

**PATHOGEN VARIATION AND QUANTIFICATION OF *PYTHIUM* SPECIES IN
BEAN FIELDS IN UGANDA**

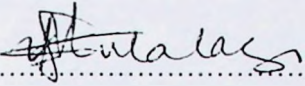
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A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of
Philosophy of Makerere University

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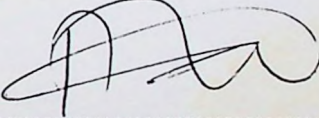
DECLARATION

This thesis is entirely my own work and has not been presented for a degree award in any University


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DEDICATION

To my family: Jackie, Genevieve, Edgar and Oscar

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SUMMARY

In Uganda, a major pathogen genus causing severe *Phaseolus* bean root rot, particularly in southwestern, is *Pythium*. But there is no information on the different *Pythium* species associated with bean root rots nor about impact of use of organic amendments on *Pythium* populations in field soils. This study was therefore, undertaken to a) identify *Pythium* species pathogenic to beans and map their distribution in major bean producing areas of Uganda, and b) develop a procedure of quantifying *Pythium* populations in field soils and c) to evaluate the impact of soil organic amendments on inoculum levels of *Pythium* spp. pathogenic on beans (*Phaseolus vulgaris* L.).

The first part of the study involved morphological, molecular and pathogenicity analyses of *Pythium* isolates collected from Kisoro and Kabale districts in southwestern Uganda and Mbale in the east. Molecular analyses using PCR-RFLP and sequencing techniques were used in the identification of *Pythium* species. Eleven *Pythium* species were found to be associated with bean root rots in Uganda. Apart from the traditional species, *P. ultimum* var *ultimum*, known to infect beans, other *Pythium* species recovered from infected beans were *P. spinosum*, *P. torulosum*, *P. salpingophorum*, *P. vexans*, *P. dissotocum*, *P. nodosum*, *P. echinulatum*, *P. pachyaule*, *P. oligandrum* and *P. deliense*.

All isolates of *P. ultimum* var *ultimum* and *P. spinosum* caused severe root rot on susceptible bean varieties CAL 96 and K20. The other isolates belonging to *P. torulosum*, *P. nodosum* and *P. pachycaule* were also pathogenic to beans varieties CAL 96 and K20. Pathogenicity tests indicated significantly more disease (8 to 9 score) when highly pathogenic *Pythium* species were used to inoculate susceptible bean varieties CAL 96 and K20 compared to the highly tolerant variety RWR 719 (score of 2 to 5).

Generally, morphological, molecular and pathogenicity analyses revealed that there is wide diversity within *Pythium* population in Uganda. The results obtained demonstrated that *P. ultimum* var *ultimum*s is responsible for the increased disease epidemics in Kabale and Kisoro districts. But, pathogenicity of *P. spinosum*, *P. nondosum* and *P. pachycaule* isolates suggest that these species also cause bean root rot under field conditions. Isolates of *P. torulosum* and *P. salpingophorum* were more variable in their pathogenicity, but the species could probably contribute to bean root rot as part of the complex of minor pathogens.

Some PCR species-specific primers with good level of specificity to detect and differentiate *P. salpingophorum*, *P. torulosum*, *P. spinosum*, *P. nodosum* and *P. ultimum*, were developed during the study. The limitation of the primers was their failure to differentiate some particular *Pythium* species with variation in pathogenicity, as was the case with *P. torulosum* and *P. salpingophorum*. Further molecular analysis of other regions of the genome is required to locate genes responsible for the variation and later design primers specific to pathogenic forms of *P. torulosum* and *P. salpingophorum*.

The second part of the study involved quantification of inoculum levels of *Pythium* spp. pathogenic to beans, using the dilution-plating method with a selective media, in combination with molecular PCR-RFLP identification procedures. Soils infested at ratios of 1:5 and 1:10 consistently produced large numbers of colonies when 1 ml of suspension from the 50 or 100 ml volumes was plated out. It was difficult to count the colonies, as in the second day they were merged. Further dilution to 200ml produced countable colonies (15 per plate; equivalent about 1500 cfu.g⁻¹). While soils infested with *Pythium* at ratios of 1:15 and 1:20 v/v consistently produced fewer colonies (about 2 per plate). The dilution plating procedure developed to estimate *Pythium* populations was plating 0.7ml (instead of 1 ml) of a suspension of two grams soil in 100ml of sterile water. The numbers of colonies growing

on each plate was about seven (equivalent to 500 cfu.g⁻¹). Two to three colony types were observed on plates containing samples from the naturally infested field soil from Kicumbi, suggesting the different possible *Pythium* species occurring in field soils. The identification using molecular techniques still differentiated the colonies into groups.

The third part of the study assessed the impact of organic amendment (farmyard manure and *Calliandra*) on *Pythium* populations in field soils. Farmyard manure and *Calliandra* significantly increased *Pythium* populations in both screen house and field experiment, by a range of 200 to 700 colony forming units in the field trial and up to 1200 in the screen house relative to the control. Interestingly, *Calliandra* increased the frequency of *P. salpingophorum* by two times but reduced that of *P. ultimum*. On the other hand farmyard manure increased frequencies of *P. ultimum* by about one and half but reduced those of *P. salpingophorum*. The mechanism by which the frequencies of particular *Pythium* species are reduced or increased is not well understood, and should be investigated.

Despite the increases in *Pythium* populations in field soils, amendments resulted into increased dry matter and bean grain yield. It is postulated that farmyard manure and *Calliandra* may be able to compensate for the negative impact of increased disease by causing a larger increase in soil fertility, thereby increasing yield. Further, both farmyard manure and *Calliandra* reduced *Pythium* population level in the follow up crop by about 50%. Then, they could be used as part of a series of management practices to reduce the impact of *Pythium* root rot on beans in a long run.

This study has therefore, a) characterized the *Pythium* species responsible for bean root rots in southwestern Uganda, b) developed *Pythium* specific primers for detecting five *Pythium* species in both plants and soil c) developed a procedure for quantifying *Pythium* inoculum levels in the soil, and d) demonstrated the beneficial effect of organic amendments

for reducing the impact *Pythium* levels in the soil. This knowledge will be useful for future development of bean varieties in the Great Lakes region of eastern Africa.

CHAPTER ONE

INTRODUCTION

1.1 Importance of beans

The common bean (*Phaseolus vulgaris* L.) is the second most important source of human dietary protein and the third most important source of calories of all agricultural commodities produced in eastern and southern Africa (Pachico, 1993). With increasing human population on limited land area, production of carbohydrates and proteins per unit area must be stepped up. Beans are able to contribute to this because they give yields in a short growing season (80-90 days). In Uganda, beans are grown twice a year, i.e., March – July and September- December which would make it possible to produce sufficient amounts of beans.

Bean production is increasing in most developing countries, but yields per unit area are still low. In Uganda, the land area planted with beans increased from 495 ha in 1990 to 630 ha in 1999 (FAO, 1999), but yield has been declining probably because of declining soil fertility and increased incidences of pests and diseases. The average national yield in the country is estimated at 349 kg/ha compared to 787 kg/ha ten years ago (FAO, 1999). Nevertheless, beans occupy an important niche in Uganda's agricultural sector and farm household economy (Opio *et al.*, 2001). Beans are a major source of both food security and income. The crop can be used as either food, sold domestically for cash or exported to earn foreign exchange. As an important pulse crop in Uganda, beans are grown throughout the country but southwestern is the largest producer, responsible for 30% of beans produced in the country (Ministry of Agriculture Animal Industry and Fisheries, 1992).

1.2 Bean production constraints

Food crops including beans are grown intensively in many areas of eastern Africa due to land shortage. As a consequence, soil fertility is declining; fallow periods are almost none existing; and crop rotation, effective in managing most diseases, is seldomly practised (Clay and Magnani, 1987). The annual bean yield losses in eastern Africa vary from 20 to 100% in bean fields (CIAT, 1990). In southwestern Uganda, bean production dropped to 20% in 1995, and this was attributed to the effect of insect pests and diseases, most specifically root rots (Opio, 1999 *Unpublished*).

Incidence and severity of root rots are associated with high intensity of bean production and less productive soils. A model has been developed to predict new areas where bean root rots are likely to emerge as a serious problem or their importance has been underestimated (Wortmann *et al.*, 1998). In Uganda, for example, predictions have been made about the possible emerging of bean root rots in new areas such as Nebbi, Apac (northern Uganda) and parts of Ntungamo (western Uganda).

1.3 Justification of the study

Root rots of beans are caused by a number of soil borne pathogens that occur either individually or as a complex of two or more pathogens depending on the environmental conditions (Rusuka *et al.*, 1997). These include; *Fusarium solani* f.sp. *phaseoli* which causes Fusarium root rot, *Rhizoctonia solani* Kühn which causes *Rhizoctonia* root rot, *Sclerotium rolfsii* Sacc. causing Southern blight, and *Pythium* spp. which cause *Pythium* root rots. In southwestern Uganda, the major pathogen causing severe root rot has been identified as *Pythium* spp. (Opio, 1998). Other pathogens include *Fusarium* spp. and

Rhizoctonia solani, which occur singly or in complex with other pathogens including *Pythium* spp. (Opio, 1998).

Four *Pythium* spp. namely, *P. ultimum* Trow, *P. irregulare* Buism, *P. aphanidermatum* (Edson) Fitz., and *P. myriotylum* Drechs, have been implicated in bean root rots, however, their importance and possible synergistic effects are not known. In fact identification of root rot pathogens using morphological or pathogenic characteristics is difficult and slow. Yet, identification of *Pythium* and other key bean root rot pathogens to species level is critical for identifying host resistance or introducing other management practices such as crop rotation. There is a need, therefore, to identify *Pythium* species responsible for root rot in Uganda and determining the levels of variation within pathogen populations, using molecular techniques (Chen, 1992; Chen *et al.*, 1992a; Wang and White, 1997).

Population levels in the soil influences incidence and severity of root rots. Some management efforts have been directed towards reducing soil inoculum to below economically damaging threshold levels. The effects of management practices can be assessed on the basis of disease severity, however, this measure alone is not a good indicator of the soil pathogen population. Environmental factors and stage of plant growth play a role in influencing disease development and severity (Hall, 1996). There is a need therefore to develop tools and procedures that are simple, fast and accurate for the quantification of *Pythium* spp in soil. This will be helpful in developing prediction models, but more importantly will facilitate determining the effects of various root rot management options (cultural, varietal and biological), and the influence of environmental factors on *Pythium* spp. soil populations.

1.4 Objectives of the study

Based on the above, the present study was undertaken with the following objectives:

- 1) to identify and differentiate *Pythium* spp. causing root rots on *Phaseolus* beans using DNA based molecular markers;
- 2) to determine the distribution of *Pythium* spp. associated with bean root rot in Uganda;
and
- 3) to develop methods of quantifying inoculum levels of *Pythium* spp pathogenic to beans and evaluating the effect of organic amendments on *Pythium* populations in field soils.

The study is based on three hypotheses:

- 1) Different *Pythium* spp. occur in Uganda and have contributed to the increased epidemics of root rot diseases in bean producing areas, particularly in southwestern Uganda;
- 2) Pathogenic *Pythium* species are widely spread and occur together in major bean producing areas; and
- 3) Organic amendments reduce *Pythium* populations in field soils.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Pythium* species as plant pathogens

Pythium species belong to Pseudofungi of class Oomycetes, order Peronosporales in the family Pythiaceae (Agrios, 1997). *Pythium* spp are ubiquitous in soil and aquatic environments, they have worldwide distribution, and life histories that range from saprotrophic, facultative parasites with extensive host ranges to highly pathogenic species with limited host ranges (Plaats-Niterink, 1981). *Pythium* species cause rot of fruit, roots or stem, pre- or post-emergence damping off of seeds and seedlings, root necrosis (Plaats-Niterink, 1981), cavity spot (White, 1986), replant diseases (Sewell, 1981), also subclinical infection in numerous crops (Stanghellini and Kronland, 1986; Cook *et al.*, 1987).

While phytopathogenic species of *Pythium* are capable of infecting a wide range of plants, members of the genus are not uniform with regard to pathogenicity; individual species vary in the extent of their host range as well as virulence on specific hosts. For example, *P. aphanidermatum* and *P. ultimum* are capable of causing serious losses of a number of economic crops in a variety of plant families (Plaats-Niterink, 1981). However, some crop plants are very susceptible to attack, especially at seedling stages, e.g. sugar beet (*Beta vulgaris*) and cucumber (*Cucumis sativus*). Cultivars of the same host species are known to vary in susceptibility. Likewise, variation in virulence among species infecting a specific host may also be found, with some *Pythium* spp. causing greater damage than others (Bruckrart and Lorbeer, 1982; Nelson and Craft, 1991; Lee and Hoy, 1992). Intraspecific variation in virulence has also been reported (Hoy and Schneider, 1988; Nelson and Craft, 1991).

Several *Pythium* spp. cause root rots in beans. Some of these species include *P. ultimum* Trow, *P. irregulare* Buism, *P. aphanidermatum* (Edson) Fitz., and *P. myriotylum* Drechs. (Abawi and Pastor Corrales, 1990). Asexually, *Pythium* produces sporangia, which may germinate directly as in *P. ultimum* or through the production of zoospores as in *P. aphanidermatum*. Sexual reproduction is characterised by the production of oospores, which helps them survive in the soil for long periods. Activities and damage by *Pythium* spp. is mainly favored by high soil moisture. Damage by *P. ultimum* to beans is severe at low soil temperature (16-20°C), whereas *P. aphanidermatum* and *P. myriotylum* cause damage at higher temperature (28°C) (Abawi and Pastor-Corrales, 1990). Thus, for the different *Pythium* species, their frequency of distribution and damage on beans are likely to be influenced by season variations. There is a need, therefore, to establish the different *Pythium* species pathogenic to beans and the variation within the pathogenic strains that are predominant at any particular growing season.

2.2 Interactions between *Pythium* spp. and other root rot causing pathogens

In eastern Africa, bean root rots are caused by a number of soil borne pathogens, namely; *Fusarium solani* f.sp. *phaseoli*, *Sclerotium rolfsii*, *Rhizoctonia solani* and *Pythium* spp. The pathogens occur either individually or as a complex of two or more pathogens depending on suitable environmental conditions (CIAT, 1992; Rusuka *et al.*, 1997). In southwestern Uganda, the major pathogen causing severe root rot has been identified as *Pythium* spp. (Opio, 1998). Other pathogens that are frequent include *Fusarium* spp. and *Rhizoctonia solani*, which may occur singly or in complex, with *Pythium* spp. (Opio, 1998). A study by Pieczarka and Abawi (1978a) reported more severe bean root rot in soil infested with *P. ultimum* and *F. solani* f. sp. *phaseoli*, which suggested a synergistic relationship between the pathogens. In the same study, *R. solani* reduced the severity of root rot incited by *P.*

ultimum suggesting an antagonistic relationship of the two pathogens. There were no interactions between *F. solani* f. sp. *phaseoli* and *R. solani*. The synergistic interaction between *P. ultimum* and *F. solani* f. sp. *phaseoli* as demonstrated by Pieczarka and Abawi (1978a) may not be operative at times when soil conditions are not favourable for *P. ultimum* activity (Pieczarka and Abawi, 1978b).

In Uganda, only limited information is available concerning the importance, and the possible synergistic effects of the four *Pythium* spp. implicated in bean root rots. Decision making in the management of *Pythium* root rot would, therefore, require identification and an understanding of the distribution of *Pythium* spp. in bean producing areas where root rots are a major constraint.

2.3 Management of *Pythium* root rots

Pythium diseases in green houses have been controlled through soil sterilisation and use of chemically treated seed. In the field seeds treated with chemicals, such as chloranil, chloroneb, mancozeb, thiram, and captan are a preventive measure. The systemic fungicide metalaxyl gives excellent control of damping off, seedling blights, and root rots caused by *Pythium* and *Phytophthora*; it is applied as soil or seed treatment (Agrios, 1997). In a field situation the entire body of the soil, at least in the surface horizon, requires treatment, which is expensive and practically impossible for smallholder farmers. Strategies to management of *Pythium* root rot on beans should therefore focus on use of host resistance and organic amendments.

In an effort to reduce the severe outbreaks of the bean root rot disease, a few resistant/ tolerant bean varieties to *Pythium* spp. have been identified and are now being grown in

areas where root rots are a serious problem in southwestern Uganda (Opio, 1998). The effectiveness of the available tolerance may be limited due to two factors; the presence of other pathogens like *Fusarium* spp. and *Rhizoctonia solani*, which occur singly or in complex (including *Pythium* spp), and the occurrence of the different *Pythium* species in the bean producing areas. The identification of *Pythium* at species level in the major bean producing areas where root rot is a problem would be very critical for identifying host resistance or introducing other management practices, particularly crop rotation of host plants, which may be resistant or susceptible.

Cultural methods such as incorporation of different types and levels of organic amendments e.g, green manure, in the management of bean root rot disease as described by Buruchara and Scheidegger (1993) and Opio (1998) results in the reduction in plant mortality, severity of bean root rots and increase in grain yield. High levels of organic matter in the soil improve physical and chemical conditions for root growth (Hall, 1996), and therefore increase the tolerance of roots to diseases or to compensate for the destructive effects of diseases (Lazarovits, 2001). They may also facilitate natural biological control through increased microbial activities in the soil that may have negative effects on the soil borne pathogens (Hall and Nasser, 1996). It is not clear whether organic amendments that reduce disease also lower population densities of the pathogens in soil.

The other cultural practices used in the control of bean root rot include; rotation with grain crops and growing of beans on raised beds especially in soils with high moisture levels such as those in valley bottoms (Abawi and Pastor-Corrales, 1990). Rotation is highly effective in reducing populations in soil of specialised pathogens of beans such as *Fusarium solani* f.sp. *phaseoli* (Hall and Phillips, 1992). Rotation has not been recommended for control of

Pythium spp, *Macrophomia phaseolina*, *Teichothecium roseum*, as these tend to have wide host ranges (Hall and Nasser, 1996).

Population levels of soil borne pathogens influence incidence and severity of root rots and some management efforts have been directed at reducing inoculum below economically damaging threshold levels. Disease severity alone is not a good measure of the effect of management practices on soil pathogen populations. Environmental factors and stage of plant growth play a critical role in disease incidence and severity. There is a need, therefore, to determine the effect of the different management practices on the inoculum levels of *Pythium* spp. in the soil.

2.4 *Pythium* species identification and differentiation

2.4.1 Classification of the genus *Pythium*

The development of an effective disease management strategy requires understanding the causal organism. *Pythium* being a major pathogen that causes disease on a wide range of crops, a number of studies have been conducted to identify *Pythium* species. The classification of the genus *Pythium* by Plaats-Niterink (1981) which has 87 species discriminated by a dichotomous key, supported by physiological characters such as daily growth rate and cardinal temperatures as supplementary diagnostic criteria. An additional 65 species were listed as excluded from the key or doubtful, principally being impossible to identify. Some isolates examined were reported to produce only sporangia or hyphal swellings and form no oogonia in single or dual cultures. These were placed into five groups: Group F, with filamentous non-swollen sporangia; Group T, with swollen turuloid

sporangia; Group G, with globose non-proliferating sporangia; Group P, with globose proliferating sporangia; and Group HS, with hyphal swellings only.

In eastern Africa, preliminary diagnosis of *Pythium* spp. has always been based on symptoms in shoots, hypocotyls, and roots that are associated with *Pythium* root rot. Identification of *Pythium* spp in the laboratory has been done according to colony characteristics and reproductive structures (Abawi and Pastor Corrales, 1990). *Pythium* isolates in the region have been identified to genus level (Rusuku *et al.*, 1997; Opio, 1998). This is partly because identification of *Pythium* spp. using morphological or pathogenic characteristics is both difficult slow, and when extracted from soil, is complicated by the presence of a wider range of both saprophytes and other pathogens. However, identification at species level is important for identifying host resistance or introducing disease management practices. There are examples of some bean varieties that show reasonable root rot resistance when grown in Rwanda, but not in Kenya implying a possible pathogen variation (Otsyula and Ajanga, 1995). There is therefore, a need to characterise *Pythium* spp. causing root rots in Uganda and determine their distribution.

2.4.1.1 Current problems in *Pythium* identification

These are related to;

- (a) The morphological features by which *Pythium* spp are distinguished may be affected by the growth media used, temperature variation and age of culture (Hendrix and Campbell, 1973)
- (b) The small number of morphological characters available in *Pythium*, which are based principally on the structure of sporangia and gametangia.

- (c) For many *Pythium* species, similarities in morphological features exist among the different groups of species. Many taxonomic characters have ranges that overlap between species.
- (d) Intraspecific morphological variation is frequently observed in different field isolates, and a large number of isolates can differ considerably from the type of species.
- (e) It is sometimes difficult to obtain all the asexual and sexual reproductive structures needed to identify an isolate. Some species produce oospores only when paired with opposite mating types. Seven heterothalic *Pythium* species have been found, and at least five of them are important plant pathogens (Plaats-Niterink, 1981). Heterothallism further complicates the identification process.
- (f) Isolates that do not reproduce sexually are the most difficult to identify. Plaats-Niterink (1981) classified these according to the types of sporangia or hyphal swellings and placed them in groups accordingly. However, it is difficult to further delineate isolates or to place them with respect to described species because variation may occur in each group in relation to sporangial size, temperature growth response, plant pathogenicity, and ecology (Piecarka and Abawi, 1978b; Martin, 1990)

2.4.2 Molecular methods for detecting and identification of plant pathogenic fungi

There are various procedures that can be used to exploit genetic differences in identification of *Pythium* species. Analysis of restriction fragment length polymorphism (RFLP) employs restriction endonucleases, which cleave DNA at recognised specific sequence. Digestion of the PCR products with endonuclease results in small fragments of DNA, which can be separated by electrophoresis in agarose gels. Variations in banding pattern are used to discriminate between isolates and species. RFLP analysis has successfully been used in detecting interspecific and intraspecific variation in a number of fungi (Carder and Barbara,

1991; Manicom and Baayen, 1993; Okoli *et al.*, 1993; Bernier *et al.*, 1994; Hopple and Vilgalys, 1994).

The amplified DNA fragments may also be sequenced to determine the primary structural sequence of nucleotides. By studying the sequences of different fungi, short regions of the ribosomal DNA can be found that are unique to particular taxa. Oligonucleotides can be made that are complementary to these regions and these can be used as specific oligonucleotide probes or as primers in a specific PCR assay for the organism. Those probes or PCR primes can be used to aid identification (Nazar *et al.*, 1991; Lea *et al.*, 1993).

Conventional PCR requires some knowledge about DNA segment of interest in order to adopt suitable primers. Random amplified polymorphic DNA (RAPD) uses short oligonucleotide of 8-12 bases as primers, which bind to complementary sites at random to produce 'fingerprints' without knowing anything about the sequence of the DNA to be amplified (Williams *et al.*, 1990). Any band that appears to be unique to particular taxa can be labelled and tested for use as specific probes. This method has been used to devise a way of differentiating between varieties of plant pathogenic fungi (Guthrie *et al.*, 1992; Kelly *et al.*, 1994; Crowhurst *et al.*, 1995).

2.4.3 Molecular identification of *Pythium* spp. by analysing Ribosomal DNA

Restriction fragment patterns from amplified Mitochondrial DNA (mtDNA) provide a useful tool in taxonomy. Variation within species in a genus such as *Phytophthora* (Forster *et al.*, 1988), *Colletotrichum* (Sreenivasaprasad *et al.*, 1992) and *Trichoderma* (Muthumeenakshi *et al.*, 1994) were high when comparisons were made using mtDNA. Restriction banding patterns of mtDNA were used to identify asexual isolates of *P. ultimum* (Martin 1990).

However, since most mutations in the mitochondria genome of *Pythium* species are due to insertion/deletions, phylogenic and evolutionary comparisions have to rely on analysis of the conserved regions of the molecule.

RFLP analysis of the mitochondrial small-subunit ribosomal RNA gene (rDNA) and the nuclear rDNA region containing the internal transcribed spacer (ITS) regions and the 5.8S rRNA gene are useful in determining relationship between fungal genera and species (Martin and Kistler, 1990; Carder and Barbara, 1991; Chen, 1992; Chen *et al.*, 1992a; Manicom and Baayen, 1994; Matsumoto and Fukumasa-Nakai, 1995). Species-specific polymorphism in transcribed ribosomal DNA of eight *Pythium* species were reported and little intraspecific variation was observed (Chen, 1992; Chen *et al.*, 1992). The extent of variation in restriction sites within the spacers of the ribosomal RNA genes has also been determined for 36 important pathogenic *Pythium* species (Wang and White, 1997). It is now possible to differentiate *Pythium* strains using RFLP analysis of the ITS region of the ribosomal DNA.

The polymerase chain reaction coupled to restricted fragment length polymorphisim analysis (PCR-RFLP) has become a useful tool in fungal taxonomy (White *et al.*, 1990; Paul *et al.*, 1998), and is currently used to identify different species of *Pythium* (Chen, 1992; Chen *et al.*, 1992a; Wang and White, 1997). Amplification of the ribosomal gene is used for the genetic identification of many organisms because they comprise of both highly conserved sequences during evolution and highly variable sequences among species and even within species. The ribosomal nuclear DNA consists of transcribed and non-transcribed regions (Chen *et al.*, 1992b). The internal transcribed spacer (ITS1) is a non-conserved region compared to ITS2. Use of sequences of the variable ITS region to elucidate the molecular variation of plant pathogens has been reported (Sreenivasaprasad *et al.*, 1992). Comparing

sequences of some fungal species, the ITS1 showed more variation than the ITS2 (Berbee *et al.*, 1995; Goodwin *et al.*, 1995; Morales *et al.*, 1995). Jasalavich *et al.* (1995) suggested that variability focused mostly at the species level in ITS1 and at genus level in ITS2. The ITS regions are small enough to be easily generated by PCR and are flanked by highly conserved sequences for which universal primers have been identified (White *et al.*, 1990).

There has been very rapid development of nucleic-acid-based detection techniques. Many of the primers have been developed from the rDNA sequences (Henson, 1992; Henson and French, 1993; Chen, 1994; Ersek *et al.*, 1994). An ideal situation for the application of PCR diagnostic test and disease epidemiology is that in which DNA sequences exist which are unique to a given pathogen. The regions are useful targets because they appear to be conserved within species, but can vary significantly among several plant pathogenic species to allow the identification of unique primer sequences (Kistler *et al.*, 1987; Nazar *et al.*, 1991; Chen *et al.*, 1992a; Xue *et al.*, 1992). The ITS sequences database of a number of known *Pythium* species is now available (Wang and White 1997; Matsumoto *et al.*, 1999; Paul, 2000; Paul and Masih, 2000). Using PCR, it is now possible to compare the ITS sequence data available with that generated from Ugandan *Pythium* isolates to aid in selecting species specific primers for the detection of *Pythium* pathogenic to beans.

2.5 Quantification of pathogenic *Pythium* in field soils

Inoculum levels of the soil-borne pathogen influence incidence and severity of root rots. Organic amendments in the soil tend to reduce root rot possibly by affecting soil inoculum levels of *Pythium* spp. (Buruchara and Scheidegger, 1993; Hall and Nasser, 1996; Opio, 1998). Unfortunately, reliable methods to determine soil inoculum levels of *Pythium* are not

yet available. Thus, tools and procedures that are simple, fast and accurate for quantification of *Pythium* populations in soil need to be developed to facilitate development of disease prediction models. This will allow determination of the effects of various root rot management options such as cultural, varietal and biological activity on *Pythium* populations. The objective or management effort is to reduce inoculum levels in the soil to below economic threshold level.

2.5.1 Dilution plating method of quantifying *Pythium* in soil

The most widely used method for quantifying a number fungus is the dilution plating/selective medium method, which is based on suspending soil particles in water and spreading on a petri plate (Nash and Snyder, 1962). Excellent selective media exist for most widely studied soil borne pathogens, including *Fusarium* (Nash and Snyder, 1962), *Pythium* (White, 1988), *Phytophthora* (Pettitt and Pegg, 1991). After about one or two days, in the case of *Pythium*, colony-forming units (cfu) per unit of soil are determined.

Dilution plating has inherent disadvantages. Mostly there is loss of spores (inoculum) which may occur at any of the various stages leading to inaccurate assessment of the level of the pathogen populations in the soil. Further, it can not distinguish closely related fungi, for example pathogenic and non-pathogenic isolates of *Pythium* spp and *Fusarium oxysporum*. The method can also overestimate fungi that sporulate heavily (Paulitz, 2000).

2.5.2 Baiting method of quantifying *Pythium* inoculum levels in soil

The method involves the use of living or non-living plant substrates, for example, leaf pieces, cotyledons, seedlings or fruit, added to a soil sample. Its used for zoosporic

pathogens such as *Pythium* and *Phytophthora* that are difficult to isolate with selective media (Paulitz, 2000). The baiting substrate is put into flooded soil and zoospores are attracted to it. The substrate can then be placed on selective media where the fungus will grow readily. The advantage is that the bait can detect low inoculum levels. In hydroponic studies with *P. aphanidemartum*, millet seeds were used to bait the pathogen out of water samples and quantify the inoculum of the pathogen (Rankin and Paulitz, 1994). The method can be semi-quantitative by developing standard curves with known zoospore concentrations.

2.5.3 Bioassay method of quantifying soil pathogen

The method is suitable for quantifying obligate soil pathogens, such as *Plasmodiophora brassica* and obligate symbionts, such as arbuscular mycorrhizial fungi (Paulitz, 2000). The method can also distinguish between pathogenic and non pathogenic species and races of formae speciales of fungi. The number can be quantified with the use of most probable number (MPN) statistics, based on presence or absence of infection in bioassay plants in serial dilutions of the soil. The advantage of the method is its sensitivity and ability to determine the inoculum potential or infectivity present in a sample, an important factor in predicting the resulting disease. The plant based bioassay method and mathematical models have been developed to determine inoculum potentials of a number of soils borne pathogens including *Rhizoctonia solani*, *Pythium irregulare* and *sclerorium rofsii* (Baker, 1971), *Fusarium solani* f.s. *phaseoli* and *Fusarium roseum* f.s *cerealis* (Baker and Drury, 1981). The method, therefore, can be useful in determining inoculum potential and later economic thresholds of pathogenic *Pythium* spp. to beans in field soils.

2.5.4 PCR based method of quantifying pathogens in the soil

Advances in molecular biology have permitted the development of rapid detection and quantification of fungal DNA without the need for different semiselective nutrients, antibiotics, and carbon sources, all which can affect fungal growth and morphology (Robb *et al.*, 1993; Nicholson *et al.*, 1998). Basing on nucleotide differences in the intervening transcribed spacer sequence (ITS1 and ITS2) of the ribosomal genes a PCR-based assay was developed for evaluation of *Verticillium* biomass (Hu *et al.*, 1993; Robb *et al.*, 1993). A fast and efficient method of fungal DNA extraction directly from soil samples has been developed and improved (Volossiuk *et al.*, 1995; Henz and Platt, 2000a). This makes it possible to employ a PCR-based assay to directly quantify the pathogen in soil.

A competitive PCR-based assay is providing a possible alternative for quantification of pathogen populations in the soil (Hu *et al.*, 1993; Mahuku *et al.*, 1995; Henz and Platt, 2000b). In competitive PCR assay an internal standard (competitor) with a known DNA sequence and mass is added to the original sample. The competitor and target are amplified together, using the same primers, and the ratio of the final mass of the competitor is compared to its starting mass, enabling the user to estimate the amount in the original target from the final amount. The limitations to using the PCR-based assay in the quantification are that PCR is an exponential process, estimating the initial amount of DNA in the sample from the amplified amount is difficult. Any small variation in the amplifying efficiency from one run to the next can result in large differences in PCR product yield. PCR can amplify dead or non-viable organisms and non-target DNA, if precautions are not taken. However, from an epidemiological point of view, the method provides an accurate estimate of potential disease risk (Henz and Platt, 2000b).

2.6 Organic amendments and soilborne pathogens

Organic materials affect bean root rots by altering the carbon:nitrogen (C:N) ratio in the soil and regulating microbial activity in the soil, or by affecting the form of nitrogen in the soil (Hall, 1996). It is also suggested that high C: N ratios in field soils reduce *Fusarium* bean root rots because the nitrogen is rapidly immobilised by soil microorganisms thus depriving *F. solani* f.sp. *phaseoli* of this essential nutrient. When soil microorganisms degrade a high nitrogen amendment, any nitrogen excess of the microorganisms' needs is released into soil solution as ammonia (NH_3) (Lazarovits, 2001). This is quickly converted to ammonium (NH_4^+) causing a rise in soil pH. As the pH rises, some NH_4^+ is converted back into NH_3 . The NH_4^+ is non-toxic even at high concentrations, whereas NH_3 is very toxic (Lazarovits, 2001). Ammonia forms only when pH rises above 8.5, this situation rarely occurs in soils with organic matter content greater than 1.7 %. Thus, generation of ammonia for disease control is limited to special soils.

Another mechanism involves production of nitrous acid (HNO_2), which may be more important for reduction of plant pathogenic organisms in soil. Conversion of NH_4^+ into nitrite (NO_2^-) and then nitrate (NO_3^-) through bacterial nitrification causes soil pH to drop. If it goes below pH 5.5, then NO_2^- takes the chemical form HNO_2 . Nitrite is non-toxic, whereas HNO_2 is very toxic to a number of soil plant pathogens. Soil pH is the crucial driving factor in determining the relative amounts of toxic (NH_3 or HNO_2) and non-toxic (NH_4^+ or NO_2^-) compounds (Hall, 1996; Lazarovits, 2001).

A number of organic materials are known to stimulate biological oxidation of ammonium to nitrate which is effective in the control of soil borne plant pathogens. For example, organic materials namely Alfalfa (*Medicago sativa*) and maize (*Zea mays*) have been reported to

reduce bean root rots (Hall, 1996). Some other organic materials, for example barley (*Hordeum jubatum*) and glucose, are known to suppress nitrification thereby increasing root rot disease. Disease suppression by organic amendments may be related more to qualitative than quantitative aspects of inoculum. Crop residues that suppress root rots may not have immediate effects on the population densities of the pathogen. For example, cellulose reduces *Fusarium* root rots but not the inoculum density of the fungus (Hall, 1996).

Stimulation of biological control agents, for example *Trichoderma*, *Bacillus*, *Gliocladium*, *Penicillium* and *Pseudomonads* may also be a mechanism by which organic amendments may reduce the effect of soil borne disease (Lazarovits, 2001). In eastern Africa, use of green manures particularly *Calandura* and Farmyard manure in root rot infested soils was found to reduce disease severity, and increased bean yield (Buruchara and Scheidegger, 1993; Otsyula *et.al.*, 1998; Opio *et al.*, 2000). It is not known whether the organic amendments have direct effect on population levels of *Pythium* pathogenic to beans.

2.7. Section conclusion

The above literature review pointed out a number of gaps. The major ones that require further investigations are:

- 1) Establishing the different *Pythium* species pathogenic to beans and the variation within the pathogenic strains predominant in eastern Africa, specifically, in Uganda.
- 2) Understanding the possible synergistic effects of *Pythium* spp. pathogenic to bean and their distribution in bean producing areas where root rots are a major constraint.
- 3) Developing a method of quantifying populations of *Pythium* spp. pathogenic to beans and evaluating the effect of organic amendments on pathogen populations in field soils

CHAPTER THREE

IDENTIFICATION, DIFFERENTIATION AND DISTRIBUTION OF *PYTHIUM* SPECIES ASSOCIATED WITH BEAN ROOT ROT IN UGANDA

3.1 Introduction

Several *Pythium* species are associated with *Pythium* root rots on beans (*Phaseolus vulgaris* L.). Some of these species include *P. ultimum*, *P. irregulare*, *P. aphanidermatum*, and *P. myriotylum* (Abawi and Pastor Corrales, 1990). In eastern Africa, bean root rots are caused by a number of soilborne pathogens, including *Fusarium solani* f.sp. *phaseoli*, *Sclerotium rolfsii*, *Rhizoctonia solani* and *Pythium* spp. The pathogens occur either individually or as a complex of two or more pathogens depending on suitable environmental conditions (CIAT, 1992; Rusuka *et al.*, 1997). In Kabale and Kisoro districts a major pathogen that causes severe root rot is *Pythium* spp. (Opio, 1998).

Pythium isolates in the region have been identified mostly to genus level (Rusuku *et al.*, 1997; Opio, 1998). This is partly because identification of *Pythium* spp. using morphological or pathogenic characteristics is both difficult and slow. However, identification at species level is important for identifying host resistance or introducing disease management practices. This study was conducted to; 1) identify and differentiate *Pythium* spp. pathogenic to *Phaseolus* beans using morphological and molecular markers, and 2) determine their distribution in Uganda.

3.2 Materials and methods

3.2.1 Study area and sample collection

Samples of beans with typical symptoms of *Pythium* spp, and soil samples from infected bean fields were collected from 117 sites in the districts Kisoro and Kabale in southwestern Uganda and Mbale and Sironko in the east (Figures 1a, b and c). This was done in April 2000. In each district samples were picked from a predetermined position using a grid distance of 5 to 10 km. In each of the bean fields sampled, at least 15 bean plants with typical *Pythium* symptoms were randomly selected following a "W" pattern. At the same time soil samples were picked from the same position infected beans were picked and later bulked to make a field sample.

Forty-one bean fields in 13 sub-counties were sampled from Kisoro district, 55 in 13 sub-counties from Kabale, 12 in six sub-counties from Mbale, and nine in four sub-counties Sironko. Each sample was put in a separate paper bags, not sealed, labelled and transported to Kawanda Agricultural Research Institute-CIAT Laboratory for *Pythium* isolation. At each field site sampled information on: a) physical location, which included; village, parish and sub-county were noted, and b) geographic locations using the geographic positioning system (GPS) were also noted to map *Pythium* distribution.

For the purpose of studying the variability and distribution *Pythium* spp. across seasons, a second collection was done in November 2000 from 69 sites. The third collection was done in November 2001 from 73 sites. Sampling was done in the same locations, although not necessarily from the same sites.

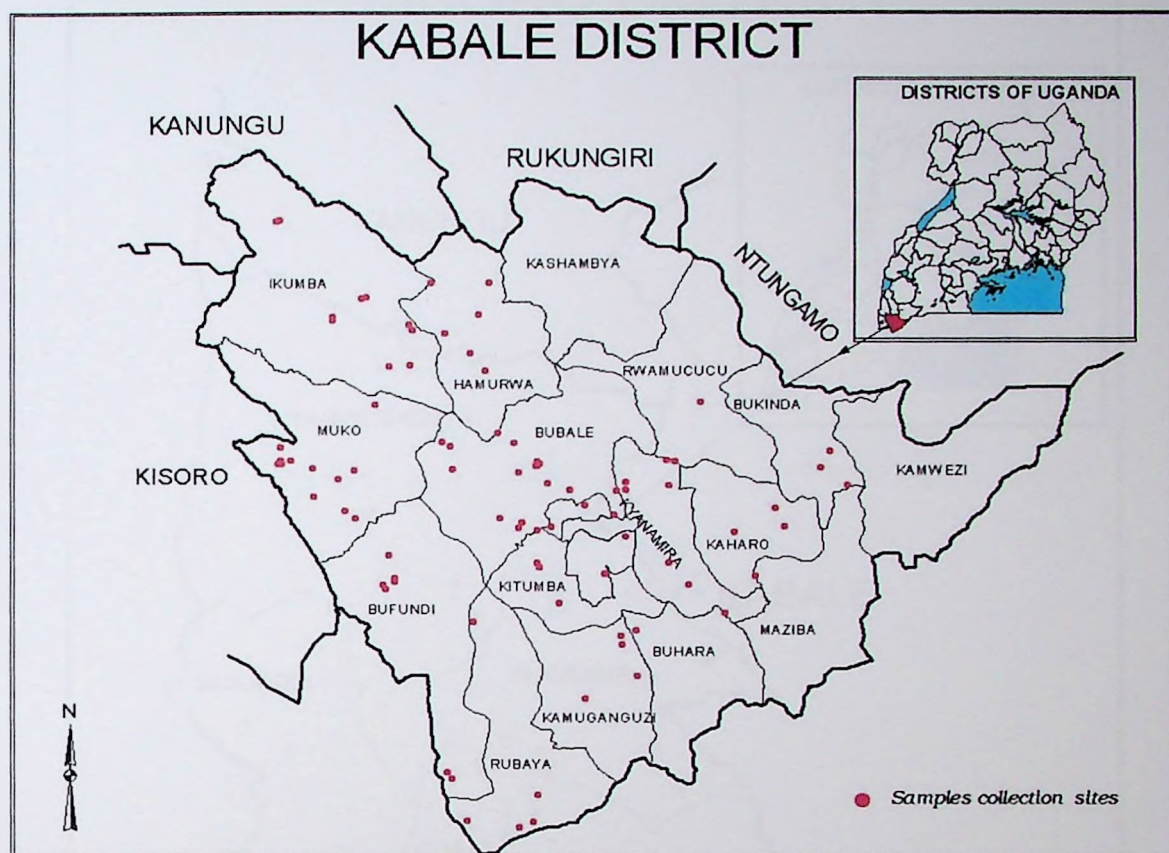


Figure 1a: Collection sites of *Pythium* bean root rot pathogen in the district of Kabale

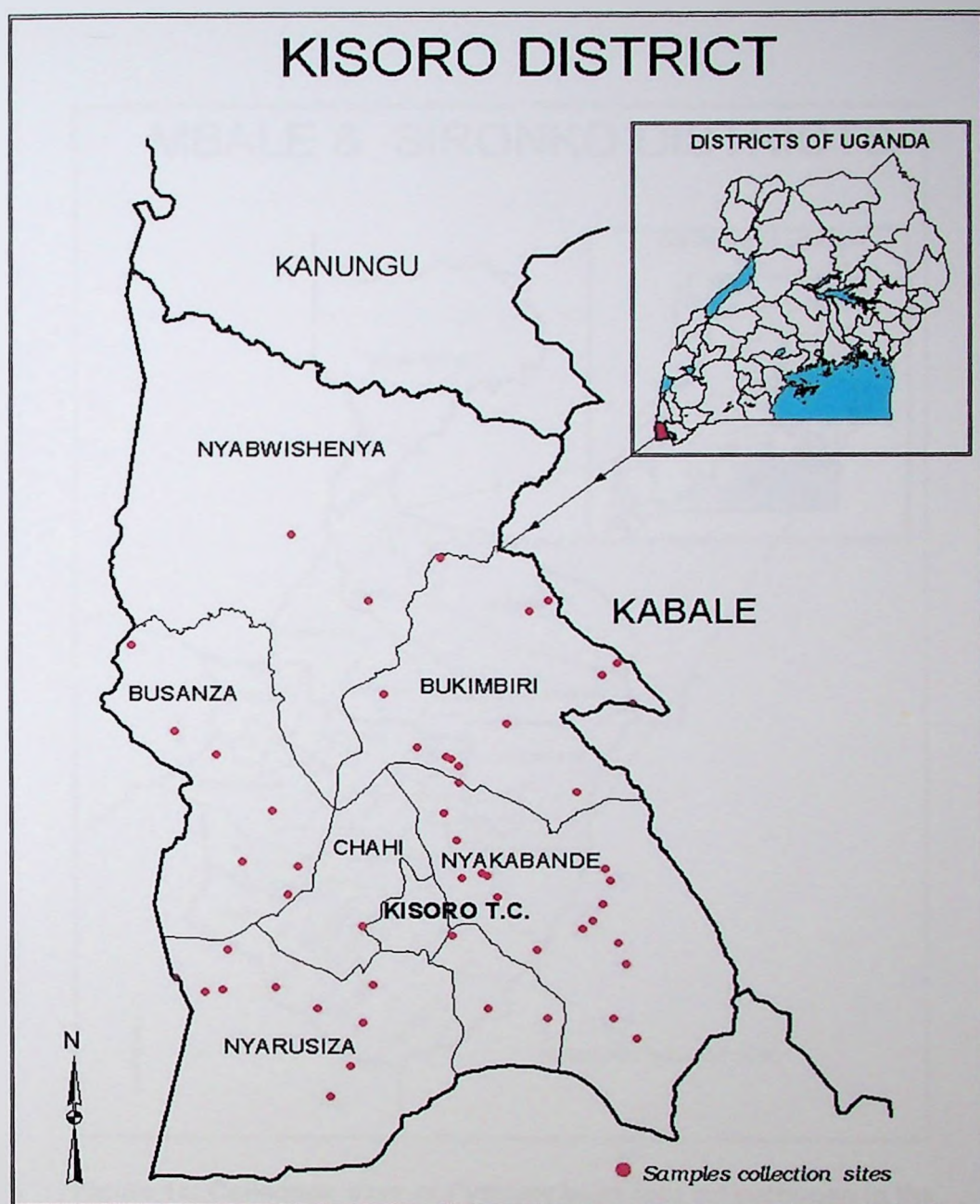


Figure 1b: Collection sites of *Pythium* bean root rot pathogen in the district of Kisoro

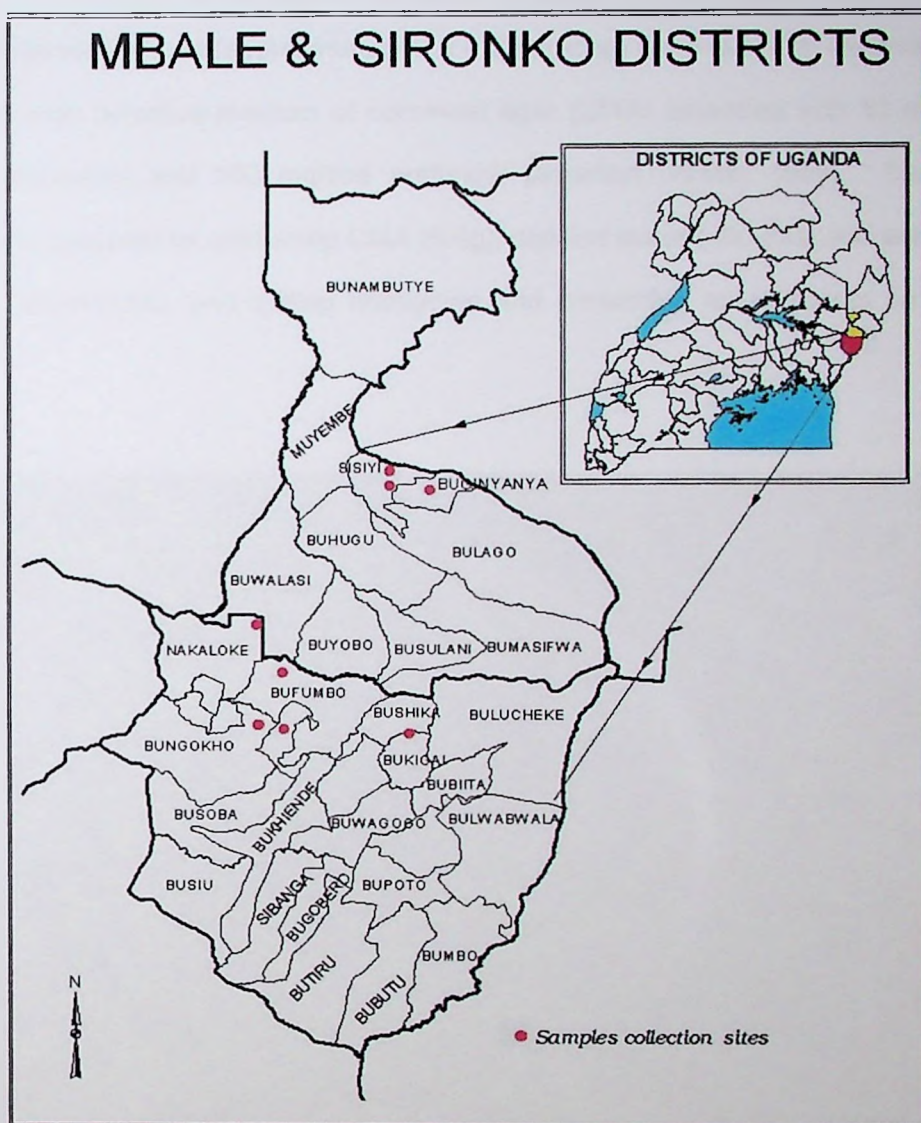


Figure 1c: Collection sites of *Pythium* bean root rot pathogen in the districts of Mbale and Sironko

3.2.2 Laboratory isolation of *Pythium* from plant tissues and soils

Infected bean root pieces (approximately 0.5 – 2 cm long) were cut from expanding lesions and plated onto selective medium of cornmeal agar (CMA) amended with 30 mg/litre antibacterial rifamycin and 100 mg/litre antifungal pimarin (White, 1988). The selective medium was prepared by combining CMA (6.8g), distilled water (400 mls) and autoclaving at 15 psi for 20 minutes, and adding rifamycin and pimarin amendments on cooling to 40°C.

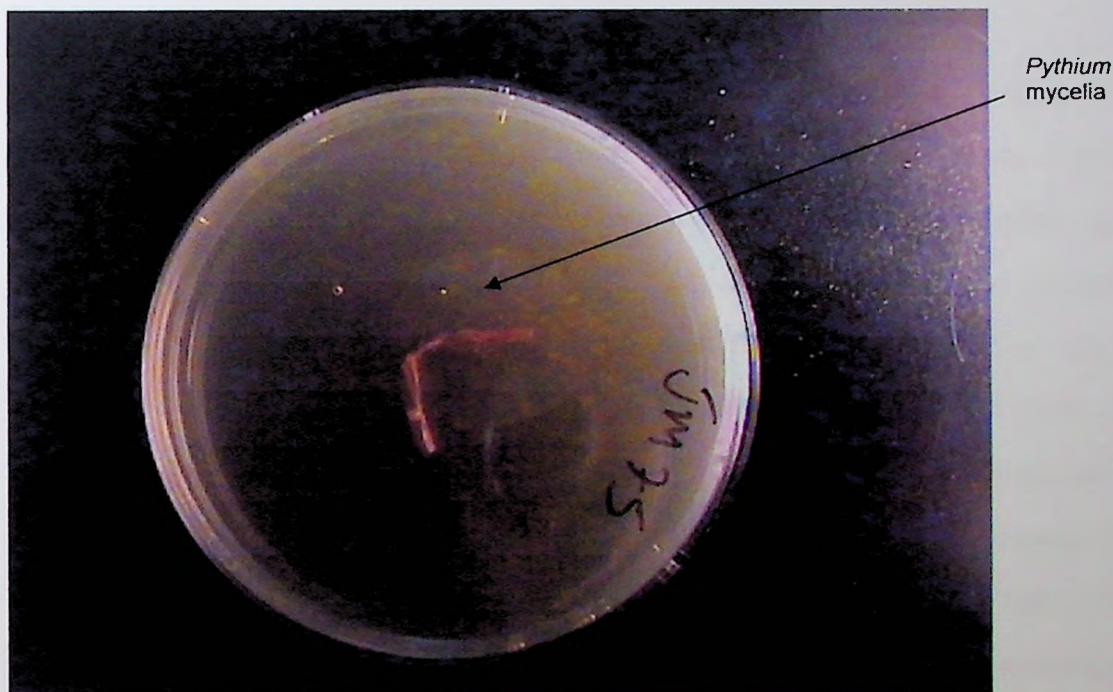


Plate 1: Two day old *Pythium* mycelia from infected bean tissue growing on selective medium

Isolations from the soil were done by first dissolving 2g of infested soil in 100 mls of water and later spreading the 0.5 mls of suspension onto selective medium of CMA. Cultures were incubated at room temperature of 20-25°C for two to five days. *Pythium* mycelia from tissue

(Plate 1) and soil isolations were later transferred onto CMA containing rifamycin and later maintained for subsequent studies. Sixty six *Pythium* isolates were recovered from the April 2000 collections, 14 from November 2000 and 65 from November 2001 collections. Thus, a total of 146 isolates were recovered. *Pythium* isolates from April 2000 collections were subjected to morphological, molecular and pathogenicity analyses for identifying different *Pythium* species pathogenic to beans in Uganda. The November 2000 collections were subjected to only molecular and pathogenicity analyses while isolates from the November 2001 collections were only subjected to molecular analysis.

3.2.3 Morphological characterisation of *Pythium*

Characterisation of the 66 *Pythium* isolates from April 2000 collections were initially done at Horticulture Research International (HRI), UK using morphological characters. *Pythium* isolates were grown on 10% V8 medium amended with 30 mg/liter of antibacterial agent rifamycin. The 10% V8 medium is good for *Pythium* to produce morphological structures used in the differentiation of a number of species (G. White, *Personal comm.*). The 10% V8 medium was prepared by combining V8 juice (40 ml), distilled water (360 ml), CaCO_3 (0.8 g), agar powder (6.0 g), and autoclaving at 15 psi for 20 minutes. Isolates were incubated at room temperature for two weeks for the *Pythium* to produce distinctive structures. The isolates were later grouped using the identification key of Plaats-Niterink (1981). The important distinctive morphological structures used in identification included: the sporangium presence, form, and size; wall thickness, and the plerotic conditions of the oospore; oogonium location, ornamentation, size, and shape; and the number, origin and morphology of the antheridia.

The 66 *Pythium* isolates were placed into 11 morphological groupings. All *Pythium* isolates in the 11 morphological groupings were subjected to PCR/RFLP and later used to inoculate beans so as to examine their pathogenicity before further identifications using ITS1 sequence analysis.

3.2.4 Molecular characterisation of *Pythium* isolates using PCR/RFLP analysis

3.2.4.1 Cultural conditions

Initially, the 66 *Pythium* isolates from April 2000 collections originally grouped using morphological structures at HRI, were first grown on water agar amended with 30 mg/L rifamycin at 18 –22°C to obtain an active culture of each isolate relatively free of bacteria. Additional 14 isolates obtained from HRI culture collection were used for comparison. After two to three days, actively growing hyphal regions were cut, and four agar blocks (6mm diameter) were placed into 25 ml of liquid medium (10% V8 juice) in a 100 ml flask. The 10% V8 medium was prepared by autoclaving a mixture of 100 ml of clarified V8 juice, 3 g of CaCO₃ and 900 ml of distilled water to make up a litre. The *Pythium* cultures were incubated at 18 –22°C for 7 days to allow the fungus form mycelia. The mycelia were filtered through a layer of 85-mm filter paper and blotted dry with paper towels. The mycelia mats were stored at -20°C until used for DNA extraction. Subsequently, isolates from the other two collections were treated in the same way to raise mycelia for DNA extraction.

3.2.4.2 DNA extraction

DNA was extracted by grinding mycelia in extraction buffer followed by isopropanol precipitation method. Fifty milligrams of *Pythium* mycelial mat of each isolate were ground

using acid purified sand in 400 μ l extraction buffer (200mM Tris, pH 8.0; 250mM NaCl; 1 mM EDTA; 1% SDS and 5.0% skimmed milk), in a 1.5 ml centrifuge tube. The crushed mycelia were placed in a water bath at 70°C for five minutes with occasional gentle mixing. After centrifugation at 16000g for five minutes, 250 μ l of the supernatant of the crushed mycelia was transferred to another centrifuge tube. DNA was precipitated by adding 250 μ l of ice-cold isopropanol. Tubes were spun at 16000g for five minutes and the supernatant was decanted. The DNA pellet was rinsed with 500 μ l of 70% ethanol to remove salts attached to the samples. Tubes were again spun at 16000g for five minutes and the supernatant decanted, later air-dried for 20-30 minutes, then the pellet was resuspended in 150 μ l of TE buffer (10 mM Tris, 1mM EDTA, pH 8.0). The concentration of genomic DNA recovered from mycelia was estimated to be 0.2 ng/ μ l of the final volume.

3.2.4.3 Polymarase chain reaction

Amplification of the ITS regions of *Pythium* isolates were performed in a programmable thermal cycler. Primers used were ITS1 (TCC GTA GGT GAA CCT GCG G) from 18S and ITS4 (TCC TCC GCT TAT TGA TAT GC) from 28S rDNA (White *et al.*, 1990). A 25 μ l reaction mixture contained 10.0 pM of each primer, 0.75 units *Taq* DNA polymarase, 0.2 mM each of the four deoxynucleotides, dATP, dCTP, dGTP, dTTP, in a PCR reaction buffer, and 2.0 pg of genomic DNA. Reactions were amplified for 40 cycles, DNA denaturation was at 94°C, 2 min 30 sec for the first cycle and 45 sec for the subsequent cycles, 1 min at 60°C for primer annealing, and at 72°C for primer extension. PCR amplifications containing no DNA template were carried out as controls to test for the presence of contamination of reaction mixtures by non-sample DNA. Efficiency of amplification was monitored by running 5 μ l of each reaction through 1% agarose gel at 100 V for 2 hrs in Tris-borate-EDTA buffer (0.89M boric acid, 0.89M Trizma base, and 20mM EDTA). A 100-bp molecular weight ladder was

used as the size standard, stained with ethidium bromide, with visualisation and photography under ultraviolet light.

3.2.4.4 Restriction analysis

To assess sequence variability among *Pythium* isolates in each of the morphological groupings, amplified DNA (8 µl) was digested for six hours at 37°C with 10 units (1 µl) of one of the following restriction enzymes: *Cfo* I, *Hinf* I and *Mbol* I supplied by GIBCOLBRIL. The digested DNA fragments were separated on 2% agarose gels with Tris-borate-EDTA buffer. Gels were stained with ethidium bromide (0.25 µl ml⁻¹) and photographed with a UVG image analysis system. The size of restriction fragments was determined by comparison of the fragment migration distances to known marker fragments (100-bp molecular size ladder).

Each of the *Pythium* isolates from Uganda and HRI culture collection was assigned a composite ITS-rDNA genotype based on the combination of the restriction site patterns obtained with the three restriction endonuclease. A data matrix was constructed from the restriction analysis to represent the variation in the restriction sites, and the presence or absence of restriction site/band was coded by scoring 1 and 0, respectively. The matrix of similarities was clustered by NTSYS computer package which showed the different RFLP groups existing in the Ugandan *Pythium* isolates. RFLP groupings were further confirmed by sequencing the ITS1 region of the ribosomal gene of representative isolates. The RFLP identifications were compared to morphological groupings and pathogenicity results in sections 3.2.3 and 3.2.6, respectively.

3.2.5 Analysis of rDNA sequences of *Pythium* isolates and identification of species

3.2.5.1 Sequencing of amplified rDNA

Forty-two *Pythium* isolates representing the different RFLP groups were selected randomly for rDNA sequences analysis. Additional 14 isolates from November 2000 were also subjected to sequencing making a total of 57 *Pythium* isolates. The primers ITS1 and ITS2 (White *et al.*, 1990) were used in the amplification of the ITS 1 regions of the ribosomal gene for each of the *Pythium* isolate selected. Amplification was done using the procedure described in section 3.2.4.3. Residual primers and dNTPS in the PCR products were removed using QIAquick™ PCR purification spin columns following the manufacturer's protocol (QIAGEN, Crawley, UK).

Direct sequencing of the PCR amplified products were carried out using ITS2 primer (White *et al.*, 1990). Sequencing reactions of the double stranded DNA templates were carried out using the ABI Prism™ Dye terminator cycle sequencing ready reaction kit (ABI/Perkin-Elmer). The products were purified by ethanol precipitation following the manufacturer protocol and nucleotide sequences were determined by the ABI 377 automated sequencing technology (Applied Biosystems).

3.2.5.2 DNA sequences analyses, identification of *Pythium* species and specific primers development

DNA sequences from ITS1 region of the ribosomal gene were edited using the Editseq program (DNASTAR Inc., Madison, Wis). ITS1 sequences of the test isolates were compared with ITS1 sequences of known *Pythium* spp. available in the *Pythium* DNA sequence database using Seqman program. Multiple alignment of the sequenced product of

ITS 1 and the 93 *Pythium* species data base sequences available was performed for comparison and primer designing. Five specific primers were designed to detect *Pythium* species found to be important as pathogens in Uganda. The specific primers were used in pair with the universal ITS 4 in PCR as described in section 3.2.4.3 to test for the specificity in the amplification of DNA fragments of identified *Pythium* species.

3.2.6 Pathogenicity of *Pythium* isolates

The 66 *Pythium* isolates recovered from soil and bean plants in the April 2000 collection and five from culture collection were initially evaluated once for their pathogenicity on beans using variety K20 at HRI. Infected sand-maize-meal media was used as the source of inoculum. A mixture of 100 ml of distilled water, 15 g of maize meal and 500 g of white sand were allowed to soak for 1 hour in a 1L Duran bottle. The bottles were then autoclaved for 1 hour at 15 psi on two consecutive days. Each bottle was inoculated with 5mm disks from a two-day old culture grown on CMA. The bottles were incubated for two weeks and shaken periodically to ensure uniform growth of inoculum.

Pre sterilised field topsoil was inoculated with 1.2g of two weeks old infested sand/maize meal per 9 g of soil. Four bean seeds were sown in a 11 cm diameter and 15 cm depth containing approximately 900 g of soil infested with one of the 66 *Pythium* isolates. Three replicate pots were prepared for each isolate with the non-infested pot as the control. The pots were maintained in the greenhouse with temperatures ranging from 19-25°C. After germination, seedlings were watered every day to provide favourable environment for the pathogen establishment and development.

Further pathogenicity tests of the *Pythium* isolates collected in April and November 2000 were done in a screen house at Kawanda Research Institute. The tests were done for the sequenced and identified isolates. Autoclaved 160 g of finger millet seeds mixed with 160 ml of water in 500-ml bottle was used to raise *Pythium* inoculum. Each isolate was inoculated onto sterile millet seed in separate bottles and kept at room temperature for two weeks for *Pythium* to grow. The finger millet seed for each isolate was inoculated in pre-sterilised top soil using a ratio of 1:10 v/v in a wooden flat of 42 cm x 72 cm replicated three times. Three varieties namely; RWR 719 highly tolerant, K20 moderate tolerant and CAL 96 highly susceptible to *Pythium* root rot were used to evaluate the pathogenicity of the isolates. Each variety was planted in two rows of 12 plants replicated in three wooden flats.

In both cases, cumulative emergence and stand were recorded at 14 and 28 days after planting, respectively. Four weeks after emergence, surviving plants were harvested. Severity of root rot was estimated based on CIAT scale of 1-9 (Abawi and Pastor Corrales, 1990), where; 1 = no root symptoms; 3 = a maximum of 10% of the hypocotyle and root tissues have lesions; 5= approximately 25% of the hypocotyle and root tissues have lesions; 7= approximately 50% the hypocotyle and root tissues have lesions and the root system suffers a considerable decay; 9 = 75% or more of the hypocotyle and root tissues have lesions and the root system suffers advanced stages of decay and considerable reduction. All isolates with mean disease score of 6.0 or above were considered to be highly pathogenic to beans. In each pathogenicity test repeat, data obtained were subjected to analysis of variance (ANOVA) using Genstat computer package (Lawes Agricultural Trust Rothamsted Experimental Station, 1995). Means were compared using Fisher's least significant difference test at 5% probability level.

3.3 Results

3.3.1 Identifications of *Pythium* spp. using morphological structure

Morphological characterisation of the 66 *Pythium* isolates from April 2000 placed them into 11 morphological groupings (Table 1). Eight groups contained at least more than one isolates and the other four had one isolate each. Each of the five groups had morphological structures similar to the indicated known *Pythium* species; Group 1: *P. oligandrum*, Group 2: *P. spinosum*; Group 7: *P. ultimum* var *ultimum*. The isolate JM 8H (KIS 4) was similar to *P. echinulatum* and JM 2HMB (MB 1) to *P. rostratum*.

Table 1: Morphological grouping of 66 Ugandan *Pythium* isolates obtained from the April 2000 collection

Isolate/strain suspected <i>Pythium</i> species	Description
Group 1 (<i>P. oligandrum</i>) 1. JM 17B MB (MB3) 2. JM 18A MB (MB4) 3. JM 18B MB (MB12)	Isolates formed ornamented oogonia in single cultures similar to <i>P. oligandram</i> with long slender spines; antheridia are lacking; it's a weak pathogenic fungus; is a micro parasite of other fungus eg. <i>P. sylvaticum</i>
Group 2 (<i>P. spinosum</i>) 4. JM 70H1(KAB 4) 5. JM 70H2 (KAB 5) 6. A 672-4 (KIS 10)	Formed aerial mycelia on media, ornamented oogonia with blunt spines; antheridia were present; sporangia and zoospores were never formed; hyphal swellings were present. The structures are close to those of <i>P. mamillatum</i> , which is zoosporic at room temperature

Group 3 7. JM 6A (KIS 2) 8. JM 66A (KAB 3) 9. JM 41A (KAB 9) 10. JM 41B (KAB 10) 11. JM 41H (KAB 11) 12. JM 65D (KAB 14) 13. JM 67A (KAB 15) 14. JM 67B (KAB 16) 15. JM69-i (KAB 17) 16. JM69-ii (KAB 18) 17. JM69-iv (KAB 19) 18. JM 89H (KAB 21) 19. JM 15 (KIS 15) 20. A 672-2A (KIS 9) 21. JM 3A (KIS 1) 22. JM 30A (KIS 8)	Isolates produced few smooth oogonia in single cultures; never produced zoospores at room temperature; oogonia are spherical; anthredia stalks not branched. Difficult group of isolates to identify using morphological structures. Isolates have structures similar to <i>P. diclinum</i> but did not test positive to zoospore production
Group 4 23. JM 12A (KIS 5) 24. JM 29B (KIS 7) 25. JM 38 (KIS 16) 26. JM 14 MB (MB 15) 27. JM 4 (KIS 13) 28. JM 75 (KAB 27) 29. JM 10 MB (MB 14) 30. JM 20 MB (MB 17) 31. A 672-5 (KIS 11) 32. JM 21 MB (MB 18)	Difficult to identify, produced lots of mycelia which are non zoosporic having round structures like sporangia or hyphal swellings on media
Group 5 33. JM 59H (KAB 2) 34. JM 84 (KAB 6) 35. JM 84H (KAB 7) 36. JM 52 (KAB 12) 37. JM 65A (KAB 13) 38. JM 78A (KAB 20) 39. JM 1 (KIS 12) 40. JM 55 (KAB 25) 41. JM 71 (KAB 26) 42. JM 91 (KAB 28) 43. JM 92 (KAB 29) 44. JM 97 (KAB 30) 45. RW 8 (KAKEN 1)	Produced smooth oogonia; had one to two antheridia on the oogonium originating at various distances below the oogonium; the oospore is plerotic; did not produce zoospores at room temperature. Structures produced by the isolates in this group are similar to those of <i>P. tracheiphilum</i> but did not test positive for oospore production to belong to this species

Group 6 46. JM 17A MB (MB 2) 47. JM 1B MB (MB 6) 48. JM 13A MB (MB 8) 49. JM 17C MB (MB 9) 50. JM 16A MB (MB 10) 51. JM 85A (KAB 8)	No oogonia formed in single cultures; produces aerial mycelia on media; it's a fast growing fungus with large mycelium; no sporangia, so did not produce zoospores in water; produced hyphal swellings which tend to be golden in colour and are not catenulated. Could belong to Group HS
Group 7 (<i>P. ultimum</i> var <i>ultimum</i>) 52. JM 8 MB (MB 13) 53. JM 90S (KAB 22) 54. JM 45 (KAB 23) 55. JM 49 (KAB 24) 56. JM 7H1(KIS 3) 57. KWD1 1	Fast growing, produced smooth oogonia in single cultures, antheridia are present and originate a distance from the oogonia from below the oogonia, colonies formed aerial mycelium, and sporangia were absent never produced zoospores. Structures are similar to those described for <i>P. ultimum</i> . The strains could be identified as <i>P. ultimum</i> var <i>ultimum</i> .
Group 8 58. JM 5 (KIS 14) 59. JM 19 MB (MB 16) 60. JM 19B MB (MB 5) 61. JM 16H MB (MB 11) 62. JM 42 (KAB 1) 63. JM 11 MB (MB 7)	Isolates produced hyphal; swellings on mycelia with mild brown colour on media; no oogonia produced; non-zoosporic. Isolates seem to belong to <i>Pythium</i> sp. Group HS
Group 9 (<i>P. echinulatum</i>) 64. JM 8H (KIS 4)	The isolate first produced mycelia only on media; in water with grass at room temperature, zoospores and ornamented oogonia were observed after 24 hours, and sporangia with a long discharge tube were produced. The characteristics are typical of <i>P. echinulatum</i>
Group 10 65. JM 29A (KIS 6)	Was zoosporic at room temp, produced smooth oogonia, on grass with sterile water, the structures were similar to <i>P. salpingophorum</i> .
Group 11 (<i>P. rostratum</i>) 66. JM 2H MB (MB 1)	The isolate exhibited chrysanthemum pattern of growth on media, cultures were translucent when seen through the plate; smooth oogonia were formed in single cultures, and are intercalary (formed in line with mycelia) often appeared in chains; the antheridia arose immediately below the oogonia. The characters were typical of <i>P. rostratum</i>

3.3.2 PCR/RFLP analysis of 80 *Pythium* isolates

The combination of ITS 1 and ITS4 primers specifically amplified the entire length of the ITS region plus a small portion of the small and large subunit rRNA genes. PCR products contained a single band. Length polymorphism which were displayed in this region, ranged from 810 (isolate, A 672-5 (KIS 11)) to 1030 (isolates in RFLP Group 1) base pairs for the 80 isolates in the RFLP groupings (Table 2).

All the amplified products of 80 isolates were digested with *Cfo* I, *Hinf* I, *Mbo* I endonuclease. *Pythium* ITS region of the tested isolates were found to be highly polymorphic (Figure 2). The three enzymes were able to separate 63 isolates into seven groups, each containing more than one isolate. In the each of the RFLP groups identified, the isolates had 95.5 % similarity (Figure 3). Seventeen isolates were not grouped, i.e., each isolated had a separate restriction pattern (Table 2, Figures 2 & 3). The five-based restriction enzyme *Hinf* I and four-based restriction enzymes *Cfo* I and *Mbo* I had one to six sites in the ITS region of the rDNA as shown in Table 2 for the different RFLP groupings and the ungrouped isolates. The sites made it possible to distinguish most isolates and group them using the three restriction enzymes (Table 2, Figure 2 & 3).

There was some relationship between morphological grouping and RFLP. But in some instances RFLP groups contained isolates from more than one morphological group as shown in Table 1 and RFLP analysis (Table 2, Figures 2 & 3).

Table 2: ITS DNA sizes (approximate base pairs) and number of restriction sites for isolates in the RFLP groupings using three endonuclease on 80 *Pythium* isolates

Lane	Isolate/strain	Base pairs	<i>Cfo</i> I	<i>Hinf</i> I	<i>Mbo</i> I
	RFLP Group 1	1030	2	4	2
1	JM 70H1(KAB 4)				
2	JM 70H2 (KAB 5)				
3	A 672-4 (KIS 10)				
4	CBS 377.72 (<i>P. spinosum</i>)				
	RFLP Group 2	820	3	3	2
5	JM 66A (KAB 3)				
6	JM 41B (KAB 10)				
7	JM 41H (KAB 11)				
8	JM 65D (KAB 14)				
9	JM 67A (KAB 15)				
10	JM 67B (KAB 16)				
11	JM69-i (KAB 17)				
12	JM69-ii (KAB 18)				
13	JM69-iv (KAB 19)				
14	JM 6A (KIS 2)				
15	JM 12A (KIS 5)				
16	JM 29B (KIS 7)				
17	JM 5 (KIS 14)				
18	JM 15 (KIS 15)				
19	JM 38 (KIS 16)				
20	JM 19 MB (MB 16)				
	RFLP Group 3	850	3	3	4
21	JM 59H (KAB 2)				
22	JM 84 (KAB 6)				
23	JM 84H (KAB 7)				
24	JM 41A (KAB 9)				
25	JM 52 (KAB 12)				
26	JM 65A (KAB 13)				
27	JM 78A (KAB 20)				
28	JM 89H (KAB 21)				
29	JM 55 (KAB 25)				
30	JM 71 (KAB 26)				
31	JM 91 (KAB 28)				
32	JM 92 (KAB 29)				
33	JM 97 (KAB 30)				
34	JM 1 (KIS 12)				
35	JM 19B MB (MB 5)				
36	JM 21 MB (MB 18)				
37	RW 8 (KAKEN 1)				
38	CBS 165.68 (<i>P. salpingophorum</i>)				
	RFLP GROUP 4	900	2	4	2
39	JM 85A (KAB 8)				
40	JM 90S KAB 22)				
41	JM 45 (KAB 23)				
42	JM 49 (KAB 24)				
43	JM 7H1 (KIS 3)				
44	JM 17A MB (MB 2)				
45	JM 1B MB (MB 6)				
46	JM 13A MB (MB 8)				
47	JM 17C MB (MB 9)				

Table 2: Continued

Lane	Isolate/strain	Base pairs	<i>Cfo</i> I	<i>Hinf</i> I	<i>Mbo</i> I
RFLP GROUP 4 continued					
48	JM 16A MB (MB 10)				
49	JM 8 MB (MB 13)				
50	HRI-HS 26				
51	HRI-P. ult.				
52	CBS 296.37 (<i>P. ultimum</i> var <i>ultimum</i>)				
RFLP GROUP 5					
		920	2	2	2
53	JM 75 (KAB 27)				
54	JM 4 (KIS 13)				
55	JM 10 MB (MB 14)				
56	JM 20 MB (MB 17)				
RFLP GROUP 6					
		880	3	4	2
57	JM 17B MB (MB3)				
58	JM 18A MB (MB4)				
59	JM 18B MB (MB12)				
60	HRI-P. olig.				
61	CBS 149.84 (<i>P. oligandrum</i>)				
RFLP GROUP 7					
		910	4	4	3
62	HRI-P. sylv.				
63	CBS 452.67 (<i>P. sylvaticum</i>)				
Un Grouped isolates					
64	JM 29A (KIS 6)	890	2	4	2
65	A 672-2A (KIS 9)	870	2	3	2
66	JM 14 MB (MB 15)	890	3	4	2
67	JM 16H MB (MB 11)	850	4	4	3
68	JM 42 (KAB 1)	910	3	3	2
69	JM 3A (KIS 1)	900	3	4	3
70	JM 8H (KIS 4)	1020	3	2	4
71	JM 30A (KIS 8)	1020	3	3	2
72	A 672-5 (KIS 11)	810	2	3	1
73	JM 2H MB (MB 1)	830	4	4	3
74	JM 11 MB (MBL 7)	940	3	3	3
75	KWD1	850	3	3	3
76	HRI-P. spin	860	4	4	3
77	HRI-P. irreg.	1010	4	4	2
78	323933 IM1 (<i>P. irregurale</i>)	810	3	4	2
79	CBS 556.67 (<i>P. echinulatum</i>)	980	2	4	5
80	CBS 172.68 (<i>P. rostratum</i>)	890	2	4	6

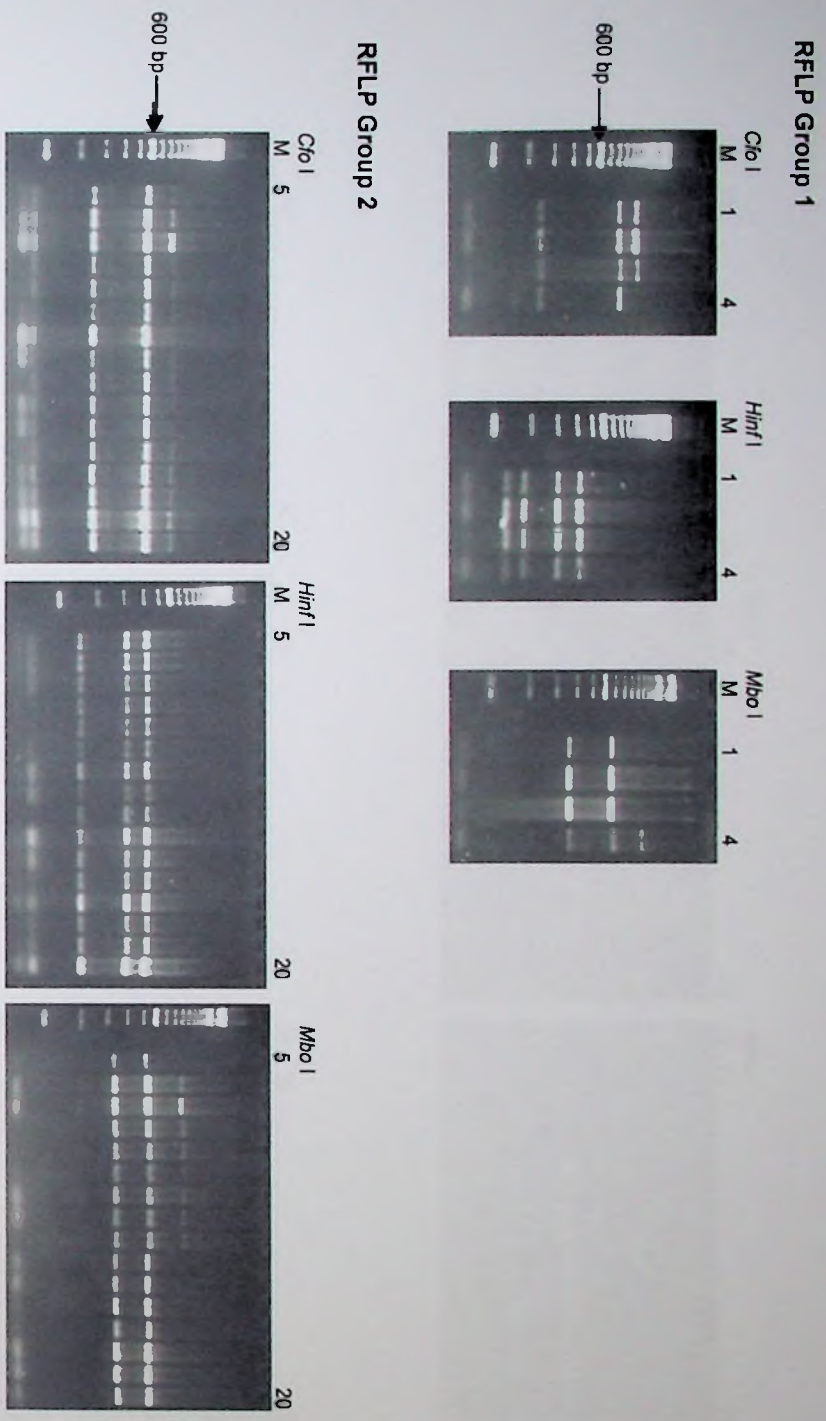
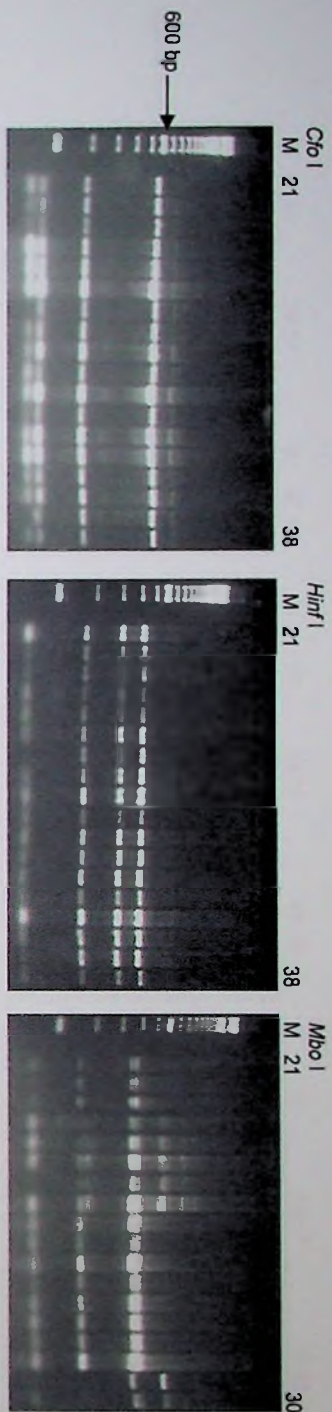


Figure 2: Agarose gels showing restriction banding patterns of ITS rDNA from 80 isolates (lanes 1 -80 identity described as in Table 2) of *Pythium* from Uganda and culture collection. Groupings 1 to 7 and ungrouped *Pythium* isolates using ITS rDNA digested with *Cfo* I, *Hinf* I and *Mbo* I. M is a 100-bp molecular size marker.

RFLP Group 3



RFLP Group 4

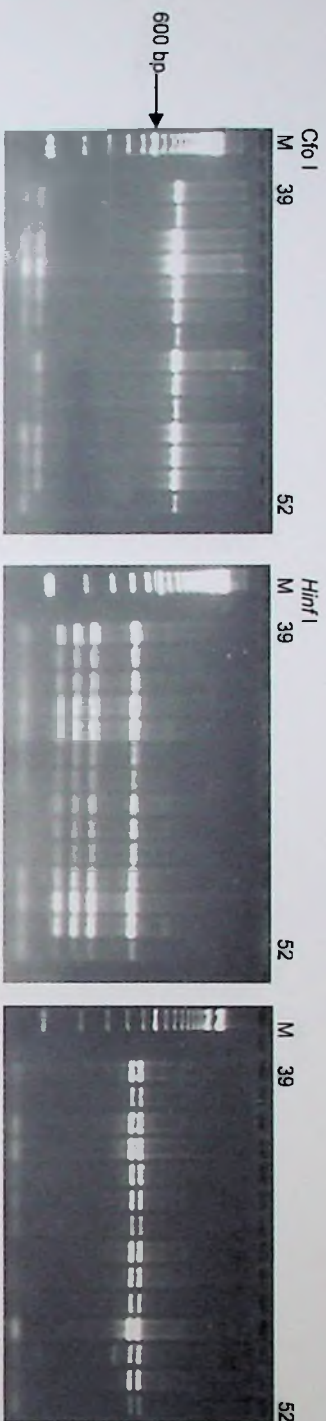
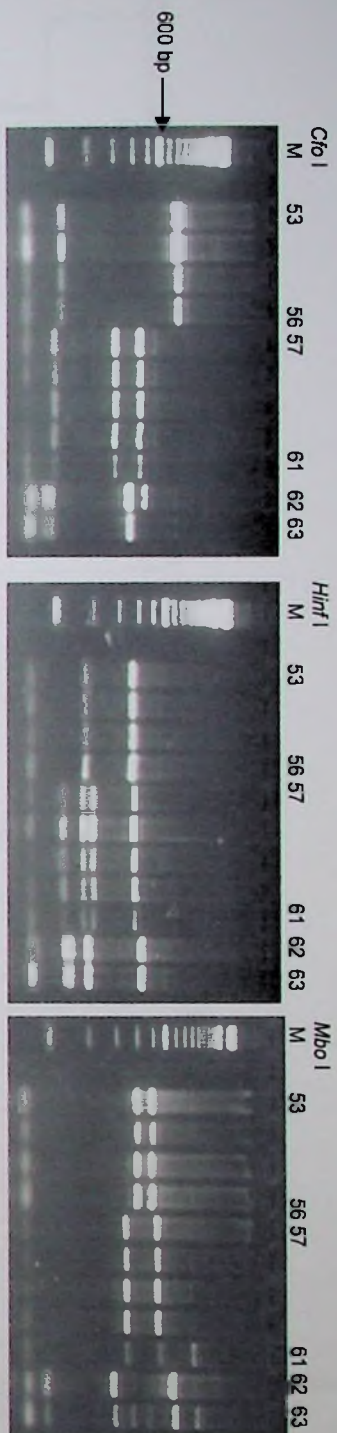


Figure 2 Continued : Agarose gels showing restriction banding patterns of ITS rDNA from 80 isolates (lanes 1 -80 identity described as in Table 2) of *Pythium* from Uganda and culture collection. Groupings 1 to 7 and ungrouped *Pythium* isolates using ITS rDNA digested with Cfo I, Hinf I and Mbo I. M is a 100-bp molecular size marker.

RFLP Group 5, 6 and 7



Ungrouped isolates

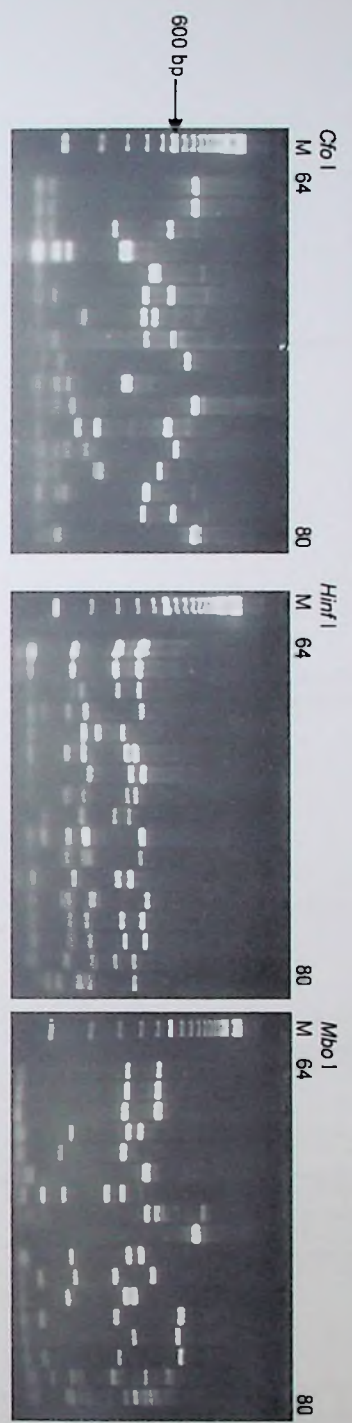


Figure 2 Continued : Agarose gels showing restriction banding patterns of ITS rDNA from 80 isolates (lanes 1 -80 identify described as in Table 2) of *Pythium* from Uganda and culture collection. Groupings 1 to 7 and ungrouped *Pythium* isolates using ITS rDNA digested with *Cfo* I, *Hinf* I and *Mbo* I. M is a 100-bp molecular size marker.

3.3.3 DNA sequences analysis, identification of *Pythium* species and the development of species specific primers

Sequence data of the ITS 1 of the rDNA were found consistent with RFLP groupings by three enzymes *Cfo* I, *Hinf* I and *Mbo* I for the 42 Ugandan and five HRI culture collections *Pythium* isolates tested. Sequences of ITS 1 for *Pythium* isolates within each of the RFLP groupings had 90 to 95 % similarity. It was possible to differentiate and confirm the identity of *Pythium* species in Uganda earlier grouped using RFLPs using multiple alignment of ITS I sequences with those in the *Pythium* database managed by Dr. Andrea Levesque in Canada. Eleven *Pythium* species were identified from the tested samples collected in Uganda during April and November 2000 (Table 3). Sequences for the 11 Ugandan *Pythium* species are indicated in Figure 4. Sample *Pythium* isolates from HRI culture collections were confirmed to their known species when their sequences were compared with those known in the database (Table 3).

All sequenced isolates distinguished by RFLPs as group 1 in Table 2 were identified as *P. spinosum*. Isolates in group 2, except JM 6A (KIS 2) were identified as *P. salpingophorum*, there rest were *P. torulosum*. Group 3 isolates were also identified as *P. salpingophorum*, with exception of isolate JM 91 (KAB 28), which was identical to *P. torulosum*. Similarly, isolates in RFLP group 4 were identified as *P. ultimum var ultimum*, except JM 7H1 (KIS 3) identified as *P. nondosum*. Group 5 isolates were identified as *P. vexans* whereas group six was *P. oligandrum*. The ungrouped isolates JM 29A (KIS 6), JM 14 MB (MB 15), JM 3A (KIS 1), JM 8H (KIS 4) and KWD1 were identified as *P. pachycaule*, *P. torulosum*, *P. nondosum*, *P. echinulatum* and *P. deliense/P. aphanidematum*, respectively.

The designed primers amplified the target DNA of respective *Pythium* species identified by sequence analysis for the five important species (Figure 5). Primer PUG 1 designed to detect *P. spinosum* amplified fragments of approximately size 900 bp when used in pair with the universal ITS 4 for isolates JM 70H1(KAB 4) and CBS 377.72. Although, the primer had a high level of specificity it also amplified an isolate CBS 452.67 (*P. sylvaticum*), with a size of less than 800 bp as shown in lane 11. Primer PUG 2 designed for *P. torulosum*, PUG 3 for *P. salpingophorum* and PUG 5 designed for *P. nodosum* also had a high level of specificity as indicated in the gel (Figure 5), except that PUG 3 amplified DNA from *P. spinosum* (CBS 377.72) from HRI culture collections. The amplified DNA *P. torulosum* was approximately 700 bp but the non target amplified DNA of *P. spinosum* was higher by approximately 100 bp. Primer PUG 4 targeting *P. ultimum* was much less specific. It amplified sample DNA from *P. sylvaticum*, *P. vexans*, *P. pachycaule*, *P. nodosum* and *Fusarium solani* f.s. *phaseoli*. Interestingly, the non target amplified samples had DNA fragments of less than 900 bp when compared with those targeted samples of isolates JM 85A (KAB 8), JM 13A MB (MB 8) and CBS 296.67.

3.3.4 Distribution of *Pythium* species associated with bean root rots in Uganda

The analyses and identifications from the sequenced test *Pythium* isolates from different parts of Uganda indicated *P. ultimum var ultimum* was the most abundant species. This specie was isolated from most samples collected in the three districts of Kabale, Kisoro and Mbale (Figures 6a, b and c). The other species common in the three districts were *P. salpingophorum*, *P. torulosum* and *P. vexans*. *P. spinosum* was identified from samples collected in Kabale and Kisoro whereas *P. oligandrum* was identified from only Mbale and Sironko samples. *P. echinulatum* was isolated only from Kisoro samples whereas *P. dissotocum* was isolated only from Kabale (Figure 6).

Table 3: *Pythium* species identified from DNA sequence analysis of 56 isolates collected in 2000 and five from HRI culture collections.

District of origin/ Isolate or strain No.	RFLP group	Identified species
Kabale district		
JM 70H1(KAB 4)	1	<i>P. spinosum</i>
JM 70H2 (KAB 5)	1	<i>P. spinosum</i>
JM 41B (KAB 10)	2	<i>P. torulosum</i>
JM 41H (KAB 11)	2	<i>P. torulosum</i>
JM 67A (KAB 15)	2	<i>P. torulosum</i>
JM 59H (KAB 2)	3	<i>P. salpingophorum</i>
JM 84 (KAB 6)	3	<i>P. salpingophorum</i>
JM 41A (KAB 9)	3	<i>P. salpingophorum</i>
JM 65A (KAB 13)	3	<i>P. salpingophorum</i>
JM 89H (KAB 21)	3	<i>P. salpingophorum</i>
JM 55 (KAB 25)	3	<i>P. salpingophorum</i>
JM 71 (KAB 26)	3	<i>P. salpingophorum</i>
JM 91 (KAB 28)	3	<i>P. torulosum</i>
JM 92 (KAB 29)	3	<i>P. salpingophorum</i>
JM 97 (KAB 30)	3	<i>P. salpingophorum</i>
JM 85A (KAB 8)	4	<i>P. ultimum</i> var. <i>ultimum</i>
JM 90S KAB 22)	4	<i>P. ultimum</i> var. <i>ultimum</i>
JM 45 (KAB 23)	4	<i>P. ultimum</i> var. <i>ultimum</i>
JM 75 (KAB 27)	5	<i>P. vexans</i>
MS 34	Not tested	<i>P. spinosum</i>
MS 38	"	<i>P. ultimum</i> var. <i>ultimum</i>
MS 41	"	<i>P. ultimum</i> var. <i>ultimum</i>
MS 44	"	<i>P. dissotocum</i>
Kabale district		
MS 46	"	<i>P. ultimum</i> var. <i>ultimum</i>
MS 47	"	<i>P. ultimum</i> var. <i>ultimum</i>
MS 50	"	<i>P. ultimum</i> var. <i>ultimum</i>
MS 52	"	<i>P. ultimum</i> var. <i>ultimum</i>
MS 58	"	<i>P. ultimum</i> var. <i>ultimum</i>
MS 61	"	<i>P. ultimum</i> var. <i>ultimum</i>
MS 66	"	<i>P. ultimum</i> var. <i>ultimum</i>
Kisoro district		
A 672-4 (KIS 10)	1	<i>P. spinosum</i>
JM 6A (KIS 2)	2	<i>P. salpingophorum</i>
JM 12A (KIS 5)	2	<i>P. torulosum</i>
JM 5 (KIS 14)	2	<i>P. torulosum</i>
JM 38 (KIS 16)	2	<i>P. torulosum</i>
JM 7H1(KIS 3)	4	<i>P. nodosum</i>
JM 4 (KIS 13)	5	<i>P. vexans</i>
JM 3A (KIS 1)	Un grouped	<i>P. nondosum</i>
JM 8H (KIS 4)	"	<i>P. echinulatum</i>
JM 29A (KIS 6)	"	<i>P. pachycaule</i>
MS 6	Not tested	<i>P. ultimum</i> var. <i>ultimum</i>
MS 12	"	<i>P. ultimum</i> var. <i>ultimum</i>
MS 15	"	<i>P. ultimum</i> var. <i>ultimum</i>

Table 3: Continued.

District of origin/ Isolate or strain No.	RFLP group	Identified species
Mbale& Sironko districts		
JM 19B MB (MB 5)	3	<i>P. salpingophorum</i>
JM 21 MB (MB 18)	3	<i>P. salpingophorum</i>
JM 17A MB (MB 2)	4	<i>P. ultimum</i> var. <i>ultimum</i>
JM 1B MB (MB 6)	4	<i>P. ultimum</i> var. <i>ultimum</i>
JM 17C MB (MB 9)	4	<i>P. ultimum</i> var. <i>ultimum</i>
JM 16A MB (MB 10)	4	<i>P. ultimum</i> var. <i>ultimum</i>
JM 8 MB (MB 13)	4	<i>P. ultimum</i> var. <i>ultimum</i>
JM 10 MB (MB 14)	5	<i>P. vexans</i>
JM 20 MB (MB 17)	5	<i>P. vexans</i>
JM 17B MB (MB3)	6	<i>P. oligandrum</i>
JM 18A MB (MB4)	6	<i>P. oligandrum</i>
JM 14 MB (MB 15)	Un grouped	<i>P. torulosum</i>
Kawanda station		
KWD1	Un grouped	<i>P. deliense/</i> <i>P. aphanidematum</i>
Culture collection		
CBS 377.72 (<i>P. spinosum</i>)	1	<i>P. spinosum</i>
CBS 165.68 (<i>P. salpingophorum</i>)	3	<i>P. salpingophorum</i>
CBS 296.37 (<i>P. ultimum</i> var. <i>ultimum</i>)	4	<i>P. ultimum</i> var. <i>ultimum</i>
CBS 149.84 (<i>P. oligandrum</i>)	6	<i>P. oligandrum</i>
CBS 452.67 (<i>P. sylvaticum</i>)	7	<i>P. sylvaticum</i>

¹Not tested: Isolates from November 2000 collection where subjected to PCR and sequencing but not RFLP analysis.

2HRI: Horticultural Research International

JM 29A (KIS 6), <i>P. pachycaule</i>	--CATTACCACACCAAAAAA--CTTTCCACGTGAACCGTTG-----TAACATATGTTTC- 49
MS 44, <i>P. dissotocum</i>	-GCATTACCACACCAAAAAA--CTTTCCACGTGAACCGTTG-----TAACATATGTTTC- 50
JM 59H (KAB 2), <i>P. salpingophorum</i>	--CATTACCACACCATAAAA--CTTTCCACGTGAACCGTTA-----AAATTATGTTTC- 49
KWD 1 <i>P. deliense</i>	--CATTACCACACCATAAAA--CTTTCCACGTGAMCCGTTG-----AAATCATGTTTC- 47
JM 12A (KIS 5), <i>P. torulosum</i>	--CATTACCACACCATAAAA--CTTTCCACGTGAACCGTTA-----CAATTATGTTTC- 49
JM 17BMB (MB 3), <i>P. oligandrum</i>	--CATTACCACACCC-TAAAAA--CTTTCCACGTGAACCGTTA-----TAACATATGTTTC- 48
JM 85A (KAB 8), <i>P. ultimum</i> var. <i>ultimum</i>	NGCHCTTCCCHCTTT--AAAA--CTGTCCACGTG-ACTGTAAG--CAAGTCTAGCGC- 50
JM 70H1 (KAB 4), <i>P. spinosum</i>	--CATTACCACACCTAAAAA--CTTTCCACGTGAACCGTTTGTACCAAGATTGTGCC 54
JM 8H (KIS 4), <i>P. echinulatum</i>	--CATTACCACACCTAAAAA--CTATCCACGTGAACCGTTTGTACCAAGATTGTGCC 54
JM 75 (KAB 27), <i>P. vexans</i>	---ATTACCACACCT-AAAAACACCCCTTCCACGTGAACCGTTTGTGTTTGCCTTCGAGT- 55
JM 3A (KIS 1), <i>P. nodosum</i>	--CATTACCACACCAAAAAA--CTTTCCACGTGAACCGTTTGTGTC--GTTTGTGCT 53
JM 29A (KIS 6), <i>P. pachycaule</i>	-----TGTG-----CTCTCT-CTCGG-- 64
MS 44, <i>P. dissotocum</i>	-----TGTG-----CTCTCTTCTCGGA- 67
JM 59H (KAB 2), <i>P. salpingophorum</i>	-----TGTG-----CCTTCT-CTCGG-- 64
KWD 1 <i>P. deliense</i>	-----TGTG-----CTCTCT-CTCGG-- 62
JM 12A (KIS 5), <i>P. torulosum</i>	-----TGTG-----CTCTCT-TTCGG-- 64
JM 17BMB (MB 3), <i>P. oligandrum</i>	-----TGTG-----CTTCGTGCGAAGAC 66
JM 85A (KAB 8), <i>P. ultimum</i> var. <i>ultimum</i>	-----TGTG-----A---CTGA-CTGGTGTGTTTTCATTTTGGAC 80
JM 70H1 (KAB 4), <i>P. spinosum</i>	TGTGCGTTGTTGGCGTGCAATTTGCTTACRCTTTGGTGTTTGTGAGTGGGTGTGGCAGTG 114
JM 8H (KIS 4), <i>P. echinulatum</i>	---GAGATGTTTGTGCGGTGTTG---TCGTATCACTGTGTATTCTGACGCGGTG 102
JM 75 (KAB 27), <i>P. vexans</i>	---GCTTTGTT---GTGCTCG---GAGCATGTTT-GGGCTTCGCTGCTGGCG 97
JM 3A (KIS 1), <i>P. nodosum</i>	---TGTGTGTT---TTTCGATC---TGCTCTCTGT-GCCTTTCGGGTGTGGAG 95
JM 29A (KIS 6), <i>P. pachycaule</i>	-GAGA-----GCTGAACGAAGGTGGGC-----TGCTTAATTGTA- 97
MS 44, <i>P. dissotocum</i>	-GAGA-----GCTGAACGAAGGTGGGC-----TGCTTAATTGTA- 101
JM 59H (KAB 2), <i>P. salpingophorum</i>	-GAGG-----GCTGAACGAAGGTGGACTGCG-A-TGCTTTATTGCGT 103
KWD 1 <i>P. deliense</i>	-GAGG-----GSTGAACGAAGGTGGSC-----TGCTTAATTGTTGG 96
JM 12A (KIS 5), <i>P. torulosum</i>	-GAGG-----GCTGAACGAAGGTAGAGCTGTC-A-TGTAAAGTGGCG 103
JM 17BMB (MB 3), <i>P. oligandrum</i>	TTGAG-----GCTGAACGAAGGTGAGTCTGC-G-T-CTATTTTGGAT 105
JM 85A (KAB 8), <i>P. ultimum</i> var. <i>ultimum</i>	ACTGG-----AACGGGAGTCAGCAGGACGAAGGTGGTCTGTTG-TAATGCAAGTTAT 132
JM 70H1 (KAB 4), <i>P. spinosum</i>	TGYGGACTGAACGAAGGTTGTGTGTTGTTATGTGYCTGCTGCACTGCTGACTTTGCATTC 174
JM 8H (KIS 4), <i>P. echinulatum</i>	TGTGGC-----AAGTATGTATGGCGCT--TGGCTGAT-CGAAGGTGCTCTCGCACTT 151
JM 75 (KAB 27), <i>P. vexans</i>	CTTGATTGTGCTGGCGGCTCAGGGCCATCAAGCGGC-GTTTGTGAGTGTGTGTCGCATAC 156
JM 3A (KIS 1), <i>P. nodosum</i>	TGTGGG---GAAGAAGCTCCAGGCTAAA---CGAAGGCTGCGAGT---TTCGTGCTT 144
JM 29A (KIS 6), <i>P. pachycaule</i>	GTCTG-----CCGATGTACTTTT-----A 116
MS 44, <i>P. dissotocum</i>	GTCTG-----CCGATGTACTTTT-----A 120
JM 59H (KAB 2), <i>P. salpingophorum</i>	CCCGGACT-----GCCGATGC-TTTTTC-----A 126
KWD 1 <i>P. deliense</i>	TCCG-----CCSATGTATTTTTC-----A 115
JM 12A (KIS 5), <i>P. torulosum</i>	TTTTG-----CCGATGTACTTTT-----A 122
JM 17BMB (MB 3), <i>P. oligandrum</i>	GCGGATTT-----GCTGATGTTATTTTA-----A 129
JM 85A (KAB 8), <i>P. ultimum</i> var. <i>ultimum</i>	GATGGACTA-----GCTGATGAACCTTTGTTTTT 161
JM 70H1 (KAB 4), <i>P. spinosum</i>	ATTGTATGGTCTTGGCGGAGTGGCGGCTACTGTGCATGCGAGTGTACTTATTTTTTCA 234
JM 8H (KIS 4), <i>P. echinulatum</i>	GATTGTGTG-----TGT-----CGGCTGACTTATTTTTCAA 182
JM 75 (KAB 27), <i>P. vexans</i>	AATTGAAA-----CGTCCAAACC---TTTTAA 180
JM 3A (KIS 1), <i>P. nodosum</i>	G-----CGGCCGATTATCTTTTCA 164
JM 29A (KIS 6), <i>P. pachycaule</i>	AACCCATTAACTAATACTGAACATACTCCGAAAACGAAAGTCTTTGGTTTTAA--TCA 174
MS 44, <i>P. dissotocum</i>	AACCCATTAACTAATACTGAACATACTCCGAAAACGAAAGTCTTTGGTTTTAA--TCA 178
JM 59H (KAB 2), <i>P. salpingophorum</i>	AACCCATT-ACCTAAWACTGATCTATACTCCTAAAACGAAAGTTTTTGGTTTTAA----- 180
KWD 1 <i>P. deliense</i>	AACCCATTACCTAATACTGATCTATACTCCAAAACGAAAGTTTCTGGTTTTAA--TCC 173
JM 12A (KIS 5), <i>P. torulosum</i>	AACCCATTACACTAATACTGAACATACTCCGAGAACGAAAGTTTTTGGTTTTAA--TCA 180
JM 17BMB (MB 3), <i>P. oligandrum</i>	ACNCTATTACTTAATACTGAACATACTCCGAATACGAAAGTTTTTGGTTTTAA--CAA 187
JM 85A (KAB 8), <i>P. ultimum</i> var. <i>ultimum</i>	AAACCCTTACCTAATACTGATTATATACTGTGGGACGAAAGTCTTGGTTTTAC-TAG- 219
JM 70H1 (KAB 4), <i>P. spinosum</i>	AACCCCATACCTAAATGACTGATTATACTGTGAGAACGAAAGTCTTGGTTTTAA--CTAG 293
JM 8H (KIS 4), <i>P. echinulatum</i>	A---CCCATTCTANAGTANGTGATTATACTGTGAAGACGAAAGTCTTTGGTTTTAA--CTNG 238
JM 75 (KAB 27), <i>P. vexans</i>	ACCCATTGATTGAAAACCTGAAGTATACTGTGGGACGAAAGTCTTGGTTTTGAAACTAG 240
JM 3A (KIS 1), <i>P. nodosum</i>	AACCCATACATTAAACACTGAAGTATACTGTGAGGACGAAAGTCTTGGTTTTAA--CTAG 223

**Figure 4: Alignment of the ITS1 sequences of 11 *Pythium* species identified from Ugandan samples. In each of the five *Pythium* species important in Uganda, blue portion indicate the Specific primer designed to detect that particular species.
N: sequence reader could not differentiate nucleotide**

JM 29A (KIS 6), <i>P. pachycaule</i>	ATAACAACCTT-----	184
MS 44, <i>P. dissotocum</i>	ATAACAACCTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTG	238
JM 59H (KAB 2), <i>P. salpingophorum</i>	-----	
KWD 1 <i>P. deliense</i>	ATAACAACCTTTCARCAGTGGATGTCTAGGCTCGC-----	207
JM 12A (KIS 5), <i>P. torulosum</i>	ATAACAACCTTTCACAG-GGATGTNTAGGCTCGC-----	213
JM 17BMB (MB 3), <i>P. oligandrum</i>	TTAACAACCTTTCAGCAG-GNAT-TCTAGGCTCGCNC-----	226
JM 85A (KAB 8), <i>P. ultimum</i> var. <i>ultimum</i>	ATAACAACCTTTCAGCAGTGGATGTCTAGGCTC-GCACATCATGAAGAACGCTGCGAACTG	278
JM 70H1 (KAB 4), <i>P. spinosum</i>	ATAACAACCTTTCANCAG-GGATGTCTAG-----	320
JM 8H (KIS 4), <i>P. echinulatum</i>	ATAGCNACTTTCAG-----	252
JM 75 (KAB 27), <i>P. vexans</i>	ATARCRACCTTTCAG-----	254
JM 3A (KIS 1), <i>P. nodosum</i>	ATAGCAACCTTTCAGCAGTGGATGTTCTAGGCTCGC-----	258
JM 29A (KIS 6), <i>P. pachycaule</i>	-----	
MS 44, <i>P. dissotocum</i>	CGATACGTAATGCGAATTGCAGAATTCAGTGAGTCATCGAAATTTTGAACGCACATTGCM	298
JM 59H (KAB 2), <i>P. salpingophorum</i>	-----	
KWD 1 <i>P. deliense</i>	-----	
JM 12A (KIS 5), <i>P. torulosum</i>	-----	
JM 17BMB (MB 3), <i>P. oligandrum</i>	-----	
JM 85A (KAB 8), <i>P. ultimum</i> var. <i>ultimum</i>	CGATA-C-GTAATGC-GAATT-GCAGAATTC-A-GTGAGTCATCG-AAATTTTGAACGCA	331
JM 70H1 (KAB 4), <i>P. spinosum</i>	-----	
JM 8H (KIS 4), <i>P. echinulatum</i>	-----	
JM 75 (KAB 27), <i>P. vexans</i>	-----	
JM 3A (KIS 1), <i>P. nodosum</i>	-----	
JM 29A (KIS 6), <i>P. pachycaule</i>	-----	
MS 44, <i>P. dissotocum</i>	CTTTCGGGTTATGCCTGGAAGTATGCCTGTGTCAATGTCCGNACATCAAACCTTGCTTTC	358
JM 59H (KAB 2), <i>P. salpingophorum</i>	-----	
KWD 1 <i>P. deliense</i>	-----	
JM 12A (KIS 5), <i>P. torulosum</i>	-----	
JM 17BMB (MB 3), <i>P. oligandrum</i>	-----	
M 85A (KAB 8), <i>P. ultimum</i> var. <i>ultimum</i>	TATTGCACCTTTC-GGGTTATGCCT-GGAA-GTATTGTC-TGTATCA-GTGT-CC-GTAAA	384
JM 70H1 (KAB 4), <i>P. spinosum</i>	-----	
JM 8H (KIS 4), <i>P. echinulatum</i>	-----	
JM 75 (KAB 27), <i>P. vexans</i>	-----	
JM 3A (KIS 1), <i>P. nodosum</i>	-----	
JM 29A (KIS 6), <i>P. pachycaule</i>	-----	
MS 44, <i>P. dissotocum</i>	TTTTTTTTGTGNATTACAAGAANAGAAATGGCAGACTGTGAAGNG-----	403
JM 59H (KAB 2), <i>P. salpingophorum</i>	-----	
KWD 1 <i>P. deliense</i>	-----	
JM 12A (KIS 5), <i>P. torulosum</i>	-----	
JM 17BMB (MB 3), <i>P. oligandrum</i>	-----	
JM 85A (KAB 8), <i>P. ultimum</i> var. <i>ultimum</i>	TCAAA-CTT-GCC-TTCTTTTTCT-GTGTA-GTCA-GGGATGGAATGTGCAG	431
JM 70H1 (KAB 4), <i>P. spinosum</i>	-----	
JM 8H (KIS 4), <i>P. echinulatum</i>	-----	
JM 75 (KAB 27), <i>P. vexans</i>	-----	
JM 3A (KIS 1), <i>P. nodosum</i>	-----	

**Figure 4 continued: Alignment of the ITS1 sequences of 11 *Pythium* species identified from Ugandan samples. In each of the five *Pythium* species important in Uganda, blue portion indicate the Specific primer designed to detect that particular species.
N: sequence reader could not differentiate nucleotide**

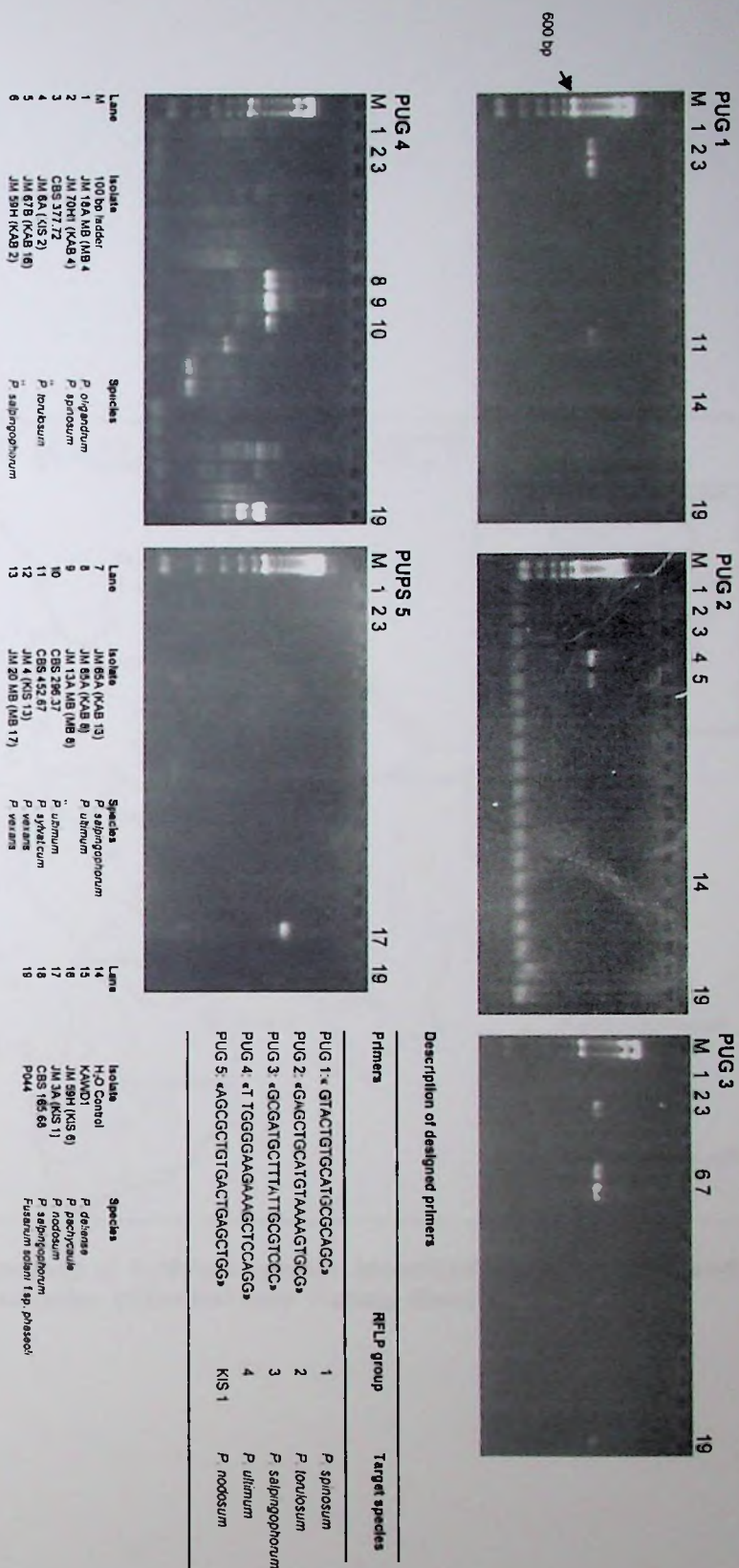


Figure 5: Agarose gels showing amplified PCR products by primers PUG 1 to 6 tested on a selection of *Pythium* isolates from different RFLP groups of identified *Pythium* species from Uganda

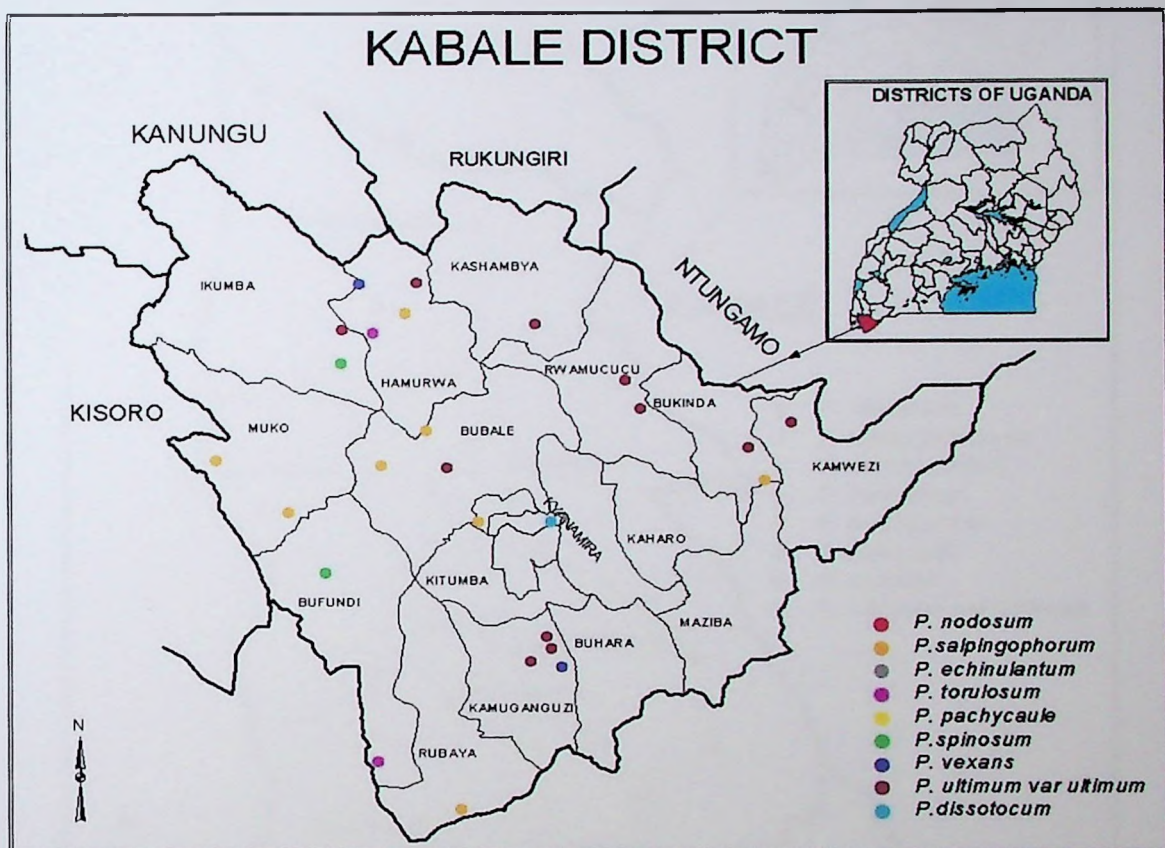


Figure 6a: A distribution map of *Pythium* species identified based on bean and soil samples collected from Kabale district

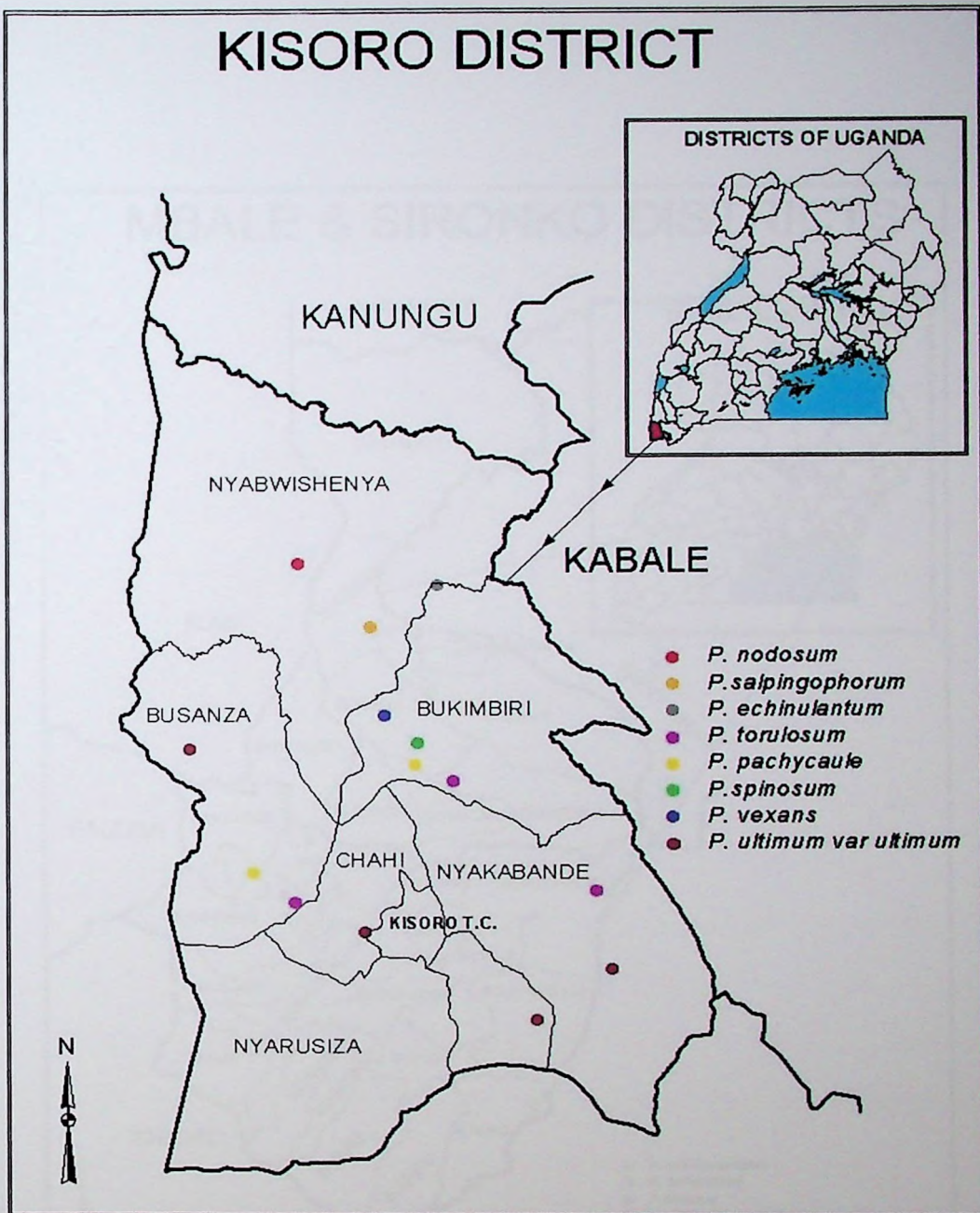


Figure 6b: A distribution map of *Pythium* species identified based on bean and soil samples collected from Kisoro district

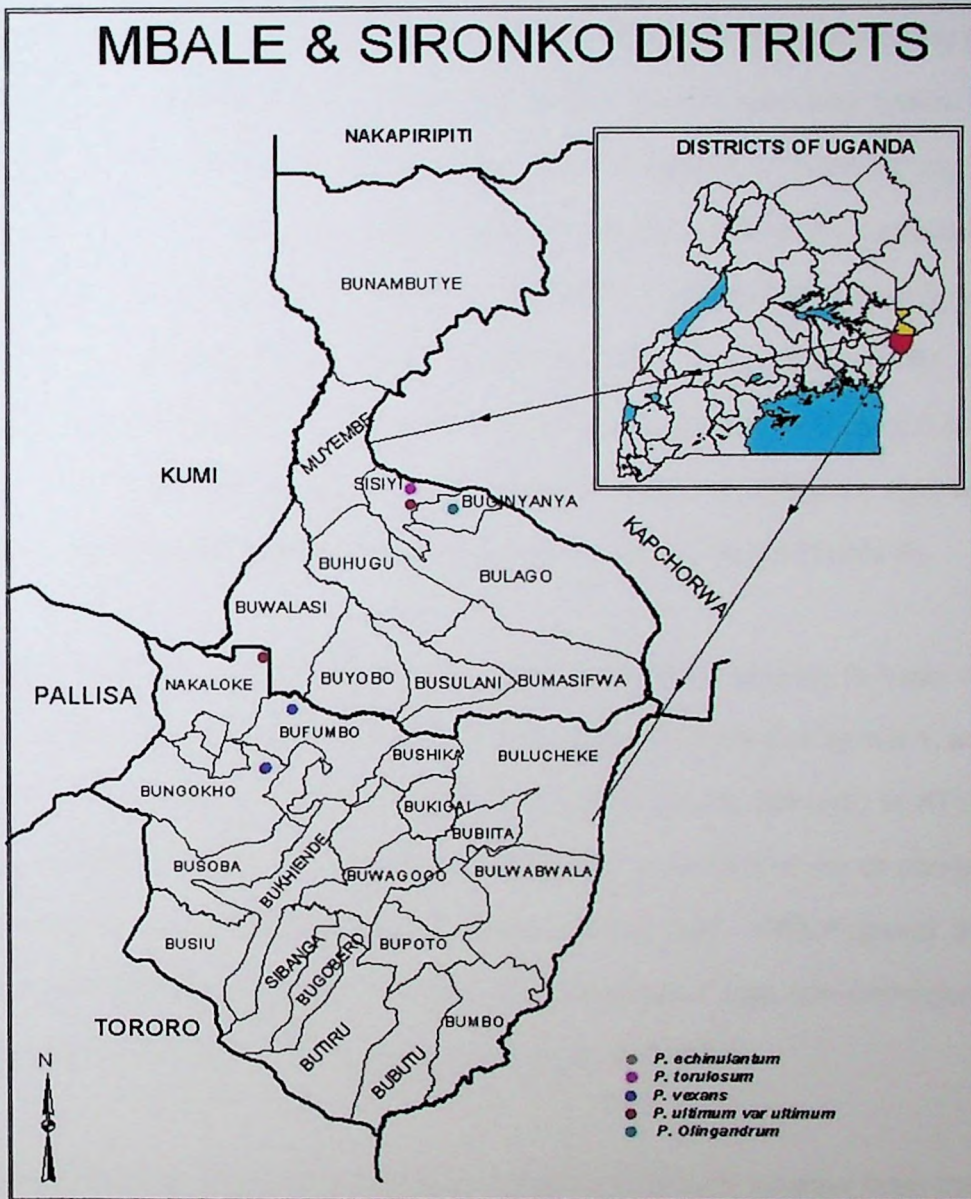


Figure 6c: A distribution map of *Pythium* species identified based on bean and soil samples collected from Mbale and Sironko districts

3.3.5 Pathogenicity of *Pythium* isolates on beans

A total of 66 *Pythium* isolates recovered from bean fields during the April 2000 collection, and five from HRI culture collections were tested for their pathogenicity on beans using variety K20 known to be susceptible to bean root rot. The amount of damage by *Pythium* on bean roots varied significant ($P < 0.01$) with the isolate used to inoculate beans. In the first *Pythium* pathogenicity trial, most isolates tested caused disease on bean variety K20. Four isolates in RFLP group 1 and 10 in RFLP group 4 caused significantly more disease, and were considered highly pathogenic (Table 5). The other highly pathogenic isolates were JM 12A (KIS 5) and JM 29B (KIS 7) in RFLP group 2, and the ungrouped JM 3A (KIS 1). Disease levels for these 17 highly pathogenic *Pythium* isolates varied from 6.3 to 7.5 on the CIAT1-9 scale. The rest of the non-pathogenic to weakly pathogenic *Pythium* isolates including the control treatment had pathogenicity scores of 1.0 to 5.4 (Table 5).

Most isolates in RFLP group 1 and 4 were highly pathogenic isolates to bean variety K20 except for culture collection isolates CBS 377.2 (*P. spinosum*) in RFLP group 1, and JM 7H1 (KIS 3), JM 17A MB (MB 2) and CBS 296. 37 (*P. ultimum var ultimum*) in RFLP group 4 which were weakly pathogenic (Table 5). RFLP group 2 had a mixture of non-pathogenic, weakly and highly pathogenic isolates on bean variety K20. RFLP group 5 had non-pathogenic and weakly pathogenic isolates whereas group 7 had non-pathogenic isolates only. The ungrouped had a mixture of the three forms of *Pythium*.

For the sequenced and identified species of Ugandan *Pythium* isolates from the April and November 2000 collection, further pathogenicity tests involved 53 isolates were conducted two more times on three bean varieties CAL 96, K20 and RWR 719 (Plate 2). The amount of disease on bean roots varied significant ($P < 0.01$) with the isolates used to inoculate each

bean type (Tables 4 and 6). Generally, disease levels for each *Pythium* isolate varied significantly ($P < 0.01$) with the variety inoculated. Thus there was a significant interaction between the different *Pythium* species available in farmers' field and bean varieties (Table 4).

Thirty eight isolates were consistently highly pathogenic to varieties CAL 96 and K20 with disease levels ranging from 6.0 to 9.0 using the 1-9 CIAT scale compared to disease levels of less than 6.0 on variety RWR 719 (Table 6). The following species and number of isolates were found to be highly pathogenic: *P. spinosum*, 4; *P. salpingophorum*, 7; *P. torulosum*, 7; *P. ultimum var ultimum*, 17; *P. nodosum*; 2; *P. pachycaule*, 1. Two isolates JM 41A (KAB 9) and JM 92 (KAB 29) identified as *P. salpingophorum* had disease scores of more than 6.0 only in the second trial. Isolates JM 6A (KIS 2), JM 71 (KB 26), JM 55 (KAB 25) and JM 19B (MB 5) also belonging to *P. salpingophorum* were consistently weakly pathogenic to all the three varieties. Isolate JM 8H (KIS 4) identified as *P. echinulatum* was also weakly pathogenic to all the three varieties.

Table 4: Mean square pathogenicity scores of *Pythium* isolates in the first trial for the different RFLP groupings on bean variety K20, second and third trials for the identified species on bean varieties CAL 96, K20 and RWR 719

Source	First trial		df	Second trial	Third trial
	df	ms			
Replication	2	3.3	2	2.9	1.7
Isolate	71	16.7**	53	32.3**	25.1**
Variety	-	-	2	383.8**	367.7**
Isolate x variety	-	-	106	3.2**	2.8**
Residual	142	0.8	322	0.8	

ns, ** denotes not significant and highly significant at $P \leq 0.01$.



Plate 2a: *Pythium* root rots from pathogenicity tests of isolates belonging to *P. ultimum* (A), JM 85A; *P. salpingophorum* (B), JM 92 on bean varieties CAL 96, K20 and RWR 719



Plate 2b: *Pythium* root rots from pathogenicity tests of *Pythium* isolates belonging to *P. torulosum* (C), JM 67A; on bean varieties CAL 96, K20 and RWR 719; *P. spinosum*, A672 (4) and *P. Pachycaule*, JM 29A (D) on bean varietie CAL 9

Table 5: Pathogenicity of *Pythium* isolates in the different RFLP groupings on bean variety K20

Isolate/strain	Disease score	Isolate/strain	Disease score
RFLP Group 1		RFLP Group 4	
JM 70H1(KAB 4)	7.5 a	JM 85A (KAB 8)	7.2 a
JM 70H2 (KAB 5)	7.2 a	JM 90S KAB 22)	6.7 ab
A 672-4 (KIS 1)	7.4 a	JM 45 (KAB 23)	6.3 abc
CBS 377.72 (<i>P. spinosum</i>)	3.5 fight	JM 49 (KAB 24)	6.3 abc
RFLP Group 2		JM 7H1(KIS 3)	5.4 bcd
JM 66A (KAB 3)	3.3fghij	JM 17A MB (MB 2)	4.3 defg
JM 41B (KAB 10)	3.0 ghijk	JM 1B MB (MB 6)	7.3 a
JM 41H (KAB 11)	1.1 l	JM 13A MB (MB 8)	6.7 ab
JM 65D (KAB 14)	1.0 l	JM 17C MB (MB 9)	7.3 a
JM 67A (KAB 15)	1.0 l	JM 16A MB (MB 10)	6.7 ab
JM 67B (KAB 16)	1.0 l	JM 8 MB (MB 13)	6.7 ab
JM69-i (KAB 17)	1.0 l	CBS 296.37 (<i>P. ultimum</i>)	3.7 efghi
JM69-ii (KAB 1)	1.0 l	RFLP Group 5	
JM69-iv (KAB 1)	1.0 l	JM 75 (KAB 27)	1.7 kl
JM 6A (KIS 2)	4.7 def	JM 4 (KIS 13)	4.3 def
JM 12A (KIS 5)	7.3 a	JM 10 MB (MB 14)	3.1 ghijk
JM 29B (KIS 7)	7.1 a	JM 20 MB (MB 17)	1.0 l
JM 5 (KIS 14)	2.0 jkl	RFLP Group 6	
JM 15 (KIS 15)	2.0 jkl	JM 17B MB (MB3)	1.0 l
JM 38 (KIS 16)	4.0 defgh	JM 18A MB (MB4)	1.0 l
JM 19 MB (MB 1)	1.0 l	JM 18B MB (MB1)	1.0 l
RFLP Group 3		CBS 149.84 (<i>P. oligandrum</i>)	1.0 l
JM 59H (KAB 2)	5.1 cde	RFLP Group 7	
JM 84 (KAB 6)	2.6 hijk	not tested	
JM 84H (KAB 7)	4.1 defg	Un Grouped	
JM 41A (KAB 9)	3.7 efghi	JM 29A (KIS 6)	1.0 l
JM 52 (KAB 12)	2.3 ijkl	A 672-2A (KIS 11)	1.0 l
JM 65A (KAB 13)	3.0 ghijk	JM 14 MB (MB 15)	1.0 l
JM 78A (KAB 20)	1.0 l	JM 16H MB (MB 11)	1.0 l
JM 89H (KAB 21)	1.0 l	JM 42 (KAB 1)	2.0 jkl
JM 55 (KAB 25)	1.0 l	JM 3A (KIS 1)	6.7 ab
JM 71 (KAB 26)	2.4 ijkl	A 672-5 (KIS 11)	1.0 l
JM 91 (KAB 28)	3.3 fghij	JM 8H (KIS 4)	1.8 kl
JM 92 (KAB 29)	1.0 l	JM 30A (KIS 8)	1.0 l
JM 97 (KAB 30)	1.0 l	JM 2H MB (MB 1)	1.0 l
JM 1 (KIS 12)	1.0 l	JM 11 MB (MBL 7)	1.0 l
JM 19B MB (MB 5)	1.0 l	KWD1	4.7 def
JM 21 MB (MB 18)	3.0 ghijk	Control	1.0 l
RW 8 (KAKEN 1)	1.0 l		
CBS 165.68 (<i>P. salpingophorum</i>)	1.0 l		

a) Means within column followed by similar letters are not significantly different at $P \leq 0.05$

b) Disease scale (1-9) 1 = no root symptoms; 3 = 10% of the hypocotyle and root tissues have lesions; 5= 25% of the hypocotyle and root tissues lesions 7= 50% the hypocotyle and root tissues have lesions and the root system suffers a considerable decay; 9= 75% or more of the hypocotyle and root tissues have lesions and the root system suffers advanced stages of decay and considerable reduction (Abawi and Pastor Corrales, 1990).

Table 6: Pathogenicity of *Pythium* species identified from sequence analysis on bean varieties CAL 96, K20 and RWR 719

Isolate/strain	Species	Second trial			Third trial		
		CAL 96	K20	RWR 719	CAL 96	K20	RWR 719
JM 70H1 (KAB 4)	<i>P. spinosum</i>	8.9 a	8.6 ab	6.3 a	8.1 abcde	8.1 abcde	4.1 bcdef
JM 70H2 (KAB 5)		6.6 efg	5.7 klm	4.1 deghi	6.7 ighi	5.7 klmn	4.0 bcdefg
A 672-4 (KIS 10)		8.3 abcd	8.3 abcde	3.7 efg hij	8.2 abcde	6.9 efg hijk	5.0 abc
MS 34	<i>P. torulosum</i>	8.7 abc	8.6 abc	3.8 efg hij	7.8 abcdef	7.5 bcdefgh	4.2 bcdef
JM 41B (KAB 10)		6.4 efg h	6.4 ijklm	4.5 cdefg	7.3 abcdefgh	6.3 hijk	4.1 bcdef
JM 41H (KAB 11)		4.0 jk	4.0 no	3.0 hijkl	7.2 cdefgh	6.1 ijkl	5.0 abc
JM 14 MB (ME 15)		6.4 efg h	6.4 ijklm	4.5 cdef	7.3 cdefgh	6.3 hijkl	4.1 bcdef
JM 67A (KAB 15)		7.8 abcde	8.0 abcdefgh	3.7 efg hij	8.3 abcd	7.9 abcd ef	5.0 abc
JM 12A (KIS 5)		8.2 abcd	8.1 abcdefg	3.9 efg hij	7.9 abcdef	8.7 ab	3.0 ighij
JM 5 (KIS 14)		9.0 a	7.8 abcdefghi	3.8 efg hij	9.0 a	8.5 abc	4.7 abcd
JM 38 (KIS 16)		9.0 a	9.0 a	4.0 efg hij	9.0 a	9.0 a	38 cdef
JM 91 (KAB 28)		8.8 ab	8.2 abcdef	4.7 bcdef	7.7 abcdef	8.0 abcdef	4.3 bcdef
JM 59H (KAB 2)	<i>P. salpingophorum</i>	7.8 abcde	7.3 bcdefghij	6.1 ab	7.3 cdefgh	6.3 hijkl	5.3 ab
JM 84 (KAB 6)		8.8 ab	8.5 abcd	5.2 abcd	8.0 abcdef	7.3 cdefgh	4.1 bcdef
JM 41A (KAB 9)		6.7 efg	5.2 lmn	4.5 cdef	5.6 jk	5.8 jklm	4.1 bcdef
JM 65A (KAB 13)		8.8 ab	8.2 abcdef	6.1 ab	7.3 cdefgh	7.3 cdefgh	3.7 cdefg
JM 89H (KAB 21)		6.0 ighi	6.0 jklm	5.0 abcde	6.0 hij	4.0 opq	4.0 bcdefg
JM 55 (KAB 25)		3.9 kl	3.0 op	3.2 ghijk	4.6 kl	4.0 opq	3.4 de fgh
JM 71 (KAB 26)		4.9 ijk	4.0 no	4.1 de fghi	4.6 kl	4.4 nop	3.0 ighij
JM 92 (KAB 29)		6.5 efg	6.8 ighij	5.5 abcd	5.2 jk	3.8 opq	3.0 ighij
JM 97 (KAB 30)		9.0 a	9.0 a	3.0 hijkl	8.5 abc	8.7 ab	3.0 ighij
JM 6A (KIS 2)		5.0 hijk	3.8 no	2.0 klmn	4.3 klm	4.7 mno	3.0 ighij
JM 19B MB (MB 5)		2.5 lmn	3.9 no	3.1 ghijkl	2.7 n	3.2 pqr	1.8 jk
JM 21 MB (ME 18)		6.0 ighi	6.0 jklm	5.0 abcde	6.0 hij	4.0 opq	3.7 cdefg
JM 85A (KAB 8)	<i>P. ultimum</i> var. <i>ultimum</i>	9.0 a	9.0 a	2.7 ijklm	8.7 ab	7.3 cdefghi	2.3 hijk
JM 90S KAB 22)		7.3 cdef	7.6 abcd	4.0 efg hij	7.0 de fgh	8.3 abcd	4.7 abcd
JM 45 (KAB 23)		6.0 ighi	6.0 jklm	2.7 ijklm	6.7 ighi	6.0 ijklm	3.3 efg hij
JM 17A MB (MB 2)		7.2 def	7.1 de fghijk	3.5 ighij	7.2 cdefgh	7.1 de fghij	3.5 de fgh
JM 1B MB (MB 6)		7.0 def	7.8 abcdefghi	3.3 ighijk	6.7 ighi	6.5 ghijkl	4.7 abcd

Table 6: Continued.

Isolate/strain	Species	Second trial			Third trial		
		CAL 96	K20	RWR 719	CAL 96	K20	RWR 719
JM 17C MB (MB 9)	<i>P. ultimum</i> var. <i>ultimum</i>	7.5 abcde	7.3 bcde ghij	4.0 e ghij	7.7 abcde f	7.3 cde ghij	3.0 ghij
JM 16A MB (MB 10)	"	6.6 e g	8.0 abcde gh	3.7 e ghij	7.7 abcde f	6.8 e ghij	3.0 ghij
JM 8 MB (MB 13)	"	6.6 e g	7.2 bcde	3.7 e ghij	6.2 gh j	7.0 de ghij	3.0 ghij
MS 38	"	7.2 def	7.3 bcde ghij	3.3 f ghijk	6.7 f ghi	7.7 abcde g	4.3 bcde f
MS 41	"	9.0 a	8.0 abc	5.8 abc	6.7 f ghi	8.1 abcde	5.8 a
MS 46	"	7.0 def	7.8 abcde ghij	3.3 f ghijk	6.7 f ghi	6.5 gh jkl	4.7 abcd
MS 47	"	7.0 def	7.0 e ghijk	1.7 lmn	7.7 abcde f	7.3 cde ghij	2.3 h jk
MS 50	"	7.5 abcde	8.2 abcde f	4.0 e ghi	7.8 abcde f	7.3 cde ghij	5.0 abc
MS 52	"	7.5 abcde	7.3 bcde fgh j	4.0 e ghi	7.7 abcde f	7.3 cde ghij	3.0 fghij
MS 58	"	7.0 dei	7.0 e fghijk	4.3 de g	8.1 abcde	7.7 abcde g	5.2 ab
MS 61	"	9.0 a	9.0 a	6.3 a	8.1 abcde	7.7 abcde g	5.2 ab
MS 66	"	8.2 abcd	6.7 gh ijk	3.7 e gh j	7.4 bcde g	6.0 i jklm	2.7 gh j
JM 75 (KAB 27)	<i>P. vexans</i>	2.0 mno	1.7 pq	1.7 lmn	3.0 mn	2.7 qr	2.7 gh j
JM 4 (KIS 13)	"	3.3 lm	2.0 pq	2.0 klmn	3.3 lmn	3.0 qr	3.0 fgh j
JM 10 MB (MB 14)	"	2.3 mno	2.7 op	2.3 klmn	2.7 n	3.3 pqr	3.0 fgh j
JM 20 MB (MB 17)	"	2.7 lmn	3.0 op	1.7 lmn	3.3 lmn	3.0 qr	2.0 j k
JM 17B MB (MB 3)	<i>P. oligandrum</i>	1.3 no	1.0 q	1.0 n	1.3	1.0 s	1.0 k
JM 18A MB (MB 4)	"	1.3 no	1.0 q	1.0 n	1.3	1.0 s	1.0 k
MS 44	<i>P. dissotocum</i>	3.0 lm	2.0 pq	1.0 n	3.0 mn	2.0 rs	1.0 k
JM 29A (KIS 6)	<i>P. pachycaule</i>	8.2 abcd	7.8 abcde fgh j	3.7 e gh j	6.9 e fgh j	6.3 h j kl	2.7 gh j
JM 3A (KIS 1)	<i>P. nondosum</i>	7.0 def	6.7 gh ijk	3.5 f gh j	7.6 bcde f	7.0 de fgh j	3.8 cde g
JM 7H1 (KIS 3)	"	7.4 bcde f	6.6 cde d	4.3 de gh	7.0 de gh	6.7 f gh j kl	4.4 abcde
JM 8H (KIS 4)	<i>P. echinulatum</i>	5.4 gh j	3.9 no	4.3 de gh	5.3 j k	4.7 mno	3.7 bcde f
KWD1	<i>P. deliense</i> /						
Control	<i>P. aphanidematum</i>	5.0 h j k 1.0 o	5.0 mn 1.0 q	1.3 mn 1.0 n	5.3 j k 1.0	5.5 lmn 1.0 s	1.3 k 1.0 k

a) Means within column followed by similar letters are not significantly different at $P \leq 0.05$. b) Disease scale (1-9) 1 = no root symptoms; 3 = 10% of the hypocotyle and root tissues have lesions; 5 = 25% of the hypocotyle and root tissues lesions 7 = 50% the hypocotyle and root tissues have lesions and the root system suffers a considerable decay; 9 = 75% or more of the hypocotyle and root tissues have lesions and the root system suffers advanced stages of decay and considerable reduction (Abawi and Pastor Corrales, 1990).

3.4 Discussion

3.4.1 Isolation of *Pythium*

Bean samples with typical symptoms of *Pythium* infection collected from 259 sites in the districts of Kisoro, Kabale, Mbale and Sironko yielded 146 isolates. The fewer numbers of *Pythium* recovered from the samples could be as a result of the presence of other pathogens especially *Fusarium* and *Rhizoctonia*, which are known to occur in complex with *Pythium* (Rusuka *et al.*, 1997; Opio, 1998). The presence of other pathogens in the complex, therefore, makes detection of *Pythium* spp. pathogenic to beans using culture media difficult. Likewise, because of the existence of a number of pathogens in the bean root rot complex it has not been easy to detect *F. solani* f.s. *phaseoli* from a number of bean and soil samples infected with root rot pathogens soil using culture media (Tusiime, 2003). However, it has been possible to detect *F. solani* f.s. *phaseoli* from at least 90% root rot infected samples collected from the districts of Kabale and Kisoro in April 2000 using molecular detection tool developed (Tusiime, 2003). Similar detection tools for *Pythium* spp. pathogenic to beans need to be developed.

3.4.2 Morphological identification of *Pythium*

Pythium isolates from Uganda were grouped using morphological features which included: sporangium presence, form, and size; the size, wall thickness, and theplerotic conditions of the oospore; oogonium location, ornamentation, size, and shape; and the number, origin and morphology of the antheridia (Plaats-Niterink, 1981). Five morphological groups had similar structures typical of known species, i.e., *P. oligandrum*, *P. spinosum*, *P. ultimum* var

ultimum, *P. echnulatum* and *P. rostrutum*. Morphological plasticity and cases of overlap of phenotypic characters for isolates in the other six morphological groups made accurate identification of isolates to species level difficult.

The identification of *Pythium* isolates to species using morphological structures require considerable time and experience (Plaats-Niterink 1981). Molecular identifications and differentiation have been developed for a number of *Pythium* species in other countries to overcome delays in identifications using morphology (Wang and White, 1997; Paul *et al.*, 1998; Lévesque *et al.*, 1998; Matsumoto *et al.*, 1999; Bailey *et al.*, 2002). In this study molecular analyses of Uganda isolates were conducted to further identify the isolates to species level.

3.4.3 Molecular characterization of *Pythium* species

3.4.3.1 PCR/RFLP analysis of 80 *Pythium* isolates

The digestion of amplified ITS region with enzymes *Cfo* I, *Hinf* I and *Mbo* I differentiated the 80 *Pythium* isolates into seven groups, but 14 of the isolates were not grouped (i.e. appeared individually). The restriction banding pattern of the ITS region for some known species from culture collection had similar ITS restriction banding pattern. The isolates include CBS 377.72 (*P. spinosum*) for RFLP group 1; CBS 165.68 (*P. salpingophorum*), RFLP group 3; CBS 296.37 (*P. ultimum* var *ultimum*), RFLP group 4; and CBS 149.84 (*P. oligandrum*), RFLP group 5. This result suggests that RFLP analysis can be used to distinguish a collection of *Pythium* isolates and, to a less extent, identify *Pythium* species if restriction banding patterns are compared with those of known species.

The Internal transcriber spacer of rDNA for the Ugandan *Pythium* and culture collection isolates was shown to be variable between, but largely conserved within RFLP groups. This finding is consistent with earlier RFLP analyses of known *Pythium* species (Chen, 1992; Chen *et al.*, 1992b; Wang and White, 1997). The present result indicates that ITS region can be used as a marker for the study of *Pythium* species by detecting species specific variation. The differentiation of *Pythium* species by RFLP analysis is a technique that can be employed to study *Pythium* populations in natural habitats (Chen *et al.*, 1992), like those collected from bean fields in Uganda.

There was some relationship between morphological grouping and RFLP. In some instances RFLP groups contained isolates from more than one morphological group. This implies that, because of morphological plasticity and cases of overlap of phenotypic characters, identification of *Pythium* using morphology to species level is difficult. More accurate methods, which include RFLP analysis and sequencing ITS region of rDNA, have been used to differentiate and identify *Pythium* to species level (Chen, 1992; Chen *et al.*, 1992b; Wang and White, 1997; Paul *et al.*, 1998; Matsumoto *et al.*, 1999; Bailey *et al.*, 2002). Similar procedures of ITS analysis were applied to Ugandan *Pythium* isolates collected from bean fields.

3.4.3.2 DNA sequences analysis and *Pythium* species identification

Previous RFLP analysis of ITS 1-ITS 4 region differentiated 63 of the test *Pythium* isolates into seven RFLP groups and the other 14 isolates were not grouped. The ITS 1 region of the rDNA is known to be more variable than ITS 2 (Berbee *et al.*, 1995; Goodwin *et al.*, 1995; Morales *et al.*, 1995). The variability is focused mostly at species level in the ITS 1 and at genus level in ITS 2 (Jasalavich *et al.*, 1995). The analysis of sequence data of the

ITS 1 were found consistent with RFLP groupings by three enzymes *Cfo* I, *Hinf* I and *Mbo* I for the 42 Ugandan and five HRI *Pythium* culture collections tested. The analysis of ITS1 sequences of the 56 Ugandan *Pythium* isolates and five HRI culture collections by aligning with ITS 1 sequences of known *Pythium* species in the data base differentiated and confirmed the identity of *Pythium* species in Uganda earlier grouped using PCR/RFLP analysis. This confirms the earlier finding that RFLP analysis and sequencing of ITS can be used to differentiate and identify *Pythium* species (Chen, 1992; Chen *et al.*, 1992b; Wang and White, 1997; Paul *et al.*, 1998; Matsumoto *et al.*, 1999; Bailey *et al.*, 2002).

The molecular analyses using RFLPs and ITS 1 sequences have revealed a wide diversity within a collection of *Pythium* species in Uganda. The results revealed that at least 11 *Pythium* species are associated with bean root rots in Uganda. Apart from the traditionally *P. ultimum* var *ultimum* known to be associated with bean root rots (Abawi and Pastor Corrales, 1990), other *Pythium* species recovered were *P. spinosum*, *P. torulosum*, *P. salpingophorum*, *P. vexans*, *P. dissotocum*, *P. nodosum*, *P. echinulatum*, *P. pachyaule*, *oligandrum* and *P. deliense*. The most abundant *Pythium* species was the traditional *P. ultimum* var *ultimum*, which is likely to be responsible for the increased incidence of bean root rots in Kabale and Kisoro districts. The other common species were *P. salpingophorum*, *P. torulosum*, *P. vexans* and *P. spinosum*.

Sequences of ITS 1 have been generated and *Pythium* species identified from a collection of Ugandan samples. The design PCR specific primers to detect and differentiate *Pythium* species pathogenic to beans had some good level of specificity. It was possible to detect the five important *Pythium* species, namely *P. salpingophorum*, *P. torulosum*, *P. spinosum*, *P. nodosum* and *P. ultimum*. Since the primers are able to amplify a specific size of the target DNA, the fragment size can be used as a marker to detect particular *Pythium* species. The

limitation is failure to differentiate a particular *Pythium* species with variation in pathogenicity as is the case of *P. torulosum* and *P. salpingophorum* discussed in section 3.4.4.

3.4.4 Pathogenicity of *Pythium* isolates on beans

All isolates of *P. ultimum* var *ultimum* known to cause root rot on beans (Abawi and Pastor Corrales, 1990) and *P. spinosum* caused severe root rot on susceptible bean varieties CAL 96 and K20. Also isolates belonging *P. torulosum*, *P. salpingophorum*, *P. nodosum* and *P. pachycaule* caused root rot on bean varieties CAL 96 and K20. Interestingly, pathogenicity tests of the four *P. spinosum* isolates suggest that this species can cause bean root rot disease under field conditions. The other species that can cause bean root rots in the field are *P. nodosum* and *P. pachycaule*. Isolates of *P. torulosum* and *P. salpingophorum* showed some level of variation in their pathogenicity, but the species could probably contribute to bean root rot as part of the complex of minor pathogens (Salt, 1979). Nevertheless, further investigations are required to compare the pathogenic variation observed with molecular variation using AFLP markers and eventually develop specific probes to target pathogenic forms of this species.

Pathogenicity tests indicate significantly more disease when highly pathogenic *Pythium* species are inoculated on susceptible bean varieties CAL 96 and K20 compared to the highly tolerant variety RWR 719. This result demonstrates the potential for host resistance in the management of different *Pythium* species causing bean root rot disease. A number of *Pythium* species, namely *P. ultimum* var *ultimum*, *P. spinosum*, *P. salpingophorum*, *P. torulosum*, *P. nodosum* and *P. pachycaule*, pathogenic to beans have now been identified from areas where root rot disease has been a problem in Uganda. Now that the different

species have been mapped out (Fig. 5), it should be possible to identify bean varieties with some good level of tolerance to specific *Pythium* species for the particular locations.

CHAPTER FOUR

EFFECT OF ORGANIC AMENDMENTS ON SOIL POPULATION LEVELS OF *PYTHIUM* SPECIES PATHOGENIC TO BEANS IN UGANDA

4.1 Introduction

Population levels of soil borne pathogens influence incidence and severity of root rots and some of the management efforts have been directed at reducing soil inoculum to below economically damaging threshold levels (Buruchara and Scheidegger, 1993; Hall and Nasser, 1996). Although the effects of management practices can be assessed on the basis of disease severity, this measure alone is not always a good indicator of soil pathogen populations. Environmental factors and stage of plant growth play a critical role in influencing disease development and severity. There are instances when epidemics of bean root rots are observed in one season and not in the next, a feature which is a source of confusion to farmers (Buruchara and Scheidegger, 1993). There is therefore a need to develop tools and procedures that are simple, fast and accurate for the quantification of pathogen populations in soil, for *Pythium*. This would assist in development of prediction models but more importantly would facilitate determining the effects of various bean roots rot management options (cultural, varietal and biological) on *Pythium* populations. These tools could also be used to study the influence of environmental factors on *Pythium* populations in field soils.

A number of methods for quantifying soil pathogen populations have been proposed for example, dilution plating for a wide range of fungal pathogens (Nash and Snyder, 1962), baiting has been used for *Pythium* and *Phytophthora*, and bioassay for quantifying

Plasmodiophora brassica and *Mycorrhizial* fungi (Paulitz, 2000), a competitive PCR-based assay for *Verticillium* (Hu *et al.*, 1993; Mahuku *et al.*, 1995; Henz and Platt, 2000b). However, there are a number of limitations in using the listed methods as described earlier in section 2.5. For example, one of the major limitations of using dilution plating is failure to distinguish pathogenic and non-pathogenic isolates of *Pythium* which require differentiation of species. There is therefore a need to develop a procedure for both identifying and quantifying the effect and relative value of different management practices on inoculum levels of pathogenic *Pythium* spp. Since dilution plating method can be used to isolate both pathogenic and non pathogenic isolates, by combining with the molecular detection tool developed in Chapter 3, it could be possible to quantify *Pythium* pathogenic species present in field soils. This study was therefore undertaken to evaluate the effect of organic amendments on inoculum levels of *Pythium* spp. pathogenic to beans in field soils using dilution-plating method in combination with molecular detection techniques.

4.2 Materials and methods

4.2.1 Validation of dilution plating method for quantification of *Pythium* in field soil

A method used at HRI to quantify *Pythium* from soil was adopted in this study with modifications. The media used was CMA amended with 30 mg/L of rifamycin and 75mg/L of pimarcin on 9-cm plastic plates. Validation of the dilution plating method technique was done using *Pythium* isolate MS 61, a *P. ultimum* var *ultimum* previously used in the pathogenicity test and found to be pathogenic to beans (varieties CAL 96 and K20). Inoculum was prepared following procedure described in section 3.2.6. The infested finger millet seed was moistened, thoroughly mixed with pre-sterilised topsoil using a ratio of 1:1

v/v; 1:5 v/v; 1:10 v/v; 1:15 v/v and; 1:20 v/v (Inoculum: soil) and allowed to settle for two days.

Two grams of each of the 10 soil sub samples containing *Pythium* inoculum were dissolved in 50, 100 and 200 ml of sterile water. Three soil samples from naturally infested bean fields were also included for comparisons. One ml of the resulting suspension was plated onto a 9 cm petri dish containing selective medium for *Pythium*. Nine plates were plated for each of the soil samples. Plates were briefly air-dried in a laminar flow bench. The plates were then incubated at 20-25°C for two days but were checked after one day for the presence of colonies.

The validation of dilution plating technique as a method of quantifying *Pythium* in soil revealed that:

- Soils infested at ratios of 1:5 and 1:10 consistently produced large numbers of colonies when 1 ml of suspension from the 50 or 100 ml volumes was plated out. It was difficult to count the colonies, because by the second day they were merged. On further dilution to 200ml the number of colonies were countable (15 –20 colonies per plate; equivalent to 1500-2000 cfu.g⁻¹)
- Soils infested at ratios of 1:15 and 1:20 v/v consistently produced fewer colonies. Colony counts ranged from zero to three for any dilution.
- Earlier work has indicated that a threshold of about 300-500 cfu.g⁻¹ is necessary for *Pythium* to cause disease (Mazzola *et al.*, 2002). The estimated thresholds are obtainable when soils are infested at ratio of 1:10 v/v (inoculum: soil). The same soils with ability to induce disease were processed using this dilution plating method but plating out 0.7ml of a suspension of two grams soil in 100ml of sterile water. The numbers of colonies growing on each plate varied between five and ten (equivalent to a

soil concentration of 250-500 cfu.g⁻¹). This dilution was adopted for the subsequent soil *Pythium* population studies.

Uniform colony types were observed for soil samples artificially inoculated with MS 61 (*P. ultimum* var *ultimum*), but 2 to 3 types of colonies were observed on plates containing samples from the naturally infested field soil from Kicumbi. The different types of colonies suggest that different *Pythium* species may occur together in field soils. Therefore, samples of different colonies were sub-cultured for further identification using molecular techniques described in Chapter three. The colony types and numbers were found to be consistent for all the ten sets tested. The method of *Pythium* quantification in soil by plating 0.7 ml of a suspension of 2 g in 100 ml of sterile water, and detection of different species using molecular techniques were adopted for the subsequent population studies of the pathogenic *Pythium* species.

4.2.2 *Pythium* populations in soils amended with farmyard manure and green manure

The first experiment set was conducted in a screen house using artificially infested soils and in the field, making use of natural infection during the season of 2002A (March – July 2002).

Screen house trial: Fresh soil was obtained from a field at Kawanda Agricultural Research Institute (KARI) with no history of bean root rot disease. The soil was kept for two weeks before inoculation with *Pythium*. Artificial inoculations was done using a *Pythium* pathogenic isolate MS 61 (*P. ultimum* var *ultimum*) with an estimated populations of about 200-300 cfu g⁻¹ of soil in each of wooden flats (42 cm x 72 cm). Seven days later, in addition to the *Pythium* inoculum, some soils in wooden flats were amended with either 300 g (equivalent to 10 tons ha⁻¹) of farmyard manure (FYM) or with green manure from *Calliandra* spp.

After 14 days of amendment incorporation, bean seeds of varieties CAL 96 (highly susceptible to *Pythium* root rot) and RWR 719 (tolerant to *Pythium* root rot) were sown in wooden flats containing each treatment; the treatments were replicated three times. The trial was planted in a split design in a randomized block design. Each variety was planted in 6 rows each of 6 plants, making a total of 36 plants in a wooden flat. *Pythium* pathogen populations were monitored from two weeks from the time amendments were added, and this was continued at two week interval for three months. The soil sampled each time were air dried for two days before being processed for *Pythium* population counts using the method described in section 4.2.1. Root rot severity and crop dry matter were recorded every two weeks beginning two weeks after sowing until crop maturity (eight weeks later). Bean grain yield was later determined at harvest. The screen house experiment was repeated in 2002B (October 2002- February 2003), and 2003A (March to June 2003).

Field trial: A field trial similar to the screen house one was set up in 2002A (March – July) to further examine the effects of soil amendments on *Pythium* populations. Two sites, Rubaya and Kicumbi, both in Kabale district were used for the trials. The gardens used had been under beans the previous season and had history of root rot disease. The trial was planted as a split plot, in randomized block design replicated three times. Varieties CAL 96 and RWR 719 were assigned to main plots and amendments to sub plots. Farmyard manure and green manure from *Calliandra* spp. (each at 10 tons ha⁻¹) was incorporated into randomly chosen plots. Control plots received no amendments. Two weeks after the amendments had been incorporated five rows of beans were sown in each plot with 50cm between rows and 10cm between seeds. The plot size at Rubaya was 2.5 m long while at Kicumbi it was 2.0 m. After a further two weeks, plant stand was recorded, and thereafter, plots were visited fortnightly to collect data on plant stand and disease severity. Plants and soil were also sampled from each plot at every visit. Plants were carefully dug up using a

garden trowel and gently shaken to dislodge soil without losing roots and dried to constant weight. Soil samples were analysed to establish *Pythium* inoculum levels using the method described in section 4.2.1. At harvest bean yield data were recorded. The field experiment was repeated in 2002B in different plots.

Follow up repeat trials: A follow up trial was set up to evaluate the effects of the amendments previously added to plots. The trial was planted with beans in the same plots used the previous season at Rubaya and Kicumbi during 2002B. A similar follow up trial was set up in the screen house during 2002B and 2003A. In the case of screen house follow up effect of FYM and *Calliandra*, each treatment soil was separated into two in wooden flats of 42 cm by 36. Effects of further addition of farmyard manure and green manure were investigated in one set of flats. In each of the cases, data obtained were subjected to analysis of variance using Genstat computer package (Lawes Agricultural Trust Rothamsted Experimental Station, 1995). Means were separated using Fisher's least significant difference test at 5% probability level.

4.3 Results

4.3.1 Effect of Farmyard manure and *Calliandra* on soil *Pythium* populations

Screen house trial

Pythium population increased during the fourth to eighth week from 250 -300 cfu g⁻¹ to as high as 700 -1000 cfu g⁻¹ for all the treatments including follow up and repeat trials (Tables 7 and 8). In the 10th to 12th week *Pythium* populations started reducing to a range of 200 – 600 cfu g⁻¹ for all trials. However, addition of FYM consistently increased *Pythium* populations compared to *Calliandra* and the control. A follow trial recorded lower colony forming units per gram of soil compared to the first experiment (Table 8). Colony counts per

gram of soil differed significantly between treatments in the six and eighth week for the first trial and 12th week for the repeat. This was also true for all the weeks for the follow up screen house trial concluded in 2002B. In the follow up experiments *Pythium* populations reduced by about 50% for the plots where farmyard and *Calliandra* were applied (Table 8). However, the numbers remained significantly higher than the control.

Further addition of FYM and *Calliandra* did not significantly reduce populations of *P. ultimum* in the screen house. Colony counts per gram of soil ranged from 298 to 464 for treatments with *Calliandra*. In the treatments with FYM colony counts ranged from 500 to 700 by 4 to 6 weeks after amendment addition. However, by 10 to 12 weeks, *Pythium* populations were lowered to 200- 300 c.f.u per gram of soil compared to initial amendment addition (Tables 7 and 8). In follow up trials, *Pythium* populations were reduced by 40 to 50% by 10 weeks, but not with further addition of amendments.

Field trial

At Rubaya, *Pythium* populations appeared to be uniform through out the season. However, treatments with farmyard manure and *Calliandra* had slightly more colony forming units per gram of soil compared to the control (Table 9). In follow up trial of 2002B soils from plots with RWR 719 had a slight lower pathogen by sixth week of counts ($< 110 \text{ cfu g}^{-1}$) when compared with variety CAL 96 with $> 114 \text{ cfu g}^{-1}$ (Table 10). At Kicumbi pathogen population trends were similar to those at Rubaya (Tables 11 and 12). However, for all experiment repeats, the number of colony forming units per gram of soil was slightly lower than those from Rubaya.

Table 7: Effect of farmyard and *Calliandra* on *Pythium* populations (cfu g⁻¹ of soil) from first screen house experiment at Kawanda, 2002A

Variety	Soil amendment	Weeks after planting (WAP)						
		start	2	4	6	8	10	12
CAL 96	<i>Calliandra</i>	305.0	381.0	643.0	745.0 ab	533.0 a	595.0	614.0
	Farmyard manure	262.0	333.0	476.0	745.0 ab	640.0 a	716.0	597.0
	Control	283.0	309.0	333.0	438.0 bc	267.0 b	376.0	531.0
RWR 719	<i>Calliandra</i>	250.0	286.0	452.0	519.0 bc	348.0 b	524.0	511.0
	Farmyard manure	309.0	405.0	571.0	1038.0 a	634.0 a	745.0	556.0
	Control	336.0	452.0	690.0	343.0 c	318.0 b	347.0	412.0

¹Within columns, means with the same letter (s) and no letters do not differ significantly at P ≤ 0.05

² Season 2002A (March to July)

Table 8: Effects of farmyard and *Calliandra* on *Pythium* populations (cfu g⁻¹ of soil) in a follow up 2002A screen house trial at Kawanda, 2002B

Variety	Soil amendment	Weeks after planting (WAP)						
		start	2	4	6	8	10	12
CAL 96	<i>Calliandra</i>	426.0 a	155.0 ab	202.0 cd	321.0 bc	143.0 d	167.0 c	286.0 ab
	Farmyard manure	524.0 a	143.0 b	750.0 a	536.0 b	236.0 c	262.0 bc	190.0 bc
	Control	171.0 b	107.0 b	155.0 d	121.0 c	119.0 d	155.0 c	357.0 a
RWR 719	<i>Calliandra</i>	267.0 b	298.0 a	333.0 bc	500.0 b	345.0 b	202.0 bc	155.0 c
	Farmyard manure	528.0 a	293.0 a	476.0 b	750.0 a	619.0 a	464.0 a	274.0 ab
	Control	150.0 b	131.0 b	417.0 b	274.0 bc	119.0 d	309.0 b	190.0 bc

¹Within columns, means with the same letter (s) do not differ significantly at P ≤ 0.05

² Season 2002A (March to July)

Table 9: Effect of farmyard manure and *Calliandra* on *Pythium* populations (cfu g⁻¹ of soil) from Rubaya, 2002A season

Variety	Soil amendment	Weeks after planting (WAP)						
		start	2	4	6	8	10	12
CAL 96	<i>Calliandra</i>	131.0	155.0	231.0	107.1	192.8	174.0	128.5
	Farmyard manure	107.0	107.0	226.0	95.2	109.5	138.0	126.1
	Control	124.0	155.0	181.0	126.1	102.3	150.0	147.6
RWR 719	<i>Calliandra</i>	102.0	107.0	126.0	102.3	164.2	114.0	149.9
	Farmyard manure	114.0	145.0	131.0	107.1	114.2	188.0	142.8
	Control	90.0	143.0	143.0	90.4	142.8	164.0	145.2

¹Within columns, means with the same letter (s) and no letters do not differ significantly at P ≤ 0.05

² Season 2002A (March to July)

Table 10: Effects farmyard manure and *Calliandra* on *Pythium* populations (cfu g⁻¹ of soil) in a follow up 2002A trial at Rubaya, 2002B

Variety	Soil amendment	Weeks after planting (WAP)						
		start	2	4	6	8	10	12
CAL 96	<i>Calliandra</i>	228.5 a	130.2 ab	148.0	115.0	161.8 a	209.0	136.0
	Farmyard manure	111.9 bc	49.0 c	131.0	134.0	166.6 a	136.0	124.0
	Control	166.6 ab	144.5 a	143.0	139.0	126.1 ab	119.0	148.0
RWR 719	<i>Calliandra</i>	78.5 c	150.2 a	198.0	75.0	21.4 c	95.0	124.0
	Farmyard manure	71.4 c	89.7 bc	183.0	108.0	95.5 b	148.0	183.0
	Control	88.1 c	106.4 ab	221.0	82.0	119.0 ab	179.0	152.0

¹Within columns, means with the same letter (s) and no letters do not differ significantly at P ≤ 0.05

² Seasons 2002A (March to July), 2002B (September to December)

Table 11: Effect of farmyard manure and *Calliandra* on *Pythium* populations (cfu g⁻¹ of soil) from Kicumbi, 2002A

Weeks after planting (WAP)								
Variety	Soil amendment	start	2	4	6	8	10	12
CAL 96	<i>Calliandra</i>	126.0	174.0	102.0	109.5	78.5	95.2	3.3 b
	Farmyard manure	102.0	121.0	71.0	109.5	102.3	95.2	154.7 a
	Control	179.0	162.0	90.0	97.6	107.1	85.7	166.6 a
RWR 719	<i>Calliandra</i>	95.0	155.0	79.0	111.9	111.9	111.9	100.0 b
	Farmyard manure	95.0	126.6	133.0	90.4	83.3	114.2	128.5 ab
	Control	119.0	126.0	95.0	95.2	119.0	119.0	78.5 b

¹ Within columns, means with the same letter (s) and no letters do not differ significantly at P ≤ 0.05

² Season 2002A (March to July)

Table 12: Effects of farmyard manure and *Calliandra* on *Pythium* populations (cfu g⁻¹ of soil) in a follow up 2002A trial at Kicumbi, 2002B

		Weeks after planting (WAP)						
Variety	Soil amendment	start	2	4	6	8	10	12
CAL 96	<i>Calliandra</i>	131.0	95.2 bc	102.0	162.0 b	107.0	178.5	78.5
	Farmyard manure	131.0	135.7 ab	95.0	102.0 b	102.0	90.4	95.2
	Control	131.0	164.2 a	159.0	109.0 b	155.0	78.5	130.9
RWR 719	<i>Calliandra</i>	124.0	71.4 cd	95.0	179.0 b	114.0	119.0	83.3
	Farmyard manure	95.0	109.5 abc	221.0	276.0 a	148.0	102.3	135.7
	Control	143.0	11.9 d	112.0	167.0 b	95.0	128.5	102.3

¹ Within columns, means with the same letter (s) and no letters do not differ significantly at P ≤ 0.05

² Seasons 2002A (March to July), 2002B (September to December)

4.3.2 Effect of organic amendments on change in frequency of existing *Pythium* species in bean fields

In the first field *Pythium* soil quantification trial at Kicumbi, visual observation on a selective media (Plate 4) separated the 60 test *Pythium* isolates into three groups, *Pythium* spp a to c (Table 13). Twelve isolates in each group which were subjected to PCR/RFLP analysis identified *P. ultimum*, *P. salpingophorum* and *P. torulosum* with frequencies of 3, 6 and 4, respectively, at the start of the experiment. It was not possible to differentiate one isolate from the rest using visual observation. Addition of FYM reduced frequency of *P. salpingophorum* from 6 to 1 by 8 WAP compared to *P. ultimum* where frequency increased to 5 by 4 WAP and later dropped to 2 by 8 WAP (Table 13). Addition of *Calliandra* favored the prevalence of *P. salpingophorum* but not *P. ultimum* and *P. torulosum*. For example, at the start, the frequency of *P. salpingophorum* was 6, but 4 WAP the frequency was still the same but reduced to 4 by 8 WAP. In the case of *P. ultimum*, the frequency at the start was only 3 but reduced to 2 by 8 WAP under the same treatment.

At Rubaya site, similar visual observations were made, with an addition group *Pythium* spp (d). The trends of frequencies of *Pythium* species were similar to those at Kicumbi when the same numbers of isolates were subjected to PCR/RFLP analysis. In the repeat trial of 2002B, one additional visual observation was made (*Pythium* spp (e)). The two species *P. salpingophorum* and *P. ultimum* had similar frequency trends to those in the first trial of 2002A (Table 14). At Rubaya, *P. torulosum* was not observed in the samples tested.

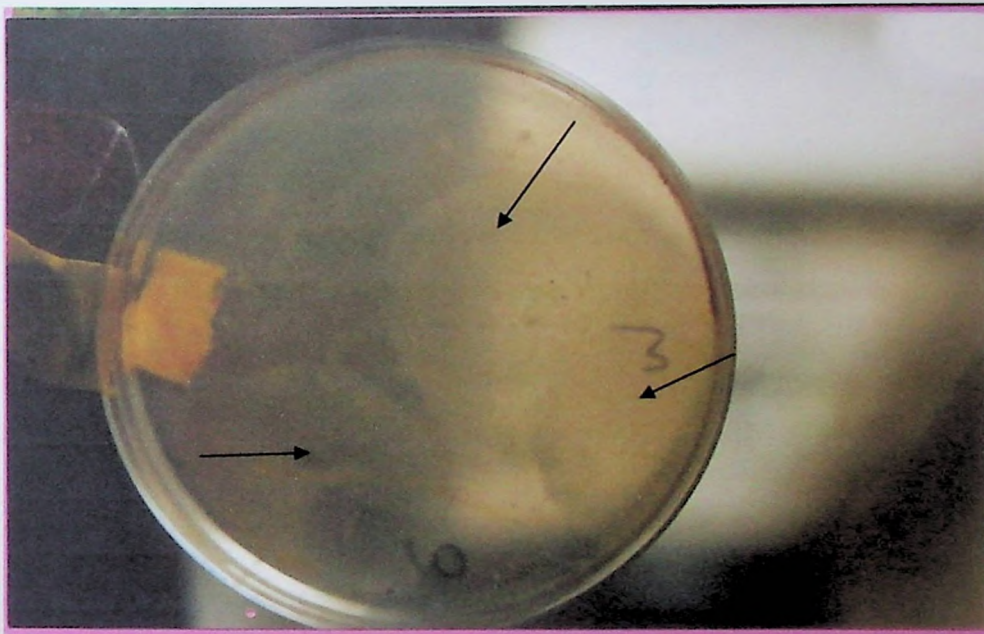


Plate 3: Colonies of *Pythium* from soil growing on selective media

Arrow indicates the different colony types growing on media

Table 13: Effect of *Calliandra* and farmyard manure on change in frequency of *Pythium* species existing in bean fields in the first trial at Kicumbi and Rubaya during 2002A

Visual grouping	PCR/RFLP identification	Calliandra				Farmyard manure				Control	
		1 st Start	WAP		1 st Start	WAP		1 st Start	WAP		
			4	8		4	8		4	8	
Kicumbi site											
Pythium spp (a)	<i>P. torulosum</i>	4	0	0	0	1	0	1	0	1	
Pythium spp (b)	<i>P. ultimum</i>	3	2	2	5	2	1	1	3		
Pythium spp (c)	<i>P. salpingophorum</i>	6	6	4	1	1	1	1	2		
	<i>Pythium</i> spp (New 1)	0	0	0	0	1	0	0	0		
Rubaya site											
Pythium spp (a)	<i>P. torulosum</i>	1	0	0	0	0	0	0	0		
Pythium spp (b)	<i>P. ultimum</i>	4	8	4	3	4	0	0	1		
Pythium spp (c)	<i>P. salpingophorum</i>	3	2	8	0	1	0	0	0		
Pythium spp (d)	<i>Pythium</i> spp (New 2)	1	0	0	1	7	1	1	2		
	<i>Pythium</i> spp (New 1)	0	0	1	0	1	0	0	0		

¹ Visuals grouping based on cultural characteristics on plates; PCR/RFLP identification of ITS using *Hinf*I enzyme and clustering done using NTSYS computer package; ¹Start= identifications made before *Calliandra* and farmyard manure were applied to the plots.

² Seasons 2002A (March to July)

Table 14: Effect of *Calliandra* and farmyard manure on change in frequency of *Pythium* species existing in bean fields in the repeat trial at Kicumbi and Rubaya during 2002B

Visual grouping	PCR/RFLP identification	Calliandra				Farmyard manure				Control	
		Start	WAP		WAP		WAP				
			4	8	4	8	4	8			
Kicumbi site											
Pythium spp (a)	P. torulosum	1	0	0	1	0	0	0	0	0	
Pythium spp (b)	P. salpingophorum	8	4	5	7	3	1	1	1	1	
Pythium spp (c)	P. ultimum	4	5	1	4	4	1	1	4	4	
Pythium spp (e)	Pythium spp (New 3)	1	0	0	0	0	0	0	0	0	
Rubaya site											
Pythium spp (b)	P. salpingophorum	1	7	4	2	2	0	0	0	0	
Pythium spp (c)	P. torulosum	2	3	2	1	2	1	1	2	2	
Pythium spp (d)	Pythium spp (New2)	0	1	0	0	0	0	0	0	0	

¹ Visuals grouping based on cultural characteristics on plates; PCR/RFLP identification of ITS using *Hinf* I enzyme and clustering done using NTSYS computer package; ¹ Start= identifications made before *Calliandra* and farmyard manure were applied to the plots.

² Seasons 2002B (September to December)

4.3.2 Effect of soil organic amendments on root rot disease progress in tolerant (RWR 719) and susceptible (CAL 96) bean varieties

Similar trends of bean root disease with symptoms of *Pythium* infection were displayed in the screen house trial and those at the two field sites in Rubaya and Kicumbi in Kabale. There was more disease progress in the susceptible variety CAL 96 than the tolerant RWR 719 in all the trials and treatments (Figures 7, 9 and 11). Disease severity ranged from 6.0 to 9.0 (CIAT 1-9 scale) in the third to eighth week after planting for the susceptible variety and 1.0 to 5.0 for the tolerant variety, except in the follow up experiments in the screen house and Rubaya sites disease level was higher (Figure 8). In plots where FYM was applied there was less bean root rot disease compared to treatments with *Calliandra* and the control. Except in the first experiment planted in the screen house disease progress was high in wooden flats with the susceptible variety CAL 96 where farmyard was incorporated in soil containing *P. ultimum* var *ultimum* (Figure 7).

In the screen house experiment, disease progress remained uniform three weeks after planting in the first trial with severity ranging from 6.0 to 8.0 for susceptible variety and 4.0 to 5.0 for a tolerant variety depending on the amendments applied (Figure 7). In follow up trials disease levels after three weeks increased to as high 9.0 in a susceptible variety and 7.0 in a tolerant one (Figure 8). In the repeat trial, disease progress (Figure 9) was similar to that in the first trial (Figure 7). Further addition of FYM and *Calliandra* did not significantly reduce disease levels as was thought (Figure 12).

Bean root rot disease progress was higher at Rubaya site in the first five weeks in all the treatments with a maximum severity score of 5.0 compared to 3.0 in Kicumbi in a tolerant

variety (Figure 7), but in a susceptible variety the score was equally higher up to 8.0 to 9.0. Similar trends in disease progress were displayed in the follow up trials (Figure 8 and 10).

In the follow up trials, application of amendments in the first trial had an impact on disease progress in both the susceptible and tolerant variety. Amendments with FYM and *Calliandra* reduced the progress of the disease by the first 8 weeks in the susceptible variety (Figure 8) compared to similar plots and period the previous season where the disease was as high as 7.0 to 8.0 (Figure 7). At Rubaya, in the follow up trial disease reduction was only in plots where FYM was applied; *Calliandra* did not significantly reduce disease in the tolerant variety RWR 719 (Figure 8). At Kicumbi site the impact of FYM and *Calliandra* on disease was expressed in a similar way, indicating the possibility of existence of uniform *Pythium* species at this location. Interestingly, disease progress at Kicumbi was as high as 8.0 to 9.0 for all the treatments in the case of a susceptible variety CAL 96 compared to a tolerant one where disease levels ranged between 3.0 and 4.

4.3.3 Percentage survival of bean plants grown in soils infested with *Pythium* but amended with farmyard manure and *Calliandra*

Screen house trial

Percentage survival for bean variety CAL 96 two weeks after planting (WAP) was 62.0, 50.5 and 60.1 for treatments of *Calliandra*, FYM and control, respectively for CAL 96 (Table 15). In the case of RWR 719, percentage of surviving beans was 71.9 85.7, 80.3 and 72.0 for *Calliandra*, FYM and control, respectively. Eight WAP, the percentage of surviving bean

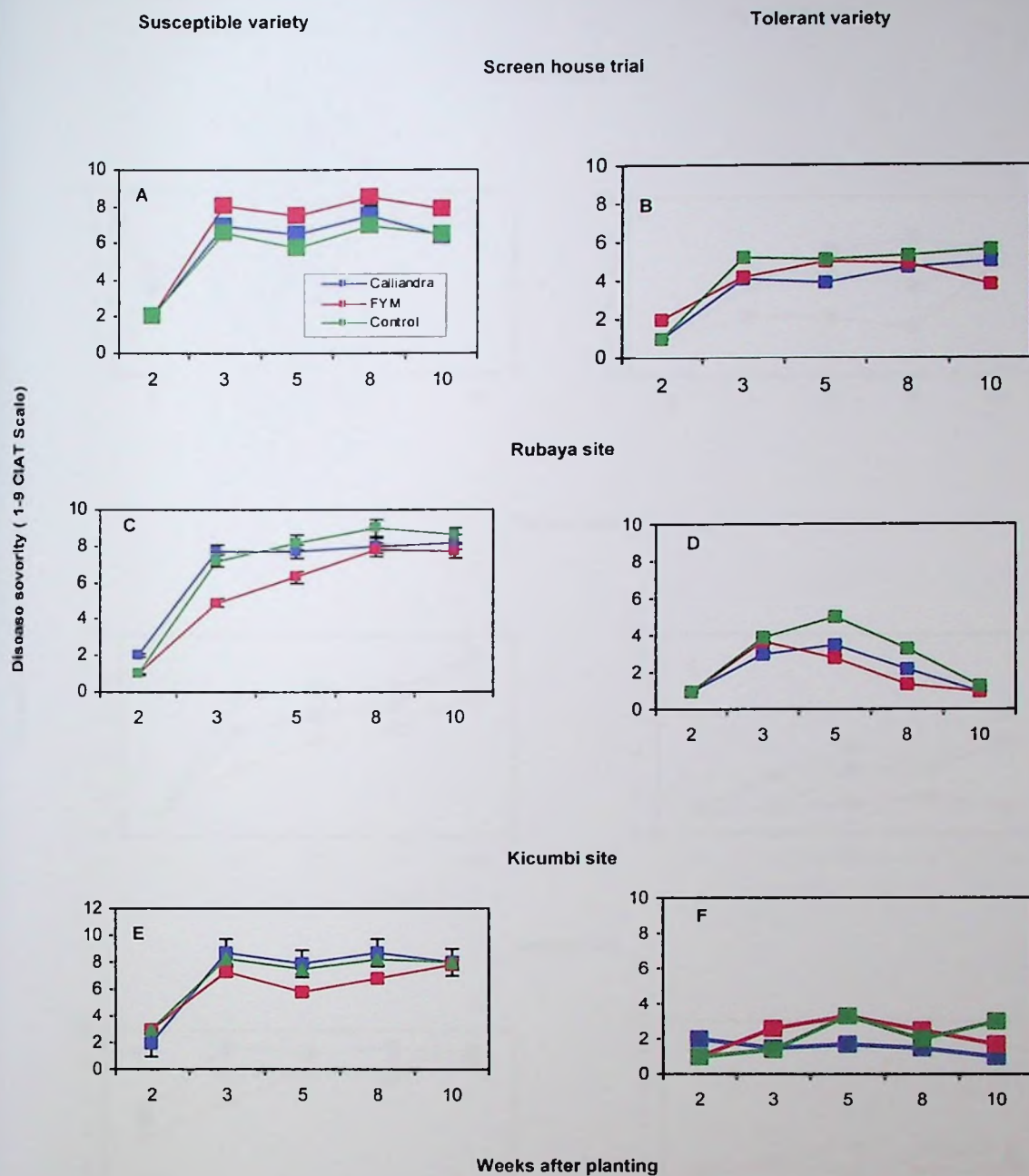


Figure 7: Effect of farm yard manure and *Calliandra* on *Pythium* root rot disease progress in the susceptible bean variety (CAL 96) and tolerant (RWR 719) for the first trial at Kawanda (screen house), Rubaya and Kicumbi 2002A
 1 Seasons 2002A (March to July),

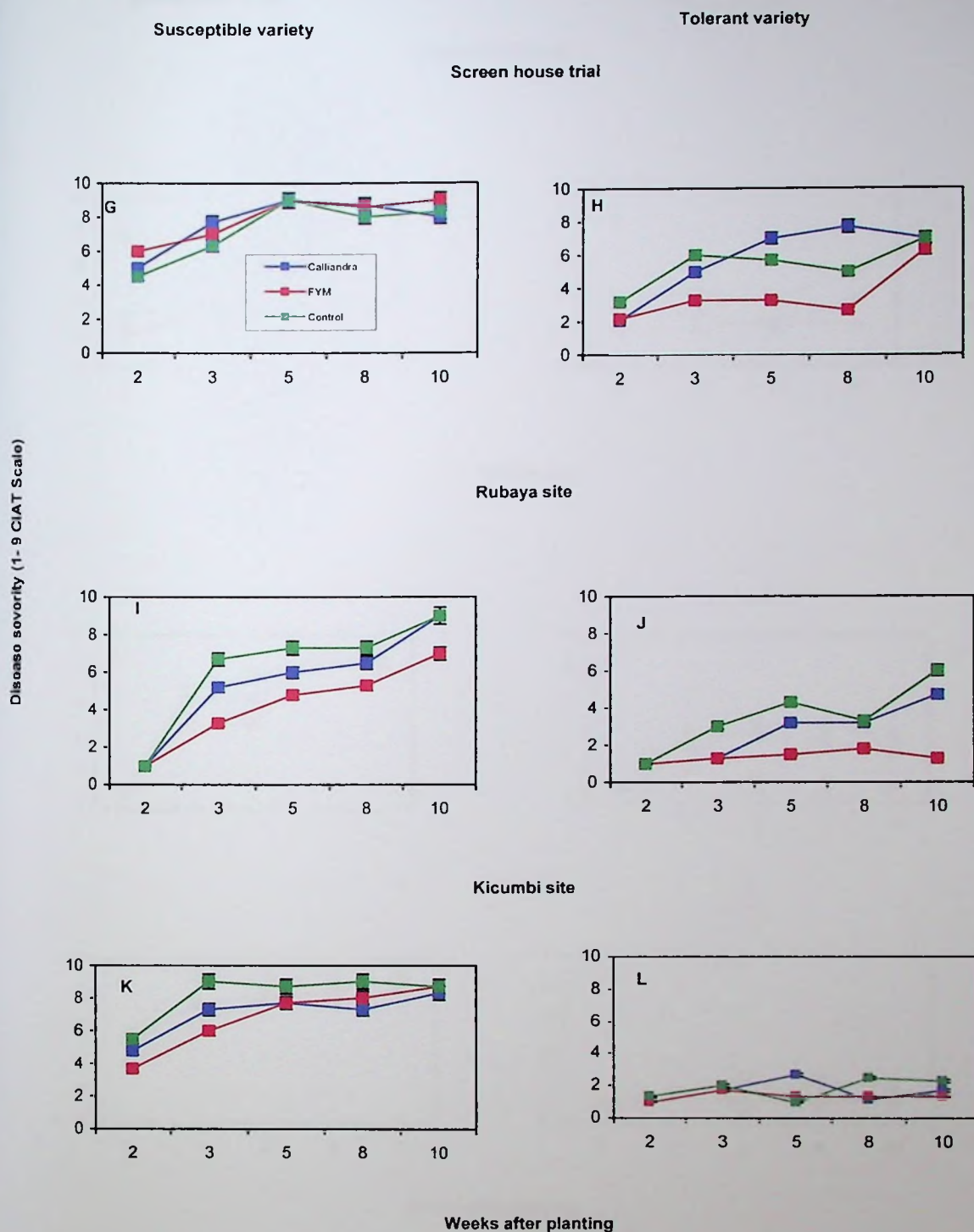


Figure 8: Effect of farm yard manure and *Calliandra* on *Pythium* root rot disease progress in the susceptible bean variety (CAL 96) and tolerant (RWR 719) in the follow up trial of 2002A at Kawanda (screen house), Rubaya and Kicumbi, 2002B

¹ 2002B (September to December)

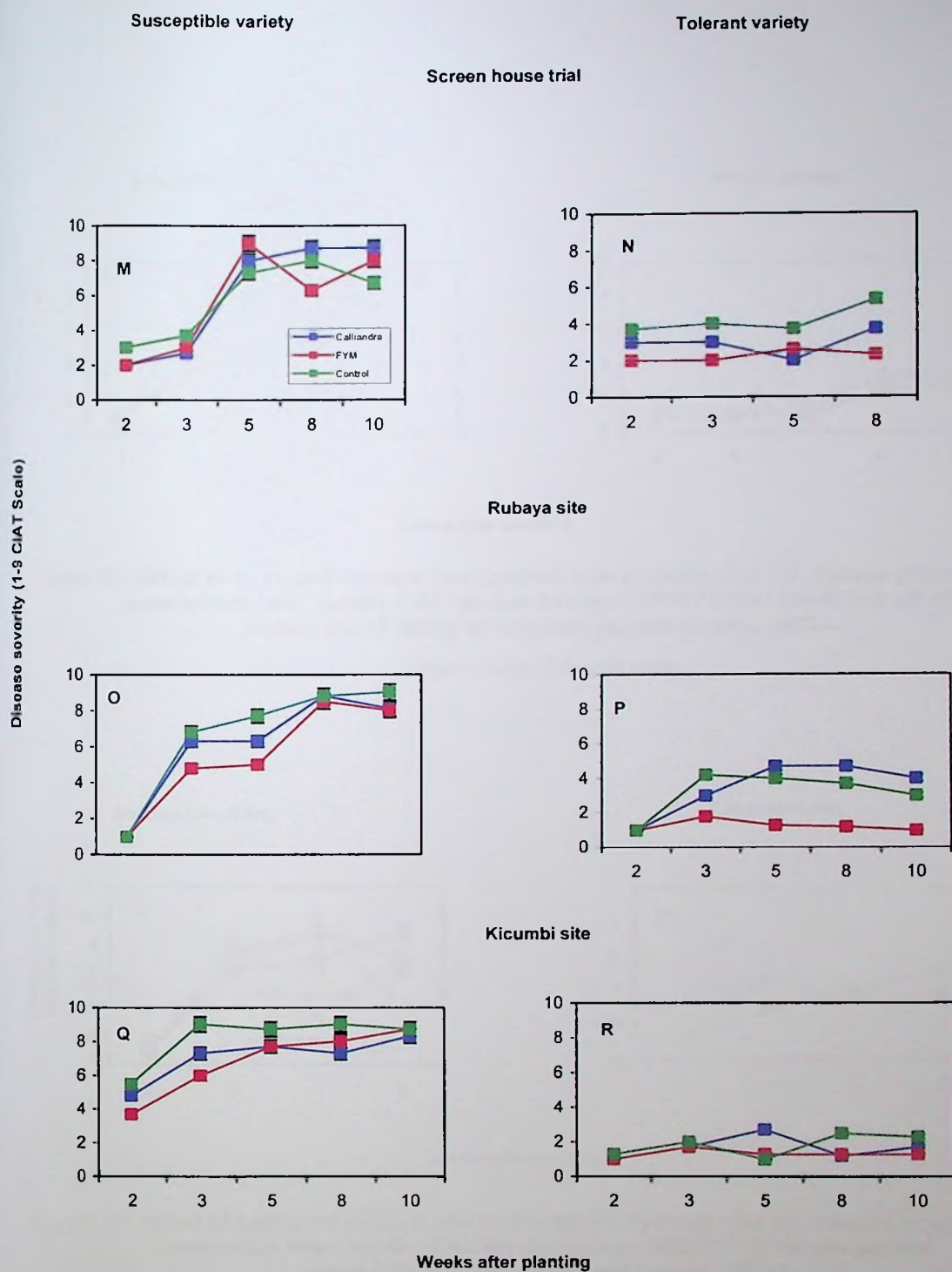


Figure 9: Effect of farm yard manure and *Calliandra* on *Pythium* root rot disease progress in the susceptible bean variety (CAL 96) and tolerant (RWR 719) for the repeat trial at Kawanda (screen house) Rubaya and Kicumbi in 2002B¹

¹ Seasons 2002B (September to December)

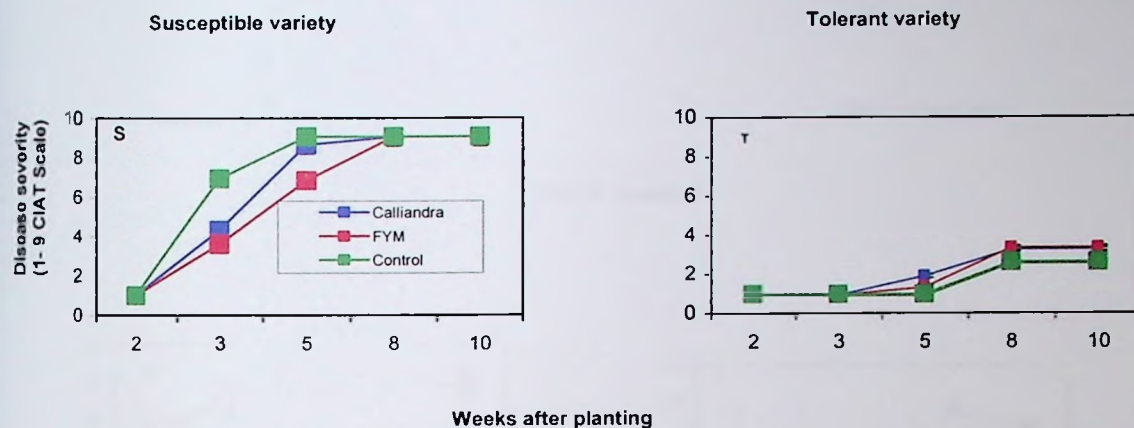


Figure 10: Effect of farm yard manure and *Calliandra* on *Pythium* root rot disease progress in the susceptible bean variety (CAL 96) and tolerant (RWR 719) for the follow up on repeat trial of 2002B at Kawanda (screen house), 2003A.

¹ Seasons 2003A (March to July))

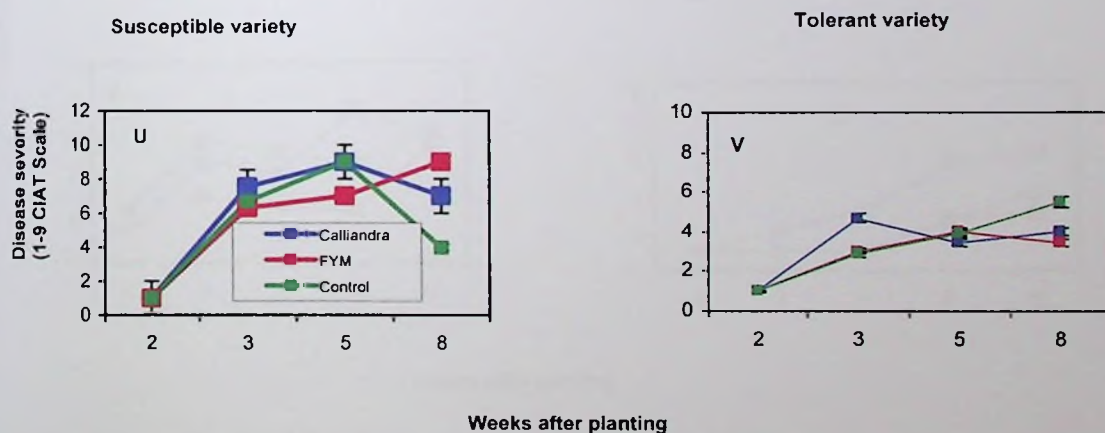


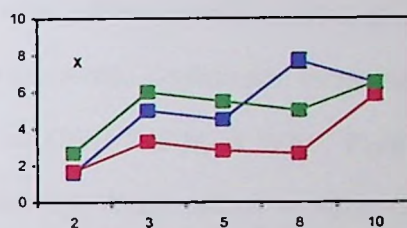
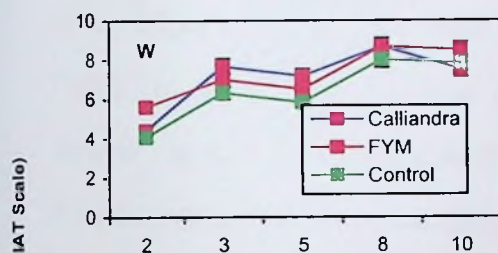
Figure 11: Effect of farm yard manure and *Calliandra* on *Pythium* root rot disease progress in the susceptible bean variety (CAL 96) and tolerant (RWR 719) for the second repeat trial at Kawanda (screen house), 2003A

¹ Seasons 2003A (March to July), 2002B (September to December)

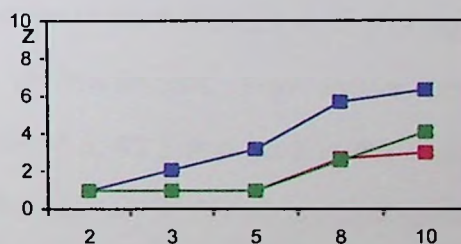
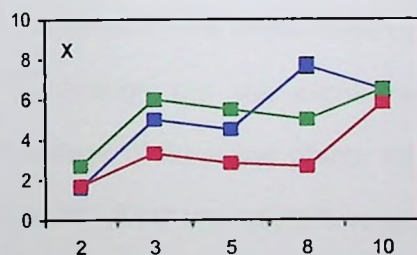
Susceptible variety

Tolerant variety

2002B season



2003A season



Weeks after planting

Figure 12: Effect of further addition of farmyard and *Calliandra* on *Pythium* root rot disease progress in the susceptible bean variety (CAL 96) and tolerant (RWR 719) for the in the follow up trials at Kawanda (screen house)

¹ Seasons 2002B (September to December), 2003A (March to July).

plants (variety CAL 96) was reduced to 32.1, 19.7 and 22.6 for treatment with *Calliandra*, FYM and control, respectively (Table 15), and the same period surviving plants for RWR 719 were reduced to a range of 42 to 49. In repeat trial, percentage survival t 2 WAP was 78.0, 76.8 and 81.0 for treatments with *Calliandra*, FYM and Control, respectively, in variety CAL 96. Eight WAP surviving plants were significantly reduced. For example, there were 21.4, 30.9 and 13.1 % reduction in CAL 96 for treatments with *Calliandra*, FYM and control, respectively. Variety RWR 719 had percentage surviving plants of 17.9, 52.4, and 36.9.

In the follow up trial, percentage survival showed similar trends to first and repeat trials (Table 16), except in the follow up repeat treatments, *Calliandra* increased percentage survival to 32.1 for variety CAL 96 and 48.8 for RWR 719 by 8 WAP. Further addition of amendments did not increase percentage of surviving bean plants in soils infested with *P. ultimum*.

Field trial

At Rubaya site, the percentage of surviving plants in the first trial was highly significant ($P \leq 0.01$) through out the eight weeks in all the treatments. Two weeks after planting, percentage of surviving plants for CAL 96 was 55.6, 62.2 and 65.3 in the treatments with *Calliandra*, FYM and control, respectively. In the case of RWR 719, percentage survival was about 47 (Table 17). Eight weeks after planting percentage survival was drastically reduced to 17.8 in treatment with *Calliandra*, 34.0 for FYM and 19.6 for control. Variety RWR 719 had percentage survival reduced to 28.7, 33.6 and 30.0 for *Calliandra*, FYM and Control, respectively.

At Kicumbi, in the first trial percentage survival 2 WAP was 70.0 to 75.0 for variety CAL 96 and 43 to 50 for RWR 719 (Table 17). Eight weeks later percentage of beans surviving was reduced to 33.1, 47.2 and 32.0 in the treatments of *Calliandra*, FYM and control for variety CAL 96. At this site percentage survival for RWR was 28.8, 27.9 and 31.7 for the respective treatments.

In follow up trials at Rubaya, percentage survival 2 WAP ranged from 70.0 to 77.8 for all treatments. In Variety CAL 96, 8 WAP surviving plants at this site were reduced to 52.5, 63.1 and 43.1% for treatments with *Calliandra*, FYM and control, respectively. Comparatively, variety RWR 719 had surviving plants reduced to 58.6, 65.6 and 61.3% for the respective treatments (Table 18). At Kicumbi, percentage survival was similar at Rubaya. By 8 WAP percentage survival was 46.7, 47.2 and 39.4 in the treatment with *Calliandra*, FYM and control, respectively, for variety CAL 96. On the other hand, variety RWR 719 had percentage of surviving plants averaging 70.0 for all treatments (Plate 4)

In the repeat trial, percentage of surviving bean plants had similar trends in all treatments as in the first trial. But at Kicumbi, the percentage of surviving plants was higher than in the first experiment. For example, in the case of variety CAL 96, surviving plants in the treatment of *Calliandra* was 37.0%, FYM had 54.5% and the control 53.7%. For tolerant variety RWR 719 the percentage of surviving plants was 65.9% for *Calliandra*, 77.9% for FYM, 71.3 for control.

Table 15: Effect of amending soil inoculated with *P. ultimum* on change in percentage survival of a susceptible (CAL 96) and tolerant (RWR 719) bean varieties from a screen house (SH) trial at Kawanda, 2002A and 2002B

Variety/ Soil amendment	First trial 2002A				Repeat 2002B			
	2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
CAL 96								
<i>Calliandra</i>	62.0	42.3	34.5	32.1	78.0 c	62.5	42.3	21.4 c
Farmyard manure	50.5	39.3	24.4	19.7	76.8 c	50.6	39.3	30.9 b
Control	60.1	42.3	31.5	22.6	81.0 bc	60.1	42.3	13.1 d
RWR 719								
<i>Calliandra</i>	85.7	67.3	54.8	48.8	91.1 a	85.7	67.3	17.9 cd
Farmyard manure	80.3	72.0	63.7	49.4	89.9 ab	80.3	72.0	52.4 a
Control	72.0	57.1	47.0	42.3	94.7 a	72.0	57.1	36.9 b

¹Within columns, means with the same letter (s) and no letters do not differ significantly at $P \leq 0.05$

²Seasons 2002A (March to July), 2002B (September to December)

Table 16: Effects of amending soil inoculated with *P. ultimum* on change in percentage survival of bean varieties CAL 96 and RWR 719 in a follow up 2002A first and 2002B repeat trial at Kawanda, 2002B and 2003A

Variety/ Soil amendment	SH Follow up on first trial 2002B				Follow up on SH repeat 2003A			
	2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
CAL 96								
<i>Calliandra</i>	67.3 c	35.7 c	19.1 c	14.3	78.0 c	73.5 c	56.5	32.1
Farmyard manure	66.1 c	20.2 d	20.2 c	14.9	76.8 c	72.4 c	55.9	19.7
Control	70.2 b	38.1 bc	25.6 bc	11.3	81.0 bc	76.5 bc	54.2	22.6
RWR 719								
<i>Calliandra</i>	80.4 a	45.8 a	31.6 ab	22.0	91.1 a	86.6 a	70.2	48.8
Farmyard manure	79.2 a	42.3 ab	35.1 a	21.4	89.9 ab	85.4 ab	57.7	49.4
Control	83.9 a	41.7 ab	29.2 ab	26.2	94.7 a	90.2 a	47.6	42.3

¹Within columns, means with the same letter (s) and no letters do not differ significantly at $P \leq 0.05$

²Seasons 2002A (March to July), 2002B (September to December)

Table 17: Effect of amending soil naturally infested with *Pythium* spp. on change in percentage survival of a susceptible (CAL 96) and tolerant (RWR 719) bean varieties in a field trial at Rubaya and Kicumbi sites in Kabale 2002A

Variety/ Soil amendment	Rubaya				Kicumbi			
	2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
CAL 96								
<i>Calliandra</i>	55.6 c	52.0 ab	31.3	17.8 b	75.5 a	62.0 a	48.1 ab	33.1 b
Farmyard manure	62.2 b	59.1 a	41.1 a	34.0 a	70.8 a	66.7 a	60.3 a	47.2 a
Control	65.3 a	58.0 a	30.0	19.6 b	75.0 a	59.2 ab	45.3 b	32.2 b
RWR 719								
<i>Calliandra</i>	46.2 e	45.1 b	40.7 a	28.7 a	50.4 b	47.9 bc	40.8 b	20.8 c
Farmyard manure	48.7 d	46.7 b	42.0 a	33.6 b	45.4 b	43.8 bc	36.3 b	27.9 bc
Control	48.9 d	47.8 b	41.3 a	30.0 b	43.4 b	41.3 c	35.8 b	31.7 b

¹ Within columns, means with the same letter (s) and no letters do not differ significantly at $P \leq 0.05$

² Seasons 2002A (March to July), 2002B (September to December)

Table 18: Effects of amending soil naturally infested with *Pythium* spp. on change in percentage survival of CAL 96 and RWR 719 bean varieties in a follow up 2002A field trial at Rubaya and Kicumbi sites in Kabale, 2002B

Variety/ Soil amendment	Rubaya				Kicumbi			
	2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
CAL 96								
<i>Calliandra</i>	69.8	69.8	59.1	52.5	83.9	83.9	50.0	46.7
Farmyard manure	72.4	72.4	69.8	63.1	86.7	86.7	72.8	47.2
Control	77.8	77.8	49.8	43.1	84.4	84.4	48.9	39.4
RWR 719								
<i>Calliandra</i>	72.5	72.5	65.3	58.6	75.5	75.5	73.6	72.8
Farmyard manure	72.5	72.5	72.2	65.6	76.1	76.1	76.0	75.3
Control	72.0	72.0	68.0	61.3	78.3	78.3	68.9	68.9

¹ Within columns, means with the same letter (s) and no letters do not differ significantly at $P \leq 0.05$

² Seasons 2002A (March to July), 2002B (September to December)



Plate 4a: Effect of manures on *Pythium* root rots in the field at Kicumbi on a susceptible variety CAL 96. Fore: Farmyard, Middle: *Calliandra*, Back: no amendments



Plate 4b Effect of manures on *Pythium* root rots in the field at Kicumbi on a tolerant variety RWR 719 Fore: *Calliandra*, Middle: Farmyard manure, Back: no amendments

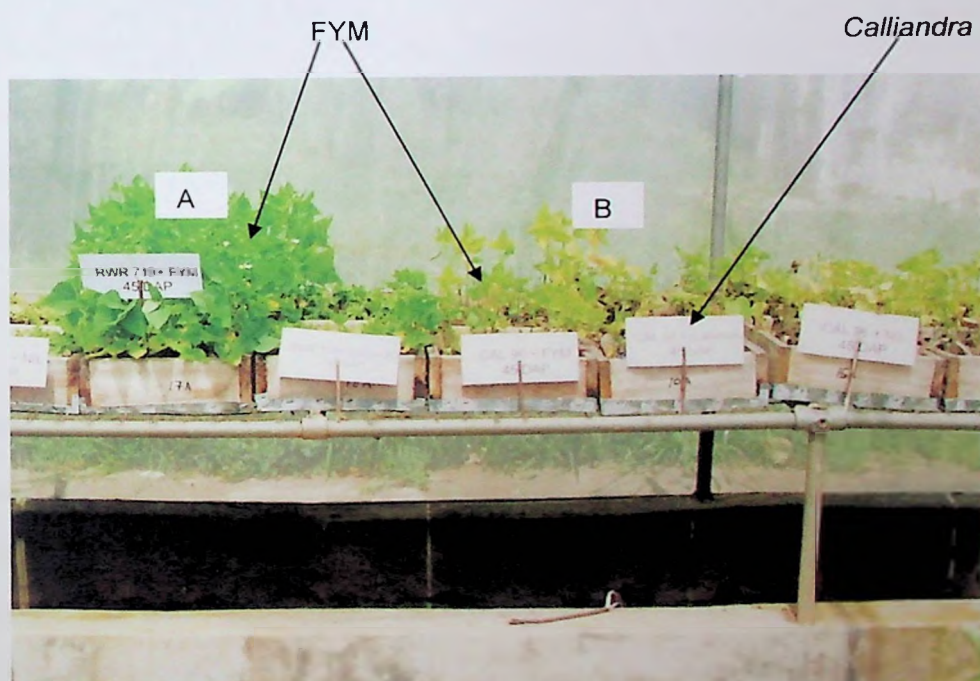


Plate 4c Effect of manures on *Pythium* root rots in the screen house at Kawanda on tolerant variety RWR 719 (A), and susceptible CAL 96 (B)

4.3.4 Effect of amending soil infested with *Pythium* on change in dry matter of beans

Screen house trial

In the first trial beans planted in the treatments with FYM produced the highest dry matter (DM) per plant. Variety CAL 96, for example, produced 1.9 g DM per plant 8 WAP in the treatment with FYM, 1.7 g with *Calliandra* and 0.7 g where nothing was applied (Table 19). RWR 719 produced highest DM per plant of 4.0 g in the same period. Interestingly, in the repeat trial of season 2002B, DM per plant increased to 7.2 and 2.4 g for variety CAL 96 in treatments with FYM and *Calliandra* by 6 WAP but dropped to 4.1 and 1.7 in the same order, compared to the control where DM per plant increased to 6.4 g by 8 WAP.

In variety RWR 719, there was a general increase in DM in the treatments with FYM producing beans with DM per plant of 4.0 g and 3.2 g for the control. In the second repeat, trends in DM change were similar to those in the first trial, except 8 WAP DM was much higher compared in other trials with values ranging from 15.7 to 26.1. In follow up trials, there was no significant increase in DM for all treatments; instead there was a reduction in the DM when compared to a range of 2 to 6.7 g (Table 19)

Field trial

At Rubaya site, DM change was significant ($P \leq 0.05$) through out the eight weeks of sampling, for all the trials. At both sites incorporating FYM produced higher DM per plant with values of 2.2 g at Rubaya and 5.0 g at Kicumbi for variety CAL 96 by 8 WAP. In the case of RWR 719 DM were 7.9 g at Rubaya and 10.5 g at Kicumbi (Table 38). A similar trend in change in DM was displayed at the two sites in the repeat trials (Table 40). In the follow up trial, there was an increment in DM per plant by 3.2 g in variety CAL 96 in

treatments with FYM at 8 WAP. In the case of variety RWR 719 there was no significant change in DM per plant.

4.3.5 Effect of amending soil infected with *Pythium* on bean yields

Generally the tolerant bean variety RWR consistently produced higher yields in all the treatments (Tables 24, 25, 26 and 27), except in season 2002A at Kicumbi where yields of the susceptible variety CAL 96 were above 2000 kg/ha (Table 24). Bean yields from FYM treatments were consistently higher than where *Calliandra* was applied and the control. In the follow up trial, all treatments with FYM and *Calliandra* resulted into significantly higher bean yields compared to the control at both Rubaya and Kicumbi (Table 25). In the repeat trial, bean yields were more in treatments with FYM and *Calliandra* (Table 26). In the screen house yields of >1.5 g/plant were only realized in treatments with FYM and only in repeat trial in the *Calliandra* treatment (Table 27)

Table 19: Effect of amending soil inoculated with *P. ultimum* on change in dry matter per plant in grams of CAL 96 and RWR 719 bean varieties from a screen house trial at Kawanda, 2002A and 2002B

Variety/ Soil amendment	First trial 2002A				Repeat 2002B			
	2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
CAL 96								
<i>Calliandra</i>	0.9	0.6	1.2	1.7 b	1.2 b	2.4 d	2.4 e	1.7 b
Farmyard manure	0.7	0.7	1.4	1.9 b	1.7 a	3.1 c	7.2 a	4.1 c
Control	0.8	1.0	0.7	0.7 b	1.2 b	3.7 a	4.3 c	6.4 b
RWR 719								
<i>Calliandra</i>	0.4	0.7	1.4	1.0 b	0.6 dc	1.6 e	3.1 d	4.0 b
Farmyard manure	0.4	1.7	2.3	4.0 a	0.7 c	3.5 b	6.4 b	8.6 a
Control	0.6	0.8	0.8	1.0 b	0.7 c	1.3 d	3.0 d	3.2 b

¹ Within columns, means with the same letter (s) and no letters do not differ significantly at $P \leq 0.05$

² Seasons 2002A (March to July), 2002B (September to December)

Table 20: Effects of amending soil inoculated with *P. ultimum* on change in dry matter per plant in grams of bean varieties CAL 96 and RWR 719 in a follow up 2002A first and 2002B repeat trials in a screen house at Kawanda, 2002B and 2003A

Variety/ Soil amendment	2002B				2003A			
	2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
CAL 96								
<i>Calliandra</i>	0.5 b	0.7 b	1.7 b	1.2	0.4 a	0.3 b	0.5 c	0.4 b
Farmyard manure	0.7 a	0.5 b	1.0 b	1.7	0.4 a	0.7 a	0.6 c	0.5 b
Control	0.4 b	0.7 b	0.7 b	1.1	0.4 a	0.2 b	0.4 c	0.3 b
RWR 719								
<i>Calliandra</i>	0.5 b	0.5	0.5 b	0.7	0.3 b	0.7 a	1.2 ab	1.8 a
Farmyard manure	0.5 b	4.8 a	4.1 a	4.6	0.3 b	0.6 a	1.7 a	1.9 a
Control	0.5 b	0.6	0.8 b	0.9	0.3 b	0.6 a	1.6 a	2.1 a

¹ Within columns, means with the same letter (s) and no letters do not differ significantly at $P \leq 0.05$

² Seasons 2002A (March to July), 2002B (September to December)

Table 21: Effect of amending soil naturally infested with *Pythium* spp. on change in dry matter per plant in grams of CAL 96 and RWR 719 bean varieties from a field trial at Rubaya and Kicumbi sites in Kabale 2002A

Variety/ Soil amendment	Rubaya				Kicumbi			
	2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
CAL 96								
<i>Calliandra</i>	0.7 c	0.8 c	1.3	1.0 c	0.7 b	0.8 c	1.1 c	2.3
Farmyard manure	1.2 a	1.0 bc	1.6	2.2 bc	1.7 a	2.1 bc	4.2 bc	5.0
Control	0.9 bc	0.6 c	0.3	1.2 c	0.8 b	0.9 c	1.2 c	2.3
RWR 719								
<i>Calliandra</i>	0.8 c	1.0 bc	5.8 a	5.1 ab	1.3 ab	5.0 a	7.7 b	14.8
Farmyard manure	1.1 ab	1.6 a	4.0 a	7.9 a	1.4 ab	4.6 a	14.8 a	10.5
Control	0.7 c	1.0 bc	1.6	2.6 bc	0.9 b	2.8 b	7.6 b	10.0

¹ Within columns, means with the same letter (s) and no letters do not differ significantly at $P \leq 0.05$

² Season 2002A (March to July).

Table 22: Effects of amending soil naturally infested with *Pythium* spp. on change in dry matter per plant in grams CAL 96 and RWR 719 of bean varieties in a follow up 2002A at Rubaya and Kicumbi, 2002B

Variety/ Soil amendment	Rubaya				Kicumbi			
	2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
CAL 96								
<i>Calliandra</i>	0.9 c	0.6 c	0.7 d	4.2 c	0.7 a	0.9 b	0.7 d	0.9 d
Farmyard manure	1.2 b	1.2 b	1.8 b	6.5 b	0.7 a	0.9 b	1.4 c	1.9 c
Control	1.5 a	0.4 d	1.3 c	2.3 d	0.5 bc	0.4 c	0.5 d	2.1 c
RWR 719								
<i>Calliandra</i>	0.4 de	0.5 cd	2.0 b	4.5 c	0.2 d	0.2 d	1.3 c	5.3 a
Farmyard manure	0.3 e	2.8 a	4.6 a	8.2 a	0.4 c	1.3 a	2.7 a	4.4 b
Control	0.7 cd	0.5 a	1.2 c	2.5 d	0.4 c	0.6 c	2.5 b	1.9 c

¹ Within columns, means with the same letter (s) and no letters do not differ significantly at $P \leq 0.05$

² Season 2002B (September to December)

Table 23: Effect of amending soil naturally infested with *Pythium* spp on change in dry matter per plant in grams of CAL 96 and RWR 719 bean varieties in a repeat field trial at Rubaya and Kicumbi sites in Kabale, 2002B

Variety/ Soil amendment	Rubaya				Kicumbi			
	2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
CAL 96								
Caillandra	1.3 bc	1.0	1.0	1.2 bc	0.8 b	0.6	0.7	2.6
Farmyard manure	2.4 a	3.6 b	6.3 b	2.6 a	1.1 a	1.0	1.4	2.2
Control	1.3 cd	0.9	1.0	0.6 d	0.5 c	0.5	1.6	1.2
RWR 719								
Caillandra	0.7 cd	1.4 c	2.5	0.7 c	0.3 d	0.7	8.6 b	7.4 b
Farmyard manure	0.6 d	5.6 a	13.1 a	1.4 b	0.5 c	0.4	11.1 a	15.7 a
Control	2.0 ab	1.7 c	1.3	1.1 bcd	0.4 cd	1.4	4.3	6.5 b

¹ Within columns, means with the same letter (s) and no letters do not differ significantly at $P \leq 0.05$

² Season 2002B (September to December)

Table 24: Bean yields (kg/ha) from a trial at Rubaya and Kicumbi in Kabale on evaluating effects of organic amendments on *Pythium* populations, 2002A season.

Variety	Soil amendment	Rubaya	Kicumbi
CAL 96	Green manure	170.0 bc	220.0
	Farmyard manure	237.0 bc	2884.0
	Control	86.0 c	303.0
RWR 719	Green manure	342.0 ab	1526.0
	Farmyard manure	516.0 a	1248.0
	Control	289.0 b	1050.0

¹ Within columns, means with the same letter (s) and no letters do not differ significantly at $P \leq 0.05$

² Seasons 2002A (March to July),)

Table 25: Bean yields (kg/ha) from a follow up trial 2002A season at Rubaya and Kicumbi in Kabale on evaluating effects of organic amendments on *Pythium* populations, 2002B season.

Variety	Soil amendment	Rubaya	Kicumbi
CAL 96	Green manure	577.0 ab	334.0
	Farmyard manure	567.0 ab	994.0
	Control	270.0 bc	506.0
RWR 719	Green manure	484.0 ac	977.0
	Farmyard manure	730.0 a	2444.0
	Control	198.0 c	1141.0

¹ Within columns, means with the same letter (s) and no letters do not differ significantly at $P \leq 0.05$

² Seasons 2002A (March to July), 2002B (September to December)

Table 26: Bean yields (kg/ha) from a repeat trial at Rubaya and Kicumbi in Kabale on evaluating effect of organic amendments on *Pythium* populations, 2002B season.

Variety	Soil amendment	Rubaya	Kicumbi
CAL 96	Green manure	360.0	809.0 b
	Farmyard manure	439.0	722.0 b
	Control	154.0	326.0 b
RWR 719	Green manure	278.0	1923.0 a
	Farmyard manure	538.0	2020.0 a
	Control	144.0	1729.0 a

¹ Within columns, means with the same letter (s) and no letters do not differ significantly at $P \leq 0.05$

² Seasons 2002B (September to December)

Table 27: Bean yields (g/plant) from a screen house evaluations on effect addition of FYM and *Calliandra* on *Pythium* population at Kawanda during 2002A for the first trial and 2002B for follow up and repeat

Variety	Soil amendment	First trial	Follow up	Repeat
CAL 96	Green manure	0.9 bc	0.1 b	23.7 a
	Farmyard manure	1.5 ab	2.7 a	37.5 ab
	Control	0.4 c	0.4 b	0.7 b
RWR 719	Green manure	1.0 bc	0.4 b	11.0 ab
	Farmyard manure	2.1 a	2.2 a	1.1 b
	Control	0.8 bc	2.3 a	1.3 b

¹ Within columns, means with the same letter (s) and no letters do not differ significantly at $P \leq 0.05$

² Seasons 2002A (March to July), 2002B (September to December)

4.4 Discussion

4.4.1 Effect of farmyard manure and *Calliandra* on soil *Pythium* population and change in frequencies of *Pythium* species

The most striking and unexpected aspect of this study was the increase in soil *Pythium* population when farmyard manure and *Calliandra* applied to reduce population to below damaging levels. Nevertheless, use of *Calliandra* produced lower soil populations of *Pythium* than farmyard manure, but all were higher than where nothing was applied. The use of organic matter containing high nitrogen have been known to significantly reduce populations of a wide range of soil-borne pathogens (Lazarovits, 2001), but this was not the case with *Pythium* populations as demonstrated by the findings of this study. Furthermore, some organic amendments, such as barley (*Hordeum jubatum*) increase bean root rot disease (Hall, 1996), and use of green manure from *Sesbania* exacerbated damping-off diseases caused by *Pythium* and *Rhizoctonia* (Campbell, 1994).

Studies by Burr and Stanghellini (1973) indicate that *P. aphanidermatum* produces oospores survival structures in naturally infested soil. Enzymatic degradation of colonized organic matter results in the liberation of dormant oospores which have an exogenous nutrient requirement for germination. Hedrix and Campbell (1973) point out that the dormant oospores take 10 to 15 days to produce infectious germ tube or zoospores, resulting into an under estimate of the initial *Pythium* population in soil. The prevalent *Pythium* species identified in Uganda namely *P. ultimum*, *P. salpingophorum*, *P. torulosum* and *P. spinosum* have got the ability to produce oospores under field conditions (Plaats-Niterink, 1981). It is possible that farmyard manure and *Calliandra* stimulated the dormant oospores of the common *Pythium* spp, hence resulting into increased *Pythium* populations in soil as demonstrated by the results of this study.

Interestingly, in follow up trials populations of *Pythium* in soil reduced by 50% compared the original soil samples. In a related work on damping-off diseases, involving *Medicago* as a green manure, the time between the incorporation of green manures and the sowing of the crop was found to be important in that the initial increased level of disease was reduced as the decomposition proceeded and antagonist populations increased (Campbell, 1994). The result from the follow up trial in this study demonstrates the possible mechanism of population reduction in soil. Since *Pythium* is known to be weak competitor for available I nutrients compared to other possible antagonistic organisms, it's likely that populations of these antagonistic populations get pronounced and deprive *Pythium* nutrients to increase in populations. Therefore, the strategy should be to delay planting after incorporation of amendments as a measure to manage *Pythium* populations in field soils.

The use of *Calliandra* increased the frequency of *P. salpingophorum* but reduced that of *P. ultimum*. Interestingly, farmyard manure instead increased frequencies of *P. ultimum* but

also reduced those of *P. salpingophorum*. The possible mechanism by which the frequencies of particular *Pythium* species are reduced but not others needs to be further explored. Nevertheless, the different organic amendments may be used to target particular *Pythium* species. There is a need to study a wide range of green manures and also compost on how they affect the abundance of the common specific *Pythium* species.

4.4.2 Effect of farmyard manure and *Calliandra* on beans grown in a *Pythium* infested soil

Generally, farmyard manure reduced *Pythium* root rot disease levels more than *Calliandra* in the field trials but not in the screen house. It is likely that farmyard has got the ability to stimulate germination of oospores of *P. ultimum* and cause disease. It's likely that under field conditions there may be interactions with antagonist populations displaying this difference in disease level expression. Disease levels were also observed in plots amended with green manures than was the case with farmyard manure (Opio, 1998). The result from this study suggests the likely benefit drawn from using farmyard manure in the management of *Pythium* root rot disease.

Interestingly, *Pythium* root rot levels were lower in a tolerant bean variety (RWR 719) in all the cases. The low levels of *Pythium* root rot on variety RWR 719 from both screen house and field demonstrated the potential impact tolerant bean varieties can have on *Pythium* pathogen populations in field soils. At Rubaya, disease levels in the susceptible variety (CAL 96) were equally higher like at Kicumbi (scores ranging 6.0-8.0), but for a tolerant variety (RWR 719) disease levels were much lower at Kicumbi (score 3.0). This result suggests differences in *Pythium* species and pathogen populations could be existing at the

two localities leading to differences in *Pythium* root rot expression on beans. Differences in reaction of bean varieties were found to show good levels of tolerance to root rots in Rwanda but not in Kenya (Otsyula and Ajanga, 1995). The implication of the finding is that breeders should target screening bean varieties over a wide range of *Pythium* species in both controlled conditions and different field spots where the root rot disease has been very rampant.

There was a general reduction in percentage survival for farmyard and *Calliandra* for screen house and field trial. But, treatments with farmyard manure had a higher a higher percentage of surviving plants, dry mater accumulation and bean yield followed by *Calliandra* and Control. The parameters were more pronounced in a tolerant variety RWR 719 that the susceptible CAL 96. The implication of the findings is that, there are benefits by farmers to be gained by growing *Pythium* root rot resistant bean varieties such as RWR 719 in soils amended with organic matter.

In general, soil amendment studies indicated that farmyard manure and green manure can give large increases in bean crop yield. Farmyard manure and *Calliandra* increased both dry matter and bean yield but also increased *Pythium* soil inoculum levels. Farmyard manure in this case may be able to compensate for the negative impact of increased disease by causing a larger increase in soil fertility, thereby increasing yield, but still stores up potential problems for the future in the form of increased pathogen numbers. However, the effect of amendments in the proceeding seasons reduced *Pythium* soil inoculum; this may be a preferred option as part of a series of management practices to reduce the impact of *Pythium* root rot in a long run.

CHAPTER FIVE

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Experiments were conducted to identify and differentiate *Pythium* species associated with bean root rots using DNA markers, and determine their distribution in Uganda. This study was also designed to develop procedures of detecting and quantifying *Pythium* species pathogenic to beans and determine the effect of organic amendments (farmyard and *Calliandra*) on *Pythium* pathogen populations in field soils.

The identification studies revealed that 11 *Pythium* species are associated with bean root rots in Uganda. They include: *P. ultimum* var *ultimum*, *P. spinosum*, *P. torulosum*, *P. salpingophorum*, *P. vexans*, *P. dissotocum*, *P. nodosum*, *P. echinulatum*, *P. pachycaule*, *oligandrum* and *P. deliense*. The study also indicated that the most widely distributed *Pythium* species were *P. ultimum* var *ultimum*, traditionally known to cause *Pythium* root rots on beans (Abawi and Pastor Corrales, 1990), *P. salpingophorum*, *P. torulosum*, *P. vexans* and *P. spinosum*. *P. ultimum* var *ultimum*, caused severe root rot on susceptible bean varieties CAL 96 and K20 with disease ratings as high as 8 (1-9 CIAT scale). The high frequency of occurrence of *P. ultimum* var *ultimum* in samples from different places, and the severe root rot it caused on the two bean varieties suggest that it is one of the species responsible for the increased root rots epidemics in southwestern Uganda. The record of occurrence of *P. spinosum*, *P. nodosum*, *P. pachycaule*, *P. torulosum* and *P. salpingophorum* on various samples, and the resultant disease in the two test cultivars, indicate that these have a potential to cause bean root rot disease. Alternatively, they may be part of the pathogen complex causing root rots in the Great Lakes region. This, however, is the first record of their occurrence in Uganda. The findings strongly support the

hypotheses that different *Pythium* species widely distributed exist in Uganda and have contributed to the increased epidemics of bean root rots, particularly in southwestern.

The findings show that *Pythium* species pathogenic to beans recorded high levels of disease on susceptible bean varieties CAL 96 and K20 (8 to 9 score on CIAT 1-9 scale) compared to the highly tolerant variety RWR 719 (2 to 5 score). In Kenya and Rwanda, similar disease levels were observed on susceptible varieties GLP 585 and Urugezi, and on tolerant MBL49-89A, SCAM 80-CM/15, AND1055 and AND1062 (Otsyula *et al.*, 1998; Buruchara and Kimani, 1999). This demonstrated the potential for host resistance in the management of different *Pythium* species causing bean root rots.

PCR/RFLP and ITS1 sequences analysis proved useful diagnostic tool for *Pythium* species. Some PCR species-specific primers with good level of specificity to detect and differentiate *Pythium* species pathogenic to beans were developed. Using these primers it was possible to detect the five important *Pythium* species, namely, *P. salpingophorum*, *P. torulosum*, *P. spinosum*, *P. nodosum* and *P. ultimum*. The limitation of the primers was their failure to differentiate some particular *Pythium* species with variation in pathogenicity as was the case with *P. torulosum* and *P. salpingophorum*. There is a need, therefore, to further analyse a bigger part of the genome using AFLP markers to locate genes responsible for the variation and later design primers specific to the pathogenic forms of *P. torulosum* and *P. salpingophorum*.

This study also attempted to develop procedures of detecting and quantifying *Pythium* species pathogenic to beans in field soils and to evaluate the effect of organic amendments on pathogen populations. The most practical quantification method adopted in this study was use of dilution plating with a selective media for *Pythium* combined with PCR/RFLP

molecular detection. The results show that farmyard manure and *Calliandra* increased soil *Pythium* population, which was not expected. But, in follow up trials populations of *Pythium* in soil was reduced by 50% a population, which is still high and can cause severe root rots on beans. A related, using *Medicago* as a green manure suggested that the time between the incorporation of green manures and the sowing of the crop is important the initial increased level of disease are reduced as the decomposition proceeds and antagonist populations increase (Campbell, 1994). The result from the follow up trial demonstrates is possible mechanism of population reduction in soil. Thus, it may be necessary to delay (after incorporation of organic amendments) planting of beans as a strategy to manage *Pythium* populations in field soils in a long run. The findings partially do not support the hypotheses that organic amendments reduce *Pythium* populations in field soils. The reduction however may occur be after subsequent seasons.

Interestingly, the use of *Calliandra* increased the frequency of *P. salpingophorum* but reduced that of *P. ultimum*. On the other hand farmyard manure increased frequencies of *P. ultimum* but reduced those of *P. salpingophorum*. The mechanisms by which the frequencies of particular *Pythium* species are reduced but not others, is not well understood, so needs to be investigated. Nevertheless, the different organic amendments could be used to target specific *Pythium* species, when the mechanism responsible for the changes in species population is well understood. Additionally, there is a need to examine the impact of a wide range of green manures and compost on the abundance of the common and important *Pythium* species pathogenic to beans. This will generate information useful in designing a package to manage populations of *Pythium* pathogenic to beans in field soil.

In general, soil amendment studies indicated that farmyard manure and *Calliandra* increased both dry matter and bean yield but also increased *Pythium* soil inoculum levels.

Farmyard manure and *Calliandra* in this case may be able to compensate for the negative impact of increased disease by causing a larger increase in soil fertility, thereby increasing yield, but still stores up potential problems for the future in the form of increased pathogen numbers. However, the effect of farmyard manure and *Calliandra* in the follow up season reduced *Pythium* soil inoculum, though not to below damaging levels. Thus organic amendments could be used option as part of a series of management practices to reduce the impact of *Pythium* root rot on beans in the long run.

Basing on the results and the observations made during the study the following conclusions can be drawn;

1. Molecular analyses using RFLPs of the rDNA and ITS 1 was an effective diagnostic tool for *Pythium* which could be used to monitor bean root rot pathogens most especially *Pythium*.
2. Apart from *P. ultimum* var *ultimum* known to cause disease on beans, *P. spinosum*, *P. nondosum*, *P. pachycaule*, *P. torulosum* and *P. salpingophorum* were identified and also found to cause root rots on beans; this is a new discovery in Uganda.
3. The dilution plating method combined with PCR/RFLP molecular detection technique procedure may be effective in monitoring the dynamics of *Pythium* species in soil.
4. Delayed planting after addition of organic amendments may reduce bean root rot pathogens, particularly *Pythium*.
5. Farmyard manure and *Calliandra* increased dry matter and bean yield despite increases in *Pythium* soil populations. The amendments probably compensate for the negative impact of increased disease by causing a larger increase in soil fertility, thereby increasing yield, but still stores up potential problems for the future in the form of increased pathogen numbers.

Basing on the results of this study the areas are recommended to further our understanding of *Pythium* species pathogen association with bean root rot disease:

1. Molecular analysis of a bigger part of the *Pythium* genome using AFLP markers to locate genes responsible for pathogenicity variation and design primers specific to the pathogenic forms, especially for *P. torulosum* and *P. salpingophorum*.
2. Understanding the impact of farming system on the epidemics of *Pythium* root rots. The role played by possible alternating hosts (weeds, crops like cabbage, sorghum, potatoes and peas) need to be examined.
3. Further examination of the impact of a wide range of green manures and compost on the abundance of the common *Pythium* species pathogenic to beans. The mechanism involved in changes in *Pythium* species and populations in soil caused by the different soil amendments should be examined.

This study has therefore, a) characterized the *Pythium* species responsible for bean root rots in southwestern Uganda, b) developed *Pythium* specific primers for detecting five *Pythium* species in both plants and soil c) developed a procedure for quantifying *Pythium* inoculum levels in the soil, and d) demonstrated the beneficial effect of organic amendments for reducing the impact *Pythium* levels in the soil. This knowledge will be useful for future development of bean varieties in the Great Lakes region of eastern Africa.

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APPENCICES

Appendix 1: Mean squares of colony forming units per gram of soil of *P. ultimum* under different amendments (farmyard manure, *Calliandra* and control) for the screen house (SH) experiment at Kawanda during 2002A, 2002B and 2003A

Source	df	start	Weeks after planting							
			2	4	6	8	10	12		
First SH trial 2002A										
Replication	2	6731.0	3682.0	10479.0	130618.0	2797.0	12000.0	169911.0		
Treatment	5	3068.0 ns	11839.0 ns	52622.0 ns	194416.0*	104120.0**	83809.0 ns	59585.0 ns		
Residual	10	5926.0	17276.0	21695.0	36436.0	8694.0	37793.0	62610.0		
Follow up on first SH trial 2002B										
Replication	2	5747.0	1133.0	5381.0	4676.0	14090.0	28110.0	30659.0		
Treatment	5	87862.0**	39155.0**	138778.0**	114001.0**	40345.0**	93406.0*	17446.0*		
Residual	10	7609.0	6656.0	8525.0	2535.0	3979.0	25391	3384.0		
Repeat of SH trial 2002B										
Replication	2	10479.0	7081.0	62521.0	33632.0	10692.0	637.0	21312.0		
Treatment	5	52622.0 ns	45995.0 ns	35799.0 ns	34440.0 ns	449575.0	30418.0 ns	40345.0**		
Residual	10	21695.0	23734.0	24711.0	19953.0	34057.0	22898.0	5764.0		
Follow up on repeat SH trial 2003A										
Replication	2	4319.0	19755.0	7293.0	2761.0	3045.0	6443.0	5169.0		
Treatment	5	36266.0**	141171.0**	61926.0*	101237.0*	9077.0*	18254.0 ns	17956.0 ns		
Residual	10	4149.0	10663.0	140902	22813.0	2535.0	8058.0	9162.0		
Second repeat SH trial 2003A										
Replication	2	7930.0	5594.0	17276.0	10196.0	5735.0	1983.0	8509.0		
Treatment	5	6911.0 ns	65834.0**	49181.0*	51319.0**	3526.0 ns	37498.0**	956446.0		
Residual	10	18381.0	8652.0	12688.0	3399.0	1487.0	5891.0	7539.0		
Further addition of amendment SH trial 2002B										
Replication	2	5747.0	6443.0	5169.0	4036.0	1487.0	283.0	2688.0		
Treatment	5	87862.0**	21142.0 ns	352447.0**	127449.0**	26297.0*	44423.0*	18933.0 ns		
Residual	10	7609.0	7378.0	4574.0	5310.0	5225.0	8185.0	5696.0		
Further addition of amendment SH trial 2003A										
Replication	2	4319.0	2620.0	7859.0	9417.0	20038.0	637.0	850.0		
Treatment	5	36266.0**	75393.0*	75917.0**	14897.0 ns	165967.0**	4588.0 ns	170102.0**		
Residual	10	4149.0	9927.0	4376.0	4829.0	10012.0	3696.0	6287.0		

¹ Treatment= farmyard manure, *Calliandra* and control; ns, * and ** denotes not significant, significant at P=0.05 and highly significant at P=0.01
² seasons when trials were conducted 2002A (March to July), 2002B (September to December), 2003A (March to July)

Appendix 2: Mean squares of colony forming units per gram of soil of *P. ultimum* under different organic amendments (FYM , *Calliandra* and control) for the field trials at Rubaya and Kicumbi sites in Kabale during 2002A and 2002B seasons

Weeks after planting (WAP)									
Source	df	start	2	4	6	8	10	12	
Rubaya first SH trial 2002A									
Replication	2	955.0	8125.0	3012.0	2200.0	2219.0	1488.0	1803.6	
¹ Treatment	5	649.0 ns	1498.0 ns	6667.0 ns	462.0 ns	3816.0 ns	2100.0 ns	309.0 ns	
Residual	10	1903	2871.0	3769.0	1001.0	1775.0	3374.0	617.4	
Follow up on Rubaya first trial 2002B									
Replication	2	462.0	20.0	139.0	487.0	4387.0	1923.0	4302.0	
¹ Treatment	5	11400.0**	3660.3*	3804.0 ns	2092.0 ns	8489.3**	4821.0 ns	1507.0	
Residual	10	1549.0	805.3	2587.0	2336.0	770.6	3582.0	2042.0	
Repeat trial at Rubaya 2002B									
Replication	2	223.8	938.0	347.0	4421.0	3436.0	535.0	2432.0	
¹ Treatment	5	358.0 ns	1654.0 ns	19900.0**	13268.0*	2376.0 ns	5397.0 ns	2601.0 ns	
Residual	10	696.0	1312.0	2975.0.0	3174.0	1732.0	1868.0	1050.0	
Kicumbi first trial 2002A									
Replication	2	1803.0	1606.0	774.0	942.6	598.4	1130.0	444.6	
¹ Treatment	5	3001.0 ns	1480.0 ns	1420.0 ns	2447.7 ns	778.0 ns	529.0 ns	4142.3*	
Residual	10	2134.0	4539.0	2142.0	913.2	658.7	1123.0	879.0	
Follow up on Kicumbi first trial 2002B									
Replication	2	3750.0	4710.0	5005.0	5647.0	4443.0	801.0	3436.0	
¹ Treatment	5	785.0 ns	8437.0**	7633.0 ns	11723.0*	1854.0 ns	3790.0 ns	1730.0 ns	
Residual	10	2968.0	1118.0	2941.0	2049.0	4488.0	1329.0	1682.0	
Repeat trial at Kicumbi 2002B									
Replication	2	5133.0	1209.0	2757.0	486.0	4015.0	11.4	1371.0	
¹ Treatment	5	5342.0*	1534.0 ns	3390.4 *	2838.0 ns	1125.0 ns	1051.7 ns	1233.0 ns	
Residual	10	1185.0	2063.0	774.2	1690.0	2549.0	772.6	1316.0	

¹Treatment= farmyard manure, *Calliandra* and control; ns, * and ** denotes not significant, significant at P<0.05 and highly significant at P<0.01
² seasons when trials were conducted 2002A (March to July), 2002B (September to December)

Appendix 3: Mean squares of root rot disease score (1 – 9 CIAT scale) on beans planted in soils inoculated with of *P. ultimum* under different organic amendments (farmyard manure, *Calliandra* and control) for the screen house (SH) experiment at Kawanda during 2002A, 2002B and 2003A

Weeks after planting						
Source	df	2	4	6	8	10
First SH trial 2002A						
Replication	2	0.5	2.7	2.4	0.6	-
Treatment	5	7.7 ns	1.6 ns	7.4*	6.1*	
Residual	10	2.5	0.9	1.4	1.7	
Follow up on first SH trial 2002B						
Replication	2	0.5	2.7	0.2	0.1	0.4
Treatment	5	7.7 ns	7.2*	16.2**	17.7**	2.9 ns
Residual	10	2.5	2.1	0.3	0.7	1.2
Repeat of SH trial 2002B						
Replication	2	1.5	0.1	0.1	2.7	-
Treatment	5	1.2*	25.6**	24.1**	18.4**	
Residual	10	0.3	0.1	1.1	1.0	
Follow up on repeat SH trial 2003A						
Replication	2	0.0	0.4	0.1	0.03	0.2
Treatment	5	0.0	17.6**	42.4**	32.4**	15.8**
Residual	10	0.0	2.0	0.4	0.5	0.6
Second repeat SH trial 2003A						
Replication	2	0.0	7.5	0.7	0.8	-
Treatment	5	0.0	11.7*	20.4**	16.0**	
Residual	10	0.0	2.1	1.2	1.4	
Further addition of amendment SH trial 2002B						
Replication	2	0.5	2.7	2.7	0.1	0.4
Treatment	5	7.7 ns	7.2*	7.2*	17.7**	3.0 ns
Residual	10	2.5	2.1	2.1	0.7	1.3
Further addition of amendment SH trial 2003A						
Replication	2	0.0	1.8	0.02	1.4	1.2
Treatment	5	0.0	16.4**	47.6**	26.2**	18.2**
Residual	10	0.0	2.3	0.14	1.1	0.5

Treatment= farmyard manure, *Calliandra* and control; ns, * and ** denotes not significant, significant at $P \leq 0.05$ and highly significant at $P \leq 0.01$

² seasons when trials were conducted 2002A (March to July), 2002B (September to December), 2003A (March to July)

Appendix 4: Mean squares of root rot disease score (1 – 9 CIAT scale) on beans planted in soils naturally infected with *Pythium* spp. under different organic amendments (farmyard manure, *Calliandra* and control) for the field trials at Rubaya and Kicumbi sites in Kabale during 2002A and 2002B seasons

		Weeks after planting				
Source	df	2	4	6	8	10
Rubaya first trial 2002A						
Replication	2	5.5	4.5	0.3	0.7	-
¹ Treatment	5	11.4*	14.6**	33.4**	45.1**	
Residual	10	2.9	1.5	1.2	0.6	
Follow up on Rubaya first trial 2002B						
Replication	2	0.0	1.5	0.2	0.1	1.2
¹ Treatment	5	0.0	13.5**	12.7**	13.7**	25.4**
Residual	10	0.0	0.1	0.6	0.2	0.9
Repeat trial at Rubaya 2002B						
Replication	2	0.0	0.4	0.0	0.1	0.2
¹ Treatment	5	0.0	11.0**	14.0**	31.7**	17.9**
Residual	10	0.0	0.9	0.7	0.1	0.4
Kicumbi first trial 2002A						
Replication	2	0.5	0.3	0.7	0.5	
¹ Treatment	5	36.2	18.9**	32.6**	34.3**	
Residual	10	1.2	0.7	1.3	1.7	
Follow up on Kicumbi first trial 2002B						
Replication	2	0.4	0.5	0.1	0.1	0.1
¹ Treatment	5	3.6*	34.0**	48.9**	51.5**	44.0**
Residual	10	0.5	0.5	0.1	0.1	0.1
Repeat trial at Kicumbi 2002B						
Replication	2	0.4	0.7	0.5	0.2	1.2
¹ Treatment	5	12.5**	31.7**	37.4**	38.9**	41.7**
Residual	10	0.3	0.3	1.0	0.3	0.2

¹Treatment= farmyard manure, *Calliandra* and control; ns, * and ** denotes not significant, significant at $P \leq 0.05$ and highly significant at $P \leq 0.01$

² seasons when trials were conducted 2002A (March to July), 2002B (September to December)

Appendix 5: Mean squares of percentage survival of a susceptible (CAL 96) and tolerant (RWR 719) bean varieties grown in soil inoculated with *P. ultimum* in a screen house trial at Kawanda

Variety/ Soil amendment	df	First trial 2002A				Repeat 2002B			
		2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
Replication	2	203.0	136.1	266.7	351.9	68.8	203.0	136.1	13.2
Treatment	5	525.3 ns	600.1 ns	678.3 ns	506.0	172.2**	525.3 ns	600.1 ns	628.5**
Residual	10	273.7	217.6	253.2	371.8	28.4	273.7	217.6	11.0
³ SH follow up on first trial 2002B									
Replication	2	68.3	50.1	131.1	37.2	68.8	68.9	69.2	351.9
Treatment	5	172.3**	246.8**	121.3**	97.1*	172.2**	171.25**	164.0 ns	506.0 ns
Residual	10	28.3	7.6	19.1	26.6	28.4	28.3	188.3	371.8
Further amendment addition 2002B									
Replication	2	68.7	124.2	164.0	165.9	68.8	248.8	160.9	130.9
Treatment	5	171.3**	373.9**	196.8*	208.5	172.2**	230.2 ns	888.6**	231.3 ns
Residual	10	28.4	23.3	35.3	24.4	28.4	121.7	135.6	102.5
Further amendments addition 2003A									

*Treatment= farmyard manure, *Calliandra* and control; ns, * and ** denotes not significant, significant at P≤0.05 and highly significant at P≤0.01

²Percentage survival for second repeat of 2003A had similar trends to repeat trial of 2002B

³seasons when trials were conducted 2002A (March to July), 2002B (September to December), 2003A (March to July)

⁴SH= Screen house

Appendix 6: Mean squares of percentage survival of CAL 96 and RWR 719 bean varieties grown in amended soil artificially infested with *Pythium* spp in a field trial at Rubaya and Kicumbi sites in Kabale during 2002A and 2002B seasons

Variety/ Soil amendment	df	Rubaya 2002A				Kicumbi 2002A			
		2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
Replication	2	6.2	12.4	51.6	37.7	236.6	204.1	348.9	120.2
¹ Treatment	5	187.2**	107.0**	91.3**	146.4**	646.8**	333.9*	250.6*	224.7**
Residual	10	8.9	15.7	12.6	10.39	64.5	87.3	63.2	28.7
Follow up on 1st trial at Rubaya 2002B									
Replication	2	5.2	5.2	73.5	74.3	283.7	283.7	203.3	26.4
¹ Treatment	5	20.9 ns	20.9 ns	206.9 ns	207.0 ns	67.9 ns	67.9 ns	462.8 ns	734.0 ns
Residual	10	25.5	25.5	88.6	88.7	85.0	84.9	219	243.1
Repeat trial at Rubaya 2002B									
Replication	2	73.5	73.5	51.6	50.8	50.2	50.2	40.8	42.0
¹ Treatment	5	206.9 ns	206.9 ns	91.3**	91.1**	99.6*	99.6*	314.4**	649.1
Residual	10	88.6	88.64	12.6	12.7	25.2	25.2	38.6	120.7
Repeat trial at Kicumbi 2002B									

¹Treatment= farmyard manure, *Calliandra* and control; ns, * and ** denotes not significant, significant at P≤0.05 and highly significant at P≤0.01

² seasons when trials were conducted 2002A (March to July), 2002B (September to December)

Appendix 7: Mean squares of dry matter per plant in grams of CAL 96 and RWR 719 bean varieties grown in amended soil inoculated with *P. ultimum* in a screen house (SH) trial at Kawanda

Variety/ Soil amendment	df	First trial 2002A				Repeat 2002B			
		2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
Replication	2	0.03	1.80	0.01	0.43	0.02	0.01	0.17	0.96
Treatment	5	0.13 ns	0.54 ns	0.94 ns	4.29**	0.54**	3.15**	11.70**	18.32**
Residual	10	0.09	0.37	0.39	0.50	0.01	0.01	0.04	0.13
SH follow up on first trial 2002B									
Replication	2	0.02	0.17	0.31	0.68	0.002	0.00	0.07	0.06
Treatment	5	0.03*	8.95**	5.60**	6.51 ns	0.01**	0.14**	0.95*	2.15**
Residual	10	0.01	0.09	0.7	1.27	0.001	0.02	0.13	0.11
Further amendment addition 2002B									
Replication	2	0.004	0.14	0.01	5.01	0.00	0.002	0.76	0.21
Treatment	5	0.08**	3.78**	3.89**	6.29 ns	0.03 ns	0.48**	3.97*	17.6**
Residual	10	0.002	0.03	0.01	2.51	0.01	0.002	0.57	0.22
Second repeat 2003A									
Replication	2	0.00	0.31	0.27	10.3				
Treatment	5	0.02*	0.48**	0.53 ns	38.6**				
Residual	10	0.003	0.05	0.39	2.3				

*Treatment= farmyard manure, *Calliandra* and control; ns, * and ** denotes not significant, significant at P<0.05 and highly significant at P<0.01
 2 seasons when trials were conducted 2002A (March to July), 2002B (September to December), 2003A (March to July)

Appendix 8: Mean squares of dry matter per plant in grams of CAL 96 and RWR 719 bean varieties grown in amended soil artificially infested with *Pythium* spp in a field trial at Rubaya and Kicumbi sites in Kabale during 2002A and 2002B seasons

Variety/ Soil amendment	df	Rubaya 2002A				Kicumbi 2002A			
		2 WAP	4 WAP	6 WAP	8 WAP	2 WAP	4 WAP	6 WAP	8 WAP
Replication	2	0.01	0.004	0.11	6.33	0.22	0.09	9.42	23.59
¹ Treatment	5	0.18*	0.29*	12.60**	21.56	0.47*	9.65**	79.53**	77.83 ns
Residual	10	0.02	0.56	1.2	3.77	0.13	0.65	8.5	27.2
Follow up on 1st trial at Rubaya 2002B									
Replication	2	0.03	0.02	0.18	0.08	0.01	0.02	0.06	0.41
¹ Treatment	5	0.69**	2.6**	5.75**	15.8**	0.09**	0.43**	2.75**	8.66**
Residual	10	0.03	0.01	0.04	0.17	0.01	0.01	0.03	0.20
Repeat trial at Rubaya 2002B									
Replication	2	0.23	0.04	1.36	0.09	0.02	0.11	0.08	1.49
¹ Treatment	5	1.39**	10.4**	69.6**	1.56**	0.30**	0.42 ns	54.85**	86.88**
Residual	10	0.16	0.06	0.78	0.10	0.01	0.12	0.37	4.08
Repeat trial at Kicumbi 2002B									

¹Treatment= farmyard manure, *Calliandra* and control; ns, * and ** denotes not significant, significant at P≤0.05 and highly significant at P≤0.01

² seasons when trials were conducted 2002A (March to July), 2002B (September to December))

Appendix 9: Mean squares of bean yields (kg/ha) as influenced by organic amendments on *Pythium* infested soil from trials at Rubaya and Kicumbi in Kabale during seasons² 2002A, 2002B

Source	df	Rubaya	Kicumbi
First trial 2002A			
Replication	2	2186.0	1400091.0
¹ Treatment	5	66828.0**	2837926.0 ns
Residual	10	11475.0	3770619.0
Follow up 2002B			
Replication	2	4449.0	2760871.0
¹ Treatment	5	12141.0*	1660607.0 ns
Residual	10	29255.0	1380981.0
Repeat 2002B			
Replication	2	24760.0	316455.0
¹ Treatment	5	74385.0 ns	1562290.0**
Residual	10	24932.0	124665.0

¹Treatment= farmyard manure, *Calliandra* and control; ns, * and ** denotes not significant, significant at $P \leq 0.05$ and highly significant at $P \leq 0.01$

² seasons when trials were conducted 2002A (March to July), 2002B (September to December)

Appendix 10: Mean squares of bean yields (g/plant) as influenced by organic amendments on *Pythium* infested soil from trials in the screen house at Kawanda during during seasons² 2002A, 2002B

Source	DF	First trial	Follow up	Repeat
First trial 2002A				
Replication	2	0.2	0.2	1072.8
¹ Treatment	5	1.1*	3.6**	668.3 ns
Residual	10	0.3	0.4	395.8

¹Treatment= farmyard manure, *Calliandra* and control; ns, * and ** denotes not significant, significant at $P \leq 0.05$ and highly significant at $P \leq 0.01$

² seasons when trials were conducted 2002A (March to July), 2002B (September to December)