	0.20
Rhizoctonia solani	ZM63	1.5	2	1.7	5.2	0.25
Phoma sp.	ZM64	0	0	0	0	0.00
Fusarium equiseti	ZM65	2	2.3	2.2	6.5	0.15
Fusarium equiseti	ZM66	0	0	0	0	0.00
Rhizoctonia solani	ZM67	6	5.5	5.7	17.2	2.50
Rhizoctonia bataticola	ZM68	6	5.9	6	17.9	0.06
Rhizoctonia solani	ZM69	5.6	5.5	4.5	15.6	0.61

Appendix 4 (continued)

Taxonomic ID	Isolate Lab ID	Lesion Length (cm)				Std
		Rep1	Rep2	Rep3	Mean	
Fusarium solani	ZM70	1.5	3	2	6.5	0.76
Fusarium solani	ZM71	6	5.5	5.7	17.2	0.25
Rhizoctonia solani	ZM72	6	5.9	6	17.9	0.06
Rhizoctonia solani	ZM73	5.6	5.5	4.5	15.6	0.61
Phoma sp.	ZM74	5	3.9	4.2	13.1	0.57
Phoma sp.	ZM75	4.5	4.8	4	13.3	0.40
Thielavia sp.	ZM76	0	0	0	0	0.00
Phoma cf. *herbarum*	ZM77	0	0	0	0	0.00
Phoma cf. *herbarum*	ZM78	0	0	0	0	0.00
Phoma cf. *herbarum*	ZM79	0	0	0	0	0.00
Phoma cf. *herbarum*	ZM80	**0**	**0**	**0**	0	0.00
Alternaria longissima	ZM81	0	0	0	0	0.00
Alternaria longissima	ZM82	0	0	0	0	0.00
Septoria arundinacea	ZM83	0	0	0	0	0.00
Alternaria longissima	ZM84	0	0	0	0	0.00
Fusarium oxysporum	ZM85	0	0	0	0	0.00
Chaetomium globosum	ZM86	0	0	0	0	0.00
Phoma glomerata	ZM87	0	0	0	0	0.00
Phoma cf. *herbarum*	ZM88	0	0	0	0	0.00
Phoma sp.	ZM89	0	0	0	0	0.00
Phoma sp.	ZM90	0	0	0	0	0.00
Phoma sp.	ZM91	0	0	0	0	0.00
Fusarium equiseti	ZM92	0	0	0	0	0.00
Fusarium equiseti	ZM93	3.7	3.5	3	10.2	0.36
Fusarium equiseti	ZM94	3.7	3.6	3.2	10.5	0.26
Fusarium equiseti	ZM95	3.4	3.9	3	10.3	0.45
Phoma sp.	ZM96	0	0	0	0	0.00
Fusarium equiseti	ZM97	4.5	4	4.3	12.8	0.25
Phoma sp.	ZM98	0	0	0	0	0.00
Phoma sp.	ZM99	0	0	0	0	0.00
Fusarium equiseti	ZM100	3.7	3.5	3.2	10.4	0.25
Rhizoctonia solani	ZM101	2	2.2	2.4	6.6	0.20
Rhizoctonia solani	ZM102	2	2.2	2.4	6.6	0.20
Rhizoctonia solani	ZM103	2	2.2	2.4	6.6	0.20

Appendix 4 (continued)

Taxonomic ID	Isolate Lab ID	Lesion Length (cm)				Std
		Rep1	Rep2	Rep3	Mean	
Rhizoctonia solani	ZM104	2	2.2	2.4	6.6	0.20
Rhizoctonia fragariae	ZM105	2	2.2	2.4	6.6	0.20
Rhizoctonia solani	ZM106	2	2.2	2.4	6.6	0.20
Rhizoctonia solani	ZM107	2	2.2	2.4	6.6	0.20
Fusarium equiseti	ZM108	4.5	4	4.3	12.8	0.25
Fusarium equiseti	ZM109	3.7	3.5	3	10.2	0.36
Fusarium oxysporum	ZM110	0	0	0	0	0.00
Phoma sp.	ZM111	0	0	0	0	0.00
Chaetomium globosum	ZM112	0	0	0	0	0.00
Chaetomium globosum	ZM113	0	0	0	0	0.00
Alternaria burnsii	ZM114	0	0	0	0	0.00
Alternaria alternata	ZM115	0	0	0	0	0.00
Phoma sp.*	ZM116	0	0	0	0	0.00
*Chaetomium globosum**	ZM117	0	0	0	0	0.00
*Fusarium oxysporum**	ZM118	1	0.5	0	1.5	0.50
Ceratobasidium sp.*	ZM119	6.7	7	6.9	20.6	0.15
Ceratobasidium sp.*	ZM120	7	7.1	6.5	20.6	0.32
*Fusarium oxysporum**	ZM121	3	3.1	2.9	9	0.10
Fusarium sp.*	ZM122	0	0	0	0	0.00
Gibberella sp.*	ZM123	4.2	4	4.1	12.3	0.10
*Phoma herbarum**	ZM124	0	0	0	0	0.00
Fusarium solani	ZM125	1.5	3	2	6.5	0.76
Fusarium oxysporum	ZM126	2.5	2.5	2.3	7.3	0.12
Fusarium oxysporum	ZM127	5	5.2	5	15.2	0.12
Fusarium oxysporum	ZM128	1.2	1.5	1	3.7	0.25
Fusarium equiseti	ZM129	3.5	0.5	2	6	1.50
Fusarium oxysporum	ZM130	0.5	2	0.7	3.2	0.81
Fusarium equiseti	ZM131	2	2.3	2	6.3	0.17
Fusarium oxysporum	ZM132	3.5	0.5	1	5	1.60
Fusarium oxysporum	ZM133	0.5	1	0.7	2.2	0.25
Fusarium oxysporum	ZM134	3	4	3.5	10.5	0.50
Fusarium oxysporum	ZM135	0.5	1	0.7	2.2	0.25
Fusarium equiseti	ZM136	4	4.1	4.7	12.8	0.38
Fusarium equiseti	ZM137	2.5	2.3	2	6.8	0.25
Fusarium equiseti	ZM138	2.5	2.4	2.7	7.6	0.15

Appendix 4 (continued)

Taxonomic ID	Isolate Lab ID	Lesion Length (cm)				Std
		Rep1	Rep2	Rep3	Mean	
Fusarium oxysporum	ZM139	3.3	3.5	3	9.8	0.25
Fusarium oxysporum	ZM140	2.4	2.6	2.3	7.3	0.15
Fusarium oxysporum	ZM141	2	2.2	2.4	6.6	0.20
Fusarium oxysporum	ZM142	3.5	0.5	1	5	1.61
Fusarium oxysporum	ZM143	3.5	0.5	1	5	1.61
Fusarium oxysporum	ZM144	2	2.2	2.4	6.6	2.00
Fusarium oxysporum	ZM145	2	2.3	2.5	6.8	0.25
Rhizoctonia solani	ZM146	2	2.2	2.4	6.6	0.20
Fusarium oxysporum	ZM147	2	2.2	2.4	6.6	0.20
Fusarium equiseti	ZM148	4	3.8	3	10.8	0.53
Sistotrema brinkmannii	ZM149	0	0	0	0	0.00
Fusarium oxysporum	ZM150	0.5	1	0.7	2.2	0.25
Fusarium equiseti	ZM151	3.5	0.5	2	6	1.50
Fusarium incarnatum	ZM152	0.5	0.8	1	2.3	0.25
Fusarium oxysporum	ZM153	0.5	1	0.7	2.2	0.25
Fusarium oxysporum	ZM154	0.5	1	0.7	2.2	0.25
Fusarium oxysporum	ZM155	3.5	0.5	1	5	1.61
Fusarium oxysporum	ZM156	4	4.1	4.7	12.8	0.38
Fusarium oxysporum	ZM157	4	4.1	4.7	12.8	0.38
Fusarium oxysporum	ZM158	0.5	0.8	1	2.3	0.25
Fusarium oxysporum	ZM159	4	4.1	4.7	12.8	0.38
Fusarium equiseti	ZM160	0	0	0	0	0.00
Fusarium equiseti	ZM161	3.7	3.5	3	10.2	0.38
Fusarium oxysporum	ZM162	2.5	2.3	2	6.8	0.25
Fusarium equiseti	ZM163	0.5	0.8	1	2.3	0.25
Fusarium oxysporum	ZM164	0.5	1	0.7	2.2	0.25
Fusarium oxysporum	ZM165	0.5	1	0.7	2.2	0.25
Fusarium oxysporum	ZM166	3.5	0.5	1.5	5.5	1.50
Fusarium oxysporum	ZM167	0.5	1	0.7	2.2	0.25
Fusarium oxysporum	ZM168	0.5	1	0.7	2.2	0.25
Fusarium equiseti	ZM169	3.5	0.5	2	6	1.50
Fusarium solani	ZM170	4.5	4	5.3	13.8	0.66
Fusarium solani	ZM171	1.5	3	2	6.5	0.76
Fusarium solani	ZM172	1.5	3	2	6.5	0.76

Appendix 4 (continued)

Taxonomic ID	Isolate Lab ID	Lesion Length (cm)				Std
		Rep1	Rep2	Rep3	Mean	
Fusarium solani	ZM173	1.5	3	2	6.5	0.76
Curvularia trifolii	ZM174	0	0	0	0	0.00
Phoma sp.	ZM175	0	0	0	0	0.00
Fusarium oxysporum	ZM176	0.5	0.8	1	2.3	0.25
Fusarium oxysporum	ZM177	4	4.1	4.7	12.8	0.38
Fusarium oxysporum	ZM178	4	4.1	4.7	12.8	0.38
Fusarium oxysporum	ZM179	0.5	0.8	1	2.3	0.25
Fusarium oxysporum	ZM180	2.5	2.3	2	6.8	0.25
Fusarium equiseti	ZM181	0	0	0	0	0.00
Fusarium equiseti	ZM182	0	0	0	0	0.00
Fusarium equiseti	ZM183	3	3.2	2.3	8.5	0.47
Fusarium oxysporum	ZM184	0.5	1	0.7	2.2	0.25
Fusarium oxysporum	ZM185	2.5	2.5	2.3	7.3	0.12
Fusarium oxysporum	ZM186	2.5	2.5	2.3	7.3	0.12
Fusarium oxysporum	ZM187	2.5	2.5	2.3	7.3	0.12
Fusarium oxysporum	ZM188	2.5	2.5	2.3	7.3	0.12
Fusarium oxysporum	ZM189	0.5	1	0.7	2.2	0.25
Fusarium oxysporum	ZM190	0	0	0	0	0.00
Fusarium oxysporum	ZM191	0	0	0	0	0.00
Fusarium oxysporum	ZM192	2	2.1	2.3	6.4	0.15
Fusarium oxysporum	ZM193	0	0	0	0	0.00
Fusarium equiseti	ZM194	0	0	0	0	0.00
Fusarium equiseti	ZM195	3.4	3.9	3	10.3	0.45
Fusarium equiseti	ZM196	4	4.1	3.9	12	0.10
Fusarium oxysporum	ZM197	0.5	0	0.5	1	0.29
Fusarium oxysporum	ZM198	0.5	0	0.5	1	0.29
Fusarium oxysporum	ZM199	0.5	0	0.5	1	0.29
Fusarium oxysporum	ZM200	0.5	0	0.5	1	0.29
Fusarium solani	ZM201	1.5	3	2	6.5	0.76
Fusarium solani	ZM202	1.5	3	2	6.5	0.76
Fusarium oxysporum	ZM203	1	2.5	1.7	5.2	0.75
Fusarium oxysporum	ZM204	0.5	0	0.5	1	0.29

*Isolates from assymptomatic(control) plants.

Appendix 5. Cross tabulations for comparisons between PCR and Pyrosequencing in 2013 and the extent of agreement between FTA cards and Tissue samples in RCR identification

	Misamfu 2013				Mt. Makulu 2013			
Pyrosequencing		PCR				PCR		
		1	0			1	0	
	1	9	4	13	1	7	1	8
	0	16	11	27	0	7	13	20
		25	15	40		14	14	28
		P(o)	0.5			P(o)	0.7143	
		P(e)	0.45625			P(e)	0.5	
		Kappa	0.08046			Kappa	0.4286	
	Misamfu 2014							
	Pyrosequencing				PCR			
FTA Card DNA		Tissue DNA						
		1	0			1	0	
	1	20	8	28	1	14	2	16
	0	3	25	28	0	9	31	40
		23	33	56		23	33	56
		P(o)	0.80357			P(o)	0.8036	
		P(e)	0.5			P(e)	0.5383	
		Kappa	0.60714			Kappa	0.5746	
	Misamfu 2015							
	Pyrosequencing				PCR			
FTA Card DNA		Tissue DNA						
		1	0			1	0	
	1	12	2	14	1	4	1	5
	0	12	34	46	0	22	33	55
		24	36	60		26	34	60
		P(o)	0.76667			P(o)	0.6167	
		P(e)	0.55333			P(e)	0.5556	
		Kappa	0.47761			Kappa	0.1375	
	Kabwe 2015							
	Pyrosequencing				PCR			
FTA Card DNA		Tissue DNA						
		1	0			1	0	
	1	2	2	4	1	13	15	28
	0	8	56	64	0	18	22	40
		10	58	68		31	37	68
		P(o)	0.85294			P(o)	0.5147	
		P(e)	0.81142			P(e)	0.5078	
		Kappa	0.22018			Kappa	0.0141	