The role of mixed cropping systems on bean root rot epidemics in south western Uganda

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Abstract (174 words)

In south western Uganda, beans are largely grown as intercrops with sorghum, maize, sweet potato and potato . Continuous cropping of beans, has increased bean root rot epidemics. Since some of the root rot causing organisms are known to affect other crops, there was need to investigate the role they may be playing in the current root rot epidemics. Surveys were carried out in Kabale district in order to establish the incidence of root rot on other crops grown in association with beans. Plant samples for isolation of *Pythium*, the main causative agent of root rot were also collected. Results indicated that potato had a high root rot incidence while maize had a low root rot incidence. Also, sorghum and peas had root rot symptoms. Out of the 142 *Pythium* isolates collected, 21 different *Pythium* species were identified by ITS-DNA sequencing. Fifteen new *Pythium* species not previously identified in the region were found. This study finds evidence that diverse crop species associated with beans may be playing a role in bean root rot epidemics.

Key words*:* *Phaseolus vulgaris*, *Pythium*, intercropping, epiphytotics, DNA sequencing, ITS primers

1.0 Introduction

In Africa, common beans (*Phaseolus vulgaris* L) are largely grown in a mixed cropping system or in rotation with other crops. This is because of land pressure. Mixed cropping also acts as an important strategy for food security by improving labour use efficiency, enhancing soil nutrients, light and rainfall because of the different demands made by the mixture of crops (Ocitti p'Obwoya, 1996). In south western Uganda, beans are grown in an intensive agricultural system together with sorghum (*Sorghum bicolor*), maize (*Zea mays* L), sweet potato (*Ipomoea batatas* L*)*, potatoes (*Solanum tuberosum* L), bananas (*Musa spp*. L) and garden peas (*Pisum satium*) (Ampaire, 2003). There are many root diseases of beans and several occur throughout many bean growing areas of the world (Abawi *et al*., 1985). The sudden increase in root rots in common beans is relatively recent and seems to be linked to intensification of farming and the decline in soil fertility (ISAR, 1990). The continuous growing of beans which is common in south western Uganda and other areas in Africa may have been associated with the root rot problem (Rusuku *et al*., 1997). Root rots in beans are caused by a complex association of pathogens mainly *Pythium* species, *Fusarium* species and *Rhizoctonia solanii* (Rusuku *et al*., 1997). Molecular analyses revealed a wide diversity within *Pythium* populations of beans in south western Uganda (Mukalazi, 2004). In other studies elsewhere root rots have been reported to occur on maize, wheat (*Triticum aestivium*), garden peas (*Pisum satium*), potato (*Solanum tuberosum*) and cowpea (*Vigna unguiculata* L) (Adandonon, 2004). The objective of this research was to evaluate the likely role/ contribution of crop species grown in association with beans in the current bean root rot epidemics in south western Uganda, so as to develop sustainable disease management strategy.

2. Materials and Methods

2.1. Diagnostic survey

A diagnostic survey was done in 16 sub counties in Kabale district in south western Uganda in April 2004, May 2005 and May 2006. A sub county is an area within a district and composed of a certain number of villages. The survey involved collecting crop samples grown together with beans. These crop samples could be with or without typical root rot symptoms. In each sub county, fields where samples were collected were separated by a 5 to 10 km distance. A 'W' sampling pattern was used in each field so as to maximize the collection of diseased crop samples. In each sampled field other information such as physical location, GPS (geographic positioning system) information, host sampled and disease symptoms observed on the crops were obtained. Data analysis was done using Statistic Programme for Social Scientists version 11.0 (SPSS) for Windows (SPSS Inc. Chertsey, England). Root rot incidence data was grouped into incidence categories for the crops sampled. Survey data was summarized using means, frequencies and percentages Cross tabulations were used to establish the strength of association between variables. Differences among crop species were compared using Fishers' protected least significant differences (LSD) at $P \leq 0.05$ (Steel *et al*., 1997).

3. Molecular analyses of *Pythium* isolates

3.1 Cultural conditions

The *Pythium* field isolates were grown on PDA at 18- 22^0C to obtain an active culture of each isolate relatively free of bacteria. After two to three days, a plug of pure culture from PDA was cut from the growing margin of cultures and placed in sterilised 20% V8 juice broth (King's Lynn Norfolk, USA) broth containing 2.5 g of $CaCO_3$ and incubated in darkness at room temperature for four days to allow the oomycete to form mycelia. Mycelia was harvested from V8 using sterilised forceps, filtered through a layer of 85- mm filter paper, blot dried of excess juice with paper towels. Subsequently the mycelia was placed in sterile micro-centrifuge tubes and kept at -20ºC.

3.2 DNA extraction

DNA was extracted from harvested mycelia according to Mahuku (2004). Mycelia were ground to a fine paste in a mortar containing Tris- EDTA- SDS (TES) extraction buffer (Sigma- Aldrich, Louis, Mo, USA) and sterilised acid-washed sea sand (BDH Laboratory Supplies, Poole, BH 15, ITD, England). Additional TES buffer containing Proteinase K (Sigma- Aldrich, Louis, Mo, USA) was added and the mixture incubated at 65ºC for 30 min. DNA was precipitated using ice-cold isopropanol (Sigma- Aldrich, Louis, Mo, USA) and the pellet was washed twice with 70% ethanol, dried and dissolved in Tris- EDTA (TE) buffer (Sigma- Aldrich, Louis, Mo, USA).

3.3 Polymerase Chain Reaction

The PCR analysis was performed using oomycete ITS specific primers (White *et al.*, 1990). PCR reaction was performed in 50 µl- reaction volumes containing 1µl of 1X PCR buffer, 4 mM $MgCl_2$, 0.0625 mM dNTP, 0.08µM of each primer, 18S (5'-TCC GTA GGT GAA CCT GCG G-3') and 28S (5'-TCC TCC GCT TAT TGA TAT GC-3'), 20 ng of DNA, and 0.02µl Taq DNA polymerase (5U/µl). Amplification was performed in a Primus 96 Plus Thermal Cycler (MWG-Biotech, Germany) programmed for 35 cycles of denaturation at 94ºC for 1 min, annealing at 68ºC for 2 min, and extension at 72ºC for 1 min, followed by a final extension for 8 min at 72ºC. The products were run on 2% agarose gels containing 5 mg/ml of ethidium bromide (Sigma- Aldrich, Louis, Mo, USA) using 1 X TBE (Tris Borate EDTA buffer) as the running buffer at a voltage of 100 Volts. At the end of electrophoresis, the agarose was placed on a UV light table and documented using a Polaroid camera (Ep-H7 0.7 x, electrophoresis hood, Polaroid, GelCam, UK) fitted with B/W 667 Polaroid films (Sigma- Aldrich, Louis, Mo, USA). Samples with amplicons below the expected size of 800 base pairs were eliminated from further analysis. PCR amplifications containing no DNA template were included as controls. Efficiency of amplification was monitored by running 12 µl of each reaction through 2 % agarose gel at 100 Volts for 2 hours in Tris-borate-EDTA buffer. A 100-bp molecular weight ladder was used as the size standard marker stained with ethidium bromide, visualised and photographed under UV light.

3.4 Analysis of ribosomal DNA sequences of *Pythium* isolates and identification of species

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 was carried out using ITS 2 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 (Applied Biosystems, CA-USA). The products were purified by ethanol precipitation following the manufacturer's protocol and nucleotide sequences were determined by the ABI 377 automated sequencing technology (Applied Biosystems, CA-USA).

3.5 DNA sequences analysis and identification

Sequences from ITS 1 region of the ribosomal gene were edited using the BioEdit program (DNASTAR Inc., Madison, Wis, USA). The ITS1 sequences were compared using blast N searches with sequence deposited at the National Center for Biotechnology Information (NCBI Gene Bank). *Pythium* sequences obtained were aligned with Clustal X (Thompson *et al.,* 1994). Consequently they were saved in Phylip format and used for phylogenetic analysis. A neighbour-joining tree was drawn using Clustal X and the boot strapping done to generate trees using 1000 replications. The Tree View software was used to view the trees (Page, 1996).

4.0 Results

4.1 Survey

Root rot incidence in each field for each crop species was determined by taking the number of crops infected with root rot over the total number (healthy and infected) crops. For the bean crop, the highest numbers of plants (45 %) were in the root rot disease incidence category of 40-59 (Table 1). In addition, potato had the most number of plants (49 %) sampled in the root rot incidence category of 40-59. However, maize and peas displayed 69 % and 77.5 % of their plants respectively in the disease category 20-39. In contrast maize and green pepper had 50 % of their plants in the root rot incidence disease category of 80-100.

The surveys were carried out for three years from 2004-2006 during the same season i.e. short rains throughout Kabale district (Figure 1). Maize, sorghum peas, potato and sweet potato were the main crops grown in Kabale district and their abundance is shown (Figure 2). Also, there were 20 different crops found in bean mixed cropping systems in south western Uganda. Each farm was found to have 1 to 6 different crops species planted. During the survey, various crop species in Kabale district were found to display symptoms characteristic of root rot (Table 2). Beans displayed 100 % root rot symptoms in 2004 and 2005 respectively. However in 2006, 11.5 % of the bean crops had root rot, while 22.5 % had yellowing of leaves. Maize had 16 % of the sampled plants with symptoms of stem rotting in 2004 but in 2005 and 2006 surveys no symptoms characteristic of root rot were observed. Moreover, sorghum had a number of symptoms which could be attributed to root rot (Table 2). Symptoms which were observed on sorghum included rotten stem, red lesions on stem, leaves with black sports, black lesions on roots and rotting roots. In addition, sorghum did not

display any root rot disease symptoms in the survey of 2005 while in the previous survey the symptoms were present. In general, symptoms found in this study on other crop species grown with beans are characteristic of root rot pathogens. The symptoms manifested on above ground plant tissue include poor seedling establishment, uneven growth, chlorosis and premature defoliation of severely infected plants (CIAT, 2005).

4.2 Molecular characterisation

Out of the 142 *Pythium* isolated characterised, 21 different *Pythium* species were identified from the samples using DNA sequences (Table 3). *Pythium spp.* were isolated from 13 different crops. The crops from which these *Pythium spp*. were isolated from were potato (*Solanum tuberosum*), sorghum (Sorghum *bicolor*), maize (*Zea mays*) and sweet potato (*Ipomoea batatas*). Thirty nine percent (39 %) of the isolates were *Pythium ultimum*, 11 % were *Pythium folliculosum*, 7 % were *Pythium acanthicum* and 7 % were *Pythium spinosum*. Other *Pythium* species accounted for less than 5.0 % (Table 3). Phylogenetic analyses revealed interesting patterns in the distribution of *Pythium spp.* The most obvious cluster or clade comprised of *Pythium spp.* that clustered together irrespective of crop species or of location of origin (Figure 2). For example *P. folliculosum* isolated from peas in Rwamucucu sub county clustered in the same clade with *P. ultimum* in Kaharo sub county. Phylogenetic analyses also showed several *Pythium spp.* Which were isolated from two different crops. For example *P. irregulare* were isolated from potato and tomato both solanaceous crops. Dual or multiple infections of both crop species were also by phylogenetic studies. For example *P. ultimum* and *P. acanthicum* were both isolated from potato, sorghum and beans. The study also revealed widespread distribution of

Pythium spp. P. ultimum the major cause of root rots was isolated from Rubaya, Bufundi, Muko and Ikumba sub counties, an area that covers 540 kms^2. Other pathogens were also isolated together with *Pythium* and these included *Fusarium oxysporum, Fusarium equiseti and Verticillium coccosporum* from millet, cabbage, potato, sorghum and bean. Other *Pythium spp.* isolated include: *P. torulosum* from potato, maize, bean and peas. *P. folliculosum* isolated from potato, sorghum, maize and peas. The study also isolated *P. irregulare* from potato and tomato.

5 Discussion

5.1 Survey

Beans and potato in this study had relatively high disease incidence in the category of 40- 59. This suggests that these two crops are affected by root rot disease. In contrast maize had a low root rot disease incidence category suggesting that it is not affected by root rot disease. Whereas peas had characteristic root rot symptoms, the majority of the pea plants were found in low root rot disease category. In general, disease incidence is affected by root damage from other soil-borne pathogens (Pieczarka and Abawi, 1978). Thus the patchy appearance of root rots especially among peas suggests that indeed, crop mixtures especially with beans have lower incidence of the disease. This may be due to the fact that beans are the preferred host compared to peas creating patchy environments in the field, with higher incidence of root rots around bean plants (Burdon, 1987; Burdon *et al.*, 1989).

In the surveys carried out in this study, beans were found to be the main crops grown by farmers followed by potato (*Solanum tuberosum*), maize (*Zea mays*), sorghum (*Sorghum bicolor*) and sweet potato (*Ipomoea batatas*). Previous studies indicated

that beans are a traditional crop grown by the Bakiga and Bafumbira people in Kabale district because they form a major source of protein in the diets. In addition, the main crops grown in Kabale were beans, sorghum, maize and sweet potato (Ampaire, 2003). This study found that cropping patterns (intercropping) were popular, consisting of two or more crops grown together. Intercropping in Kabale district is a traditional practice done to satisfy the need for other food requirements (Ampaire, 2003). Intercropping systems also provide for deliberate design and manipulation of land and plant populations to optimise the use of spatial, temporal and physical resources both above- and below ground. This is achieved by maximizing positive interactions and minimizing negative ones (Silwana and Lucas, 2002).

This study also shows that other crops in bean based cropping systems are affected by root rots for example, sorghum, peas and potato. Symptoms observed on sorghum in this study included rotten stem, red lesions on stem, leaves with black spots, black lesions on roots and rotting roots. In previous studies, symptoms on sorghum have included stunting and black roots, characteristic of *Pythium* root rot (Vincelli and Hershmann, 2002). In another field study carried out in Kabale district, sorghum plants were found to display symptoms of stunted growth, purple leaves and dark-red to black root lesions (CIAT, 2004). These symptoms found in this study on other crops grown in association with beans are characteristic of root rot pathogens infection. Root rots are characterised by above ground symptoms such as poor seedling establishment, uneven growth, chlorosis and premature defoliation of severely infected plants (CIAT, 2005). T the problem of poor re-establishment and poor seasonal production in long term sub terranean clover pastures has been recognised for some time (Paulitz and Adams, 2003). These independent studies

provide support for the hypothesis that other crop species may provide alternative hosts to *Pythium* root rot pathogens.

In this study, sorghum did not display any root rot disease symptoms in the survey of 2005 while in the previous survey the symptoms were present. This suggests that root rot disease is seasonal and may be dependent on presence of suitable environmental conditions as well as differences in time of sowing and plant population, which in some instances may aid escape from disease. Also it is known that growing crops in mixtures is important to overcome disease epidemics (Skelsey *et al.,* 2005). Studies elsewhere indicate that increased host diversity can contribute to a reduction of disease progress (Garret *et al*., 2001; Andrivon *et al*., 2003). There is evidence of potential benefits of host diversity in reducing potato late blight in temperate regions such as France and the United States where late blight epidemics tend to develop from obvious foci (Garret and Mundt, 1999). Part of the mixture effect might be due to differences in the spatio temporal pattern of disease spread, with disease progressing mainly along the rows in mixed-cultivar plots (lines of least resistance) as opposed to spatial spread in direct correspondence with the mean wind direction.

DNA sequence analyses showed diverse clades and sub groups that did not correspond to host or origin of occurrence of the *Pythium* isolates. These results are similar to those of Cilliers *et al*. (2000) and Harlton *et al*. (1995). Cilliers *et al*. (2000) compared ITS regions among isolates of *Sclerotium rolfsii* and reported that there was no apparent clustering according to host or geographic origin. Similarly, Harlton *et al.* (1995) found that unique individual were not necessarily correlated to the host nor restricted in geographical range. Results however show that there is a close affinity of

the *Pythium* species. In addition diversity exists among the species but there is no grouping based on host or geographic origin. In this study, using ribosomal DNA sequences of the ITS region, 21 *Pythium* species were found to be associated with root rot in other crops commonly included in intercrops of beans. Of these, six had been found previously to be associated with bean root rot (Mukalazi, 2004). These species are *Pythium spinosum*, *Pythium oligandrum*, *Pythium torulosum*, *Pythium vexans*, *Pythium pachycaule* and *Pythium ultimum*. However fifteen were new additions identified for the first time. Other studies elsewhere made use of restriction fragment polymorphisms of the ITS regions of ribosomal DNA and 36 *Pythium* species were distinguished (Wang and White, 1997). *P. ultimum* was found to be the most abundant of all the *Pythium* species in this study. It was isolated from potato, sorghum, maize, bean, sweet potato, cabbages, peas, wheat, bananas and yams. Previous work in Kabale district identified *P. ultimum* as the most abundant *Pythium* species where beans are grown (Mukalazi, 2004). *P. ultimum* is also mainly pathogenic to dicotyledons (Bruno and Griffith, 2004). Other *Pythium* species isolated in this study included *P. torulosum* from potato, maize, bean and peas; *P. folliculosum* isolated from potato, sorghum, maize and peas. In other studies elsewhere, *P. torulosum* and *P. folliculosum* have been isolated from monocotyledons, bryophytes, green algae, soil and occasionally from dicotyledons and conifers (Levesque *et al*., 2004). This study also found *P. irregulare* isolated from potato and tomato. In previous work this pathogen had also been isolated from the same hosts in Florida (Alferi *et al*., 1994). In addition *P. irregulare* was amongst 10 species of *Pythium* isolated from roots of pepper plants from various fields in Florida (Chellemi *et al*., 2000).

In conclusion, this study for the first time finds evidence suggesting the role of diverse crop species commonly included in bean intercrops on root rot epiphytotics in south western Uganda. Potato, sorghum, maize and peas were found to have typical root rot symptoms. Also, 15 new *Pythium* species were identified from diverse crop species which were intercrops of beans. In addition, *Fusarium* species and *Verticillium coccosporum* were also isolated though in low numbers compared to *Pythium*. Bean root rot thus appears to be caused by a complex of fungal and oomycete pathogens. Their control will require a number of mechanisms among others, the need to deploy non hosts. This is challenging given the fact that intercropping is used by farmers as an insurance against crop failure.

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Table 1: Mean frequencies of root rot disease incidence found on diseased crop species in Kabale encountered during this study

	[a] Root rot disease incidence categories					
Crop species	**0-19**	**20-39**	**40-59**	**60-79**	**80-100**	**LSD (p≤ 0.05)**
Beans	15.0	19.5	45.0	17.0	34.0	**56.12**
Cabbage	37.5	109.5	28.0	133.5	16.5	**116.15**
Cauliflower	7.5	45.0	21.0	59.5	38.5	**51.53**
Green pepper	0.0	0.0	0.0	100.0	50.0	**136.02**
Potato	49.0	36.0	9.5	29.0	19.5	**13.30**
Maize	25.0	69.0	6.5	0.0	50.0	**106.94**
Peas	16.5	77.5	46.0	0.0	31.5	**65.66**
Sorghum	28.5	28.5	33.5	18.0	18.0	**52.20**

LSD = Least Significance Difference test computed at P≤ 0.05.
a = Root rot incidence categories is based on the number of crops infected with root rot divided by total number of crops (healthy and infected).

Table 2: Frequencies of root rot symptoms found on diseased crop species in Kabale district encountered during this study

	2004 [a]		2005 [b]		2006 [c]	
Crops	Root rot symptoms found on crops	Percentage number of crops with symptoms	Root rot symptoms found on crops	Percentage number of crops with symptoms	Root rot symptoms found on crops	Percentage number of crops with symptoms
Beans	Root rot	100.0	Root rot	100.0	Root rot	11.5
	Yellowing of leaves	100.0	Yellowing of leaves	100.0	Yellowing of leaves	22.5
Maize	Yellow and red spots on leaves	83.3	No disease symptom	100.0	No disease symptoms	100.0
	Stem rotting	16.0				
Sorghum	Rotten stem	15.4	Stunting	25.0	Stunted plants	14.3
	Red lesions on stem	46.2	Not diseased	66.7	Black roots	14.3
	Leaves with black spots	15.4	Black purple leaves	8.3	Drying of leaves	28.6
	Black lesions on roots	7.7				
	Rotting roots	15.4				
Peas	Reduced root system	3.0	Not diseased	90.9	Drying of roots	26.7
	Root rot				Yellowing stem	20.0
	Wilted leaves	5.0				
		1.0				
potato	Wilted leaves	51.3	Not diseased	65.4	Wilting leaves	55.0
	Rotten stems	7.7	Tubers rotting	3.8	Rotting roots	10.0
	Rotten roots	7.7	Wilting leaves	30.8	Yellowing of leaves	3.3
	Black spots on leaves	17.9				
Sweet potato	Reduced root system	100.0				
Cabbage	Yellow leaves	100.0	Rotting roots	100.0	Drying of leaves	11.1
					Dry roots	11.1
					Shrunken leaves	33.3
					Rotten roots	11.1

[a] = The study covered 15 sub counties
[b] = The study covered 18 sub counties
[c] The study covered 8 sub counties

Table 3: *Pythium* species identified from other crops in south western Uganda using rDNA regions

Plant family	Crop Species	*Pythium* species																				Total
		P.ult	*P.acan*	*P.spinosum*	*P.torul*	*P.folli*	*P.olig*	*P.parvum*	*P.irre*	*P.glome*	*P.hetero*	*P.rost*	*P.arrh*	*P.macr*	*P.mamil*	*P.orth*	*P.conid*	*P.erinac*	*P.perip*	*P.vexans*	*P.pach*	
Leguminosae	Beans	2	1	-	1	5	-	-	-	-	1	-	-	-	-	1	5	-	-	-	-	9
	Peas	2	-	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5
Graminaceae	Sorghum	15	3	4	-	5	4	2	-	-	1	-		1	-	-	-	1	-	1	2	40
	Maize	11	-	1	2	3	2	-	-	-	-	-	4	-	-	-	-	1	-	1	-	27
	Millet	-	-	-	-	-	-	2	-	-	-	-	2	-	-	-	-	-	-	-	-	4
	Wheat	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
Solanaeceae	Potato	15	5	3	2	1	1	-	2	2	1	1	-	1	1	-	-	-	-	-	-	35
Convolvulaceae	Sweetpot*	1	-	-	-	-	-	-	-	-	2	1	-	-	-	-	-	-	1	-	-	5
Solanaeceae	Tomatoes	-	-	1	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Brassicaceae	Cabbage	5	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	7
Musaceae	Bananas	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Dioscoreaceae	Yams	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
	Weed	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Total		55	10	10	6	15	7	6	3	2	5	2	6	2	1	1	5	2	1	2	2	

*Sweetpot= Sweetpotato

Figure captions

Figure 1: A map showing south western Uganda where farms were surveyed in this study.

Figure 2: *Pythium* species isolated from other crops in south western Uganda. The tree was rooted with *Fusarium* species collected during this study and the dendogram was generated using Clustal X program (Thompson, 1994) with bootstrapping of 1000 replications.