A series of 231 samples of bean plants affected by bean root rot were collected from different areas of Rwanda in order to characterize the causal agents. The collected samples were used to isolate 96 typical Pythium colonies which were classified into 16 Pythium species according to their respective molecular sequences of the ribosomal ITS fragments. Inoculation assays carried out on a set of 10 bean varieties revealed that all identified species were pathogenic on common bean. However, the bean varieties used in this investigation showed differences in their reaction to inoculation with the 16 Pythium species. In fact, the varieties CAL 96, RWR 617-97A, URUGEZI and RWR 1668 were susceptible to all the Pythium species while the varieties G 2331, AND 1062, MLB 40-89A, VUNINKINGI, AND 1064 and RWR 719 showed a high level of resistance to the all Pythium species used in our study. This high level of resistance to Pythium root rot disease found in diverse varieties of common bean grown in Rwanda constitutes a real advantage to be exploited as source of resistance in breeding programs aiming to increase resistance to the disease in the most popular bean varieties grown in Rwanda.

Key words: Bean, characterization, molecular, Phaseolus, Pythium, root rot.

INTRODUCTION

Common bean (Phaseolus vulgaris L.) is the second most important source of human dietary proteins and the third most important source of calories (Sarikamis et al., 2009; Widers, 2006; Bennink, 2005). According to Miklas et al. (2006), this crop has a high nutritional value with important protein contents (~22%), minerals (calcium, copper, iron, magnesium, manganese, zinc), and vitamins necessary to warrant the food security of people in the developing countries. 

P. vulgaris is the most widely distributed Phaseolus species as it is grown on all the continents with a broad range of adaptation to various environmental conditions (Baudoin et al., 2001; Broughton et al., 2003; Melotto et al., 2005).

The crop production is hampered by several constraints among which are bean root rot caused by Pythium spp. This disease is considered as being the most damaging in East and Central Africa including Rwanda where beans are grown intensively (Nderitu et al., 1997; Wortmann et al., 1998). The bean root rot disease caused by Pythium spp. can lead to total yield losses when susceptible varieties are grown under favourable environmental conditions for the pathogen development (Buruchara and Rusuku, 1992; Otsyula et al., 2003; Rachier et al., 1998). The disease is characterized by above ground symptoms such as poor seedling establishment, uneven growth and premature defoliation of severely infected plants (Abawi et al., 1985; Abawi and Ludwig, 2005;
sustainable management of the resistant varieties. In fact, from a better knowledge about the composition of the disease, the disease is also characterized by lower leaf yellowing (similar to nitrogen deficiency), stunting, leaf browning and plant death (Pankhurst et al., 1995; Ampaire, 2003).

The Pythium inducing agents produce several zoospores that enable them to rapidly and continuously re-infest growing roots. Consequently, crops can be exposed to repeated ‘waves’ of Pythium infections throughout the cropping season, rather than the slower inoculum build-up shown by some of the other fungal root diseases (Alfieri et al., 1994; Pacumbaba et al., 2008). Methods for controlling Pythium include metalaxyl-based fungicides that are usually applied as seed dressings. However, different research works revealed that when applied in this manner, the fungicide only offers a minimum protection. In different other crops, although the seed dressing protection resulted in only about 20% control of the disease in the first 2 to 3 months of crop growth, substantial yield increases were observed in cereals (5 to 20%), canola (5 to 30%) and pulses (5 to 50%) (Salih and Agreeb, 1997; Louise and Paul, 2006). In Africa, the combination of organic amendments, raised beds and resistant varieties has been shown to be more efficient than the strict use of single control method in reducing the severity of root rots as well as yield losses (Buruchara and Scheidegger, 1993; Voland and Epstein, 1994).

For an efficient and practical control of the Pythium root rot of bean, the use of resistant varieties is considered as the most viable option in East Africa region (Otsyula and Ajanga, 1994; Garret et al., 2001). However, selection and sustainable use of resistant varieties has to take into account diversity of causal agents.

The traditional bean growing system in Rwanda is mainly based on the use of mixed varieties in the different bean growing areas of the country. In these conditions, improving the resistance to this bean root rot disease has to take into account the fact that as farmers do not accept easily pure varieties which they introduce progressively in their own mixtures.

As the use of resistant varieties to control Pythium root rot disease in beans is considered as a recommendable control method under African conditions, the present work was undertaken to characterize Pythium agents inducing root rot symptoms on common bean in Rwanda. That step is fundamental prior to development of a breeding strategy aiming at improving the resistance to that disease as it facilitates determining the conditions of a sustainable management of the resistant varieties. In fact, from a better knowledge about the composition of bean Pythium populations in Rwanda, it would become possible to identify and exploit sources of resistance to a maximum of Pythium pathotypes found in the country. Moreover, the deployment strategy which can improve sustainability of the released varieties would also be adapted according to the data revealed through analysis of Pythium populations.

The investigations cover different components: (1) Collecting Pythium isolates; (2) Mapping the geographical distribution of collected isolates; (3) Characterizing the collected isolates by molecular profiles, and (4) Determining their pathogenicity properties through inoculation of common bean varieties.

MATERIALS AND METHODS

Collection of samples and purification of the inducing Pythium agents

Bean root samples showing root rot symptoms were collected from all the districts of Rwanda covering 3 altitude levels: Low (900 to 1400 m), intermediate (1400 to 1650 m) and high (1650 to 2300 m). Practically, the collected samples were taken along transects in micro sites separated from each other by 5 km. In each of the sampled fields, 5 plants were randomly uprooted based on the presence of Pythium like symptoms prevailing on leaves (yellowing), roots and stems. Once the samples were collected, the isolation procedure described by White (1988) was used to isolate the Pythium agents related to the observed symptoms. A selective medium was prepared by mixing corn meal agar, CMA (17 g) and distilled water (1000 ml) before autoclaving through incubation at 121°C for 20 min. The antibiotic preparation [Rifamycin (0.03 g/L) and Pimaricin (0.02 g/L)] was then added after heat sterilization when the medium was cooling (around 40°C). Isolations were accomplished by first washing soil from the plant tissues in a jet-stream of tap water, rinsing twice in sterile distilled water, blotting dry on new paper towel, and placing infected root pieces (approximately 0.5 to 2 cm long) cut from expanding lesions on the prepared selective medium (CMA). Petri plates with plant samples were observed after incubation for 24 and 48 h at room temperature (20 to 25°C). The Pythium mycelia developing from the plant tissues were transferred on potato dextrose agar (PDA) slants.

DNA extraction

Prior to DNA extraction, the fungal mycelial tissues were previously multiplied in liquid V8 medium (20% of V8 juice broth in distilled water) (King’s Lynn Norfolk, USA) containing 2.5 g of CaCO3. After 14 days of incubation under darkness at 25°C, the fungal tissues were harvested by separating the mycelium and the liquid medium. DNA was extracted from the harvested mycelia according to the procedure described by Mahuku (2004). Mycelia were ground to a fine paste in a mortar containing extraction buffer (0.2 M Tris-HCl [pH 8], 10 mM EDTA [pH 8], 0.5 M NaCl, 1% SDS) and sterilized acid-washed sea sand. Additional TES buffer containing protease K was added and the mixture incubated at 65°C for 30 min. DNA was precipitated using ice-cold isopropanol and the pellet was washed twice with 70% ethanol, dried and dissolved in TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA).

Polymerase chain reaction

PCR analysis was performed using Oomycete ITS (Internal Transcribed Sequence) region primers to differentiate Pythium from other closely related fungi (White et al., 1990). The PCR reaction was performed in 50 µl final reaction volume containing 5 µl of 10X PCR buffer, 8 µl of 25 mM MgCl2, 2.5 µl of 1.25 mM dNTP, 0.2 µl of each primer (20 µM) [18S (5'-TCC GTA GGT GAA CCT GCG G-3') and 2.5 µl of 10X PCR buffer, 8 µl of 25 mM MgCl2, 2.5 µl of 1.25 mM dNTP, 0.2 µl of each primer (20 µM) [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 0.2 µl Taq DNA polymerase (5 U/µl) (Roche Molecular Systems, Inc., USA). Amplification was performed in a BIO RAD My Cycler thermal cycler programmed for initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 1 min, and extension at 72°C for 1.5 min. At the end of the amplification reaction, a final extension step was achieved at 72°C for 7 min. The products were run on 2% agarose gels containing 5 mg/ml of ethidium bromide in a TBE (1 time concentrated) as the running solution. The electrophoretic migration was carried out during 2 h under a 100 V voltage. The amplified products were visualized and photographed under UV light. To estimate the size of the PCR products, a 100 bp molecular ladder (Bioneer Inc, Korea) was used. The negative controls were based on reactions where the DNA solutions were replaced by water.

Sequencing the amplified DNA and Pythium identification

All the PCR products having a size of 800 bp were submitted to sequencing procedure. For that, residual primers and dNTPs were removed using QIAquick™ PCR purification spin columns following the manufacturer’s protocol (Karp et al., 1998). Direct sequencing of the PCR amplified products was carried out using ITS 2 primers (White et al., 1990). The sequencing analysis was carried out in an institution (Macrogen) of the South Korean Republic.

Sequences obtained from the ITS region of the ribosomal DNA gene were edited using the Editseq program (DNASTAR Inc., Madison, WIs). The ITS sequences of the analysed isolates were compared with ITS sequences of known Pythium species available in the public databases using Segmann program (DNASTAR), by performing a nucleotide-nucleotide blast search at the National Center for Biotechnology Information (NCBI) website: http://www.ncbi.nlm.nih.gov/BLAST.

Multiple alignments of the sequenced ITS product was performed for comparison. Pythium sequences obtained were aligned with Clustal X (Thompson et al., 1994). Consequently, sequences 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 TreeView software was used to view the trees.

Pathogenicity analysis of the Pythium species

Trials aiming to investigate capacity to induce root rot and the severity of the related symptoms for the different isolated Pythium agents were carried out through inoculation assays. On the other side, the data generated through these assays were used to determine sources of resistance to the root rot disease among the bean varieties available in Rwanda. These experiments were performed in a screen house at the National Agricultural Research Laboratories, Kawanda. This site is located at 0°25’05” N and 32°31’54” E at 1190 m above sea level (masl), average rainfall is 1224 mm per annum and average daily temperatures are 15.3°C (minimum) and 27.3°C (maximum).

Inoculum of the various Pythium species (one isolate was randomly selected for each identified Pythium species) was multiplied by plating mycelia on autoclaved millet grains (100 g) mixed with 200 ml of water in 500 ml bottles. After two weeks of incubation under darkness at 25°C, pre-sterilized soil was mixed with the infested millet at a ratio of 1:10 v/v in wooden trays of 42 x 72 cm. Each tray contained 10 plants of each bean variety used in this evaluation analysis. The trays were set up in a Completely Randomized Block Design (CRBD) with three replications for each Pythium species. The inoculum was applied to the following bean varieties locally grown in Rwanda (G 2331, Uringezi, R617-97A, WWR 1668, Vuninkingi, RWR 719), plus a set of 4 varieties provided by CIAT and already known as being resistant to Pythium in other regions (CAL 96, MLB-40-89A, AND 1064 and AND 1062).

After germination, the seedlings were watered two times per day to provide a favourable environment for the pathogen establishment and development. Three weeks after emergence of the seedlings, the surviving plants were uprooted and washed with water to remove soil. Severely of root rot symptoms was then assessed using the CIAT visual scale whose scores vary from 1 to 9 (Abawi and Pastor- Corrales, 1990), where 1 = no root rot symptoms; 3 = a maximum of 10% of the hypocotyls and root tissues having lesions; 5 = approximately 25% of the hypocotyls and root tissues having lesions and the root system suffering a considerable decay; 9 = 75% or more of the hypocotyls and root tissues having lesions and the root system suffering advanced stages of decay and considerable reduction. Isolates that had an average disease score of 1 to 2 were considered as being non pathogenic while those with an average score of 3 to 5 were considered moderately pathogenic and those with an average score of 6 to 9 were considered to be highly pathogenic. Evaluation of the disease symptom importance was performed on 10 plants per each variety in each of the three replicates.

RESULTS

Sample collection and characterization of the isolated agents

231 samples collected were used to isolate the Pythium spp. Figure 1 represents the map of Rwanda showing the places where the samples were collected during our survey. On the CMA culture medium, we observed development of fungal colonies after a minimum of 24 h of incubation. From the 231 samples, 96 isolates were isolated, purified and submitted to further molecular characterization tests. The difference between the number of collected samples and the number of identified Pythium species is probably due to the fact that root rots are caused by one or more soil-borne pathogens acting either alone or as a complex of two or more pathogens depending on environmental conditions. Table 1 shows geographical location and isolates codes of different Pythium species isolated in Rwanda. Based on these data, it becomes clear that the Pythium bean root rot disease is widely distributed in the Rwanda as several Pythium species were isolated from samples presenting root rot symptoms collected in 25 districts of Rwanda. For that, it can be hypothesized that the causing Pythium agents can be found in all the agro-ecological zones of Rwanda. Moreover, there is no clear relationship between the occurrence of Pythium species and the altitude. Distribution of Pythium vexans can be given as a clear example of that situation as an isolate of this species was found at an altitude of 1329 m while another isolate of this species was found at an altitude of 1696 m.

The PCR reaction allowed amplifying the fungal ITS fragments of 800 bp. It is known that the ITS fragment of Pythium is of 800 bp (Mahuku et al., 2007). In summary, only 96 isolates of the 231 samples had the Pythium expected specific size of ITS fragment (800 bp); these
Figure 1. Map of Rwanda showing the places where the samples have been collected during our root rot survey. Different colors show four provinces (North, South, East, West and Kigali City which has orange color and located in the center of the country). The green spots represent the places where the samples have been collected.

Table 1. Geographical location and isolates codes of different *Pythium* species collected in Rwanda.

| Latitude       | Longitude       | Altitude (m) | Temperature (°C) | District | Isolates code | *Pythium* species       |
|----------------|-----------------|--------------|-----------------|---------|---------------|------------------------|
| 02°39'21,1“    | 029°45'23,4“    | 1697         | 17.1            | Huye    | 07HYEa        | *Pythium torulosum*    |
| 02°35'5,8“     | 029°43'26“      | 1697         | 17.2            | Huye    | 07HYEb        | *P. torulosum*         |
| 02°38'59,3”    | 029°46'38,1“    | 1697         | 18.3            | Huye    | 8HYE a        | *Pythium macrosporum*   |
| 02°39'21,1“    | 029°45'23,4“    | 1689         | 14.2            | Gisagara| 12 GIS        | *Pythium rostratilifingens* |
| 02°33'14,9“    | 029°44'24,7“    | 1741         | 21.7            | Huye    | 20HYE         | *Pythium spinosum*      |
| 02°13'41,5“    | 029°47'26,9“    | 1723         | 21.2            | Ruhango | 07RNGO        | *Pythium diclinum*      |
| 02°10'19,9“    | 029°45'58,9“    | 1810         | 22.3            | Ruhango | 9 MUH         | *Pythium conidiophorum* |
| 02°04'15,1“    | 029°43'32,2“    | 1876         | 21.0            | Muhanga | 14 MUH        | *P. torulosum*         |
| 02°05'15,7“    | 029°20'6,5“     | 1589         | 21.0            | Karongi | 29 KNGIb      | *Pythium folliculosum*   |
| 02°06'7,2“     | 029°19'54,1“    | 1581         | 20.0            | Karongi | 29 KNGIc      | *Pythium ultimum*       |
| 02°06'7,2“     | 029°19'43“      | 1565         | 20.3            | Karongi | 30 KNGI       | *P. torulosum*         |
| 02°08'52,2“    | 029°17'46,6“    | 1626         | 19.6            | Karongi | 33 KNGI       | *Pythium dissotocum*    |
| 02°08'5,5“     | 029°19'23,2“    | 1584         | 21.3            | Karongi | 38 KNGIb      | *P. ultimum*            |
Table 1. Contd.

| Latitude    | Longitude   | X   | Y   | Location  | Lat.  | Long.  | Species                  |
|-------------|-------------|-----|-----|-----------|-------|--------|--------------------------|
| 02°12'12.9" | 029°15'4.3" | 1716 | 19.4 | Karongi   | 130 KNGI | Pythium vexans          |
| 02°16'10.2" | 029°23'1.5" | 1560 | 20.0 | Nyamasheke | 37 NSKE | P. vexans               |
| 02°22'31"   | 029°05'8"   | 1598 | 22.3 | Nyamasheke | 42 NSKE | P. spinosum             |
| 02°24'26.6" | 029°16'2.6" | 1598 | 22.3 | Nyamasheke | 42 NSKE | P. spinosum             |
| 02°32'14.9" | 028°53'1.6" | 1659 | 22.1 | Rusizi    | 49 RSZ | P. rostratiginges      |
| 02°33'11"   | 028°53'1.6" | 1659 | 22.1 | Rusizi    | 49 RSZ | P. rostratiginges      |
| 02°19'48.1" | 029°46'1.9" | 1774 | 23.9 | Nyanza    | 58 NGBE | P. folliculosum         |
| 02°19'6.7"  | 029°49'29"  | 1584 | 24.6 | Nyanza    | 75 NGBE | P. vexans               |
| 02°20'49.1" | 029°52'12.5" | 1598 | 23.9 | Nyanza    | 79 NGBE | Pythium folliculosum    |
| 02°19'56.3" | 029°53'16.1" | 1422 | 23.4 | Nyanza    | 82 NGBE | P. vexans               |
| 02°18'49.4" | 029°54'5.4" | 1437 | 23.6 | Nyanza    | 87 NGBE | P. folliculosum         |
| 02°18'35.5" | 029°55'2.7" | 1435 | 23.9 | Nyanza    | 88 NGBE | P. vexans               |
| 01°57'30.4" | 030°05'8.4" | 1408 | 23.2 | Gasabo    | 97 GSB | P. vexans               |
| 01°58'7.7"  | 030°10'6.1" | 1366 | 22.2 | Gasabo    | 97 GSB | P. vexans               |
| 01°58'40.9" | 030°10'5.4" | 1354 | 23.2 | Gasabo    | 98 GSB | P. vexans               |
| 01°59'17.5" | 030°11'3.9" | 1345 | 23.9 | Gasabo    | 98 GSB | P. vexans               |
| 01°58'53.5" | 030°12'5.8" | 1326 | 23.6 | Gasabo    | 101 GSB | P. vexans               |
| 01°54'29.6" | 030°26'3.8" | 1514 | 25.3 | Rwamagana | 108 RWM | P. vexans               |
| 01°54'6.4"  | 030°29'4.8" | 1598 | 25.4 | Kayonza   | 110 KYNZA | P. rostratiginges   |
| 01°55'11.1" | 030°29'3.9" | 1563 | 26.3 | Kayonza   | 111 KYNZA | P. rostratiginges   |
| 02°11'14.5" | 030°31'4.9" | 1663 | 25.6 | Ngoma     | 117 NGM | P. vexans               |
| 02°09'52"   | 030°31'1.8" | 1679 | 25.6 | Ngoma     | 120 NGM | Pythium chamaehyphon    |
| 02°07'39.2" | 030°30'4.9" | 1669 | 25.6 | Ngoma     | 122 NGM | P. indigoferae          |
| 02°08'40.7" | 030°34'3.0" | 1684 | 25.0 | Ngoma     | 124 NGM | P. vexans               |
| 02°12'57.2" | 030°23'5.4" | 1330 | 20.5 | Ngoma     | 126 NGM | P. vexans               |
| 2°13'39.1"  | 030°33'1.7" | 1373 | 17.8 | Ngoma     | 128 NGM | P. vexans               |
| 2°15'41.4"  | 030°38'5.4" | 1570 | 18.1 | Kirehe    | 133 KKRHa | P. vexans            |
| 2°16'23"    | 030°40'5.4" | 1627 | 20.5 | Kirehe    | 133 KKRHa | P. vexans            |
| 1°8'3.7"    | 030°19'1.5" | 1359 | 28.9 | Nyagatere | 143 NGTR | P. conidiophorum      |
| 1°8'20.6"   | 030°19'1.0" | 1359 | 28.3 | Nyagatere | 145 NGTR | P. rostratiginges    |
| 1°24'22.6"  | 030°16'2.6" | 1370 | 27.0 | Nyagatere | 149 NGTR | P. vexans            |
| 1°24'58.7"  | 030°16'4.8" | 1375 | 26.4 | Nyagatere | 151 NGTR | P. vexans            |
| 1°25'41.9"  | 030°16'2.0" | 1374 | 27.4 | Nyagatere | 153 NGTR | P. ultimum           |
| 1°24'45.3"  | 030°18'4.6" | 1438 | 28.3 | Nyagatere | 158 NGTR | P. vexans           |
| 1°44'24.5"  | 030°07'4.2" | 1518 | 23.7 | Gicumbi   | 162 GCM | P. vexans           |
| 1°38'50.5"  | 030°07'4.3" | 2098 | 25.5 | Gicumbi   | 166 GCM | P. diclinum         |
isolates were submitted for sequencing analysis. These products were submitted to the sequencing operation to generate sequence data in view of classifying the different isolates in comparison with the Pythium spp. reference sequences. During the alignment analyses, a series of 17 sequences were found to be uncorrelated to Pythium sequences available in the data base. In these conditions, only 79 isolates were classified, after comparison using blast N searches with sequence deposited at the National Center for Biotechnology Information (NCBI Gene Bank) to establish their respective relationships with known Pythium species, as being Pythium agents belonging to various species (Figure 2).

Analyses of ITS sequences revealed that the 79 isolates belong to 16 different Pythium species. Table 2 contains the number of isolates classified in each Pythium species per district in Rwanda.

On the side of Pythium species geographical distribution, P. vexans was shown to be the most widespread in the country as its presence was revealed with 23 isolates distributed in 13 districts (Table 2). The species Pythium indigoferae was found in samples from 6 districts, while the species Pythium torulosum, Pythium ultimum and Pythium rostratifingens were found in only 4 districts. The remaining Pythium species identified among the samples collected in Rwanda were distributed in low number of districts with the species Pythium cucurbitacearum, Pythium arrhenomanes, Pythium pachycaule and Pythium rostratum being the less widespread as having been found in only one district for each species.

Pathogenicity

Table 3 illustrates the severity of the root rot disease caused by the different Pythium species as a consequence of their inoculation on the bean varieties.

The root rot symptoms were observed 21 days after sewing beans on the contaminated soil substrate. After that incubation period, there was an important development of root rot symptoms on the susceptible variety (CAL 96) whatever the inoculated isolate while the symptoms appearing on the resistant variety (RWR 719) remained very moderate in all the cases. The morphological aspect of the root rot symptoms development on the bean plants is illustrated by the pictures presented in the Figure 3. The disease symptoms appearing on the root system of the susceptible variety were also associated with a significant decrease of the plant size. As the root rot symptoms were visible only when the bean plants were growing on previously contaminated substrate, it was concluded that the observed symptoms resulted from the microorganisms used to contaminate the growing substrate.

Given the artificial inoculation with the different Pythium species conducted to development of the root rot symptoms, it was considered that each of the species used in the present study were pathogenic on bean. Table 3 presents the results of disease severity assessment carried out on all the bean varieties used in the present study.

Globally, it can be noticed that for all the Pythium species, the variety CAL 96 was highly susceptible while the variety RWR 719 was shown to be highly resistant whatever the inoculated isolate. Based on these data, it was concluded that all the Pythium species isolated in Rwanda and tested through this biological assay were pathogenic on beans. These data confirmed also that the root rot symptoms previously observed on the sampled materials were due to Pythium agent. Moreover, there was an important variability of the bean variety reaction following inoculation with the different Pythium species isolates. In fact, for a given Pythium species, it was
| Code       | Species                                      |
|------------|----------------------------------------------|
| 98GSBiP    | Pythium v. vexans                            |
| 29KNGiP    | Pythium folliculosum                         |
| 79NYAP     | Pythium torulosum                            |
| 30KNGiP    | Pythium folliculosum                         |
| 84NYAP     | Pythium torulosum                            |
| 87NYAP     | Pythium torulosum                            |
| 178NGGEp   | Pythium torulosum                            |
| 14MUH     | Pythium torulosum                            |
| 37GNSKbP   | Pythium torulosum                            |
| 07HYEaP    | Pythium v. vexans                            |
| 07HYEbP    | Pythium torulosum                            |
| 9MUH      | Pythium conidiophorum                        |
| 43NGTbP    | Pythium conidiophorum                        |
| 7007HYPaP  | Pythium v. vexans                            |
| 7007HYPbP  | Pythium torulosum                            |
| 709MUH     | Pythium conidiophorum                        |
| 43NGTbP    | Pythium conidiophorum                        |
| 54RSZiP    | Pythium arrenonales                          |
| 120NGMP    | Pythium cham aehyphon                        |
| 171GCMBP   | Pythium cham aehyphon                        |
| 133KRHaP   | Pythium v. vexans                            |
| 98GSBP     | Pythium v. vexans                            |
| 124NGMP    | Pythium indigoferae                          |
| 162GCMBP   | Pythium vexans                               |
| 110KYNZAP  | Pythium vexans                               |
| 97GSSBP    | Pythium vexans                               |
| 126NGMP    | Pythium vexans                               |
| 128NGMP    | Pythium vexans                               |
| 95BGSRP    | Pythium vexans                               |
| 88NYAP     | Pythium vexans                               |
| 108RMWP    | Pythium vexans                               |
| 158NGTbP   | Pythium vexans                               |
| 133KRMHP   | Pythium vexans                               |
| 124NGMP    | Pythium indigoferae                          |
| 162GCMBP   | Pythium vexans                               |
| 110KYNZAP  | Pythium vexans                               |
| 97GSSBP    | Pythium vexans                               |
| 117NGMP    | Pythium indigoferae                          |
| 96BGSRP    | Pythium vexans                               |
| 101GSSBP   | Pythium vexans                               |
| 149NGTbP   | Pythium vexans                               |
| 93BGSRP    | Pythium vexans                               |
| 151NGTbP   | Pythium indigoferae                          |
| 75NYAP     | Pythium indigoferae                          |
| 180NGGEp   | Pythium vexans                               |
| 182NGGEp   | Pythium vexans                               |
| 177NGGEaP  | Pythium vexans                               |
| 130KRNZP   | Pythium vexans                               |
| 173GCMBP   | Pythium cucubitacearum                       |
| 184NGKDP   | Pythium indigoferae                          |
| 20HYEP     | Pythium spinosum                             |
| 46RSZb1P   | Pythium spinosum                             |
| 46RSZb2P   | Pythium spinosum                             |
| 46RSZb49P  | Pythium rostratifingens                      |
| 64NGBE64NGBE| Pythium rostratifingens                     |
| 111KYNZAP  | Pythium rostratifingens                      |
| 89NYAP     | Pythium indigoferae                          |
| 153NGTbP   | Pythium ultimum                             |
| 204MNZEP   | Pythium diclinum                            |
| 38KNGbP    | Pythium ultimum                             |
| 77NGEBP    | Pythium pachycala                           |
| 46RSZiP    | Pythium spinosum                             |
| 56RSZiP    | Pythium dissotocum                          |
| 43NSKEP    | Pythium conidiophorum                        |
| 07RNGOP    | Pythium diclinum                            |
| 166GCMBP   | Pythium indigoferae                          |
| 33KNZP     | Pythium dissotocum                          |
| 183RNDO    | Pythium dissotocum                          |

Figure 2. Phylogenetic relationship of *Pythium* spp. from Rwanda based on the ITS ribosomal DNA sequences. The codes following number are relative to district of origin (RNDO: Rulindo; KNG: Karongi, GCMB: Gicumbi, RNGO: Ruhango, RSZI: Rusizi, NSKE: Nyamasheke, NGBE: Nyamagabe, KNGI: Karongi, MNZE: Musanze, NGTR: Nyagatare, NYA: Nyanza, KYNZA: Kayonza, HYE: Huye, NGGE: Nyarugenge, BGS: Bugeesa, GSB: Gasabo, NGM: Ngoma, KRH: Kirehe, MUH: Muheanga, RW: Rwamagana). The isolates codes are followed by different *Pythium* species. The dendrogram was generated using Clustal X program.
Table 2. Distribution of the *Pythium* species isolated from the different bean samples affected by root rot symptoms per districts in Rwanda.

| District       | *P. indigoferae* | *P. chamaehyphon* | *P. torulosum* | *P. cucubitaeoretum* | *P. diclinum* | *P. arthromanes* | *P. pachycapulare* | *P. ultimum* | *P. vexans* | *P. folliculosum* | *P. macrosporum* | *P. rostratilingens* | *P. spinosum* | *P. dissolocum* | *P. rostratum* | Total |
|----------------|------------------|-------------------|----------------|----------------------|---------------|------------------|-------------------|---------------|-------------|------------------|------------------|------------------------|---------------|--------------|------------------|-------|
| Huye           | 2                |                   |                |                      |               |                  |                   | 1             | 1           | 1                | 5                |                         | 2             |              |                   | 5     |
| Gisagara       |                  |                   |                |                      |               |                  |                   | 1             | 2           |                  |                  |                         | 3             |              |                   | 3     |
| Nyanza         | 2                | 1                 |                |                      |               |                  |                   | 1             | 2           |                  |                  |                         | 1             | 1            |                   | 2     |
| Karongi        | 2                |                   |                |                      |               |                  |                   | 2             | 1           | 1                | 1                |                         | 6             | 1            |                   | 7     |
| Muhanga        | 1                |                   |                |                      |               |                  |                   | 1             | 1           |                  |                  |                         | 2             |              |                   | 2     |
| Ruhango        |                  |                   |                |                      |               |                  |                   | 1             | 1           |                  |                  |                         | 1             |              |                   | 1     |
| Nyamasheke     |                  |                   |                |                      |               |                  |                   | 1             | 1           |                  |                  |                         | 2             |              |                   | 2     |
| Rusizi         |                  |                   |                |                      |               |                  |                   | 2             | 2           | 2                |                  |                         | 7             |              |                   | 7     |
| Nyamagabe      | 1                | 1                 |                |                      |               |                  |                   | 1             | 1           | 1                |                  |                         | 4             |              |                   | 4     |
| Bugesera       | 1                |                   |                |                      | 1             |                  |                   | 3             | 1           |                  |                  |                         | 5             |              |                   | 5     |
| Gasabo         | 3                |                   |                |                      |               |                  |                   | 3             | 3           |                  |                  |                         | 3             |              |                   | 3     |
| Rwamagana      |                  |                   |                |                      |               |                  |                   | 1             | 1           |                  |                  |                         | 2             |              |                   | 2     |
| Kayonza        |                  |                   |                |                      |               |                  |                   | 1             | 1           |                  |                  |                         | 2             |              |                   | 2     |
| Ngoma          | 4                |                   |                |                      |               |                  |                   | 2             | 1           |                  |                  |                         | 5             |              |                   | 5     |
| Kirehe         |                  |                   |                |                      |               |                  |                   | 1             | 1           |                  |                  |                         | 2             |              |                   | 2     |
| Gatsibo        |                  |                   |                |                      |               |                  |                   | 1             | 1           |                  |                  |                         | 4             |              |                   | 4     |
| Ngoma          | 4                | 1                 |                |                      |               |                  |                   | 1             | 3           | 1                |                  |                         | 5             |              |                   | 5     |
| Nyagatare      | 1                | 1                 |                |                      | 1             |                  |                   | 1             | 1           | 2                |                  |                         | 5             |              |                   | 5     |
| Rurindo        |                  |                   |                |                      |               |                  |                   | 1             | 1           | 1                |                  |                         | 2             |              |                   | 2     |
| Gicumbi        | 1                | 1                 | 1              |                      |               |                  |                   | 1             | 1           |                  |                  |                         | 4             |              |                   | 4     |
| Nyarurugenge   | 1                |                  |                |                      | 1             |                  |                   | 1             | 3           | 1                |                  |                         | 5             |              |                   | 5     |
| Gakenke        |                  |                   |                |                      |               |                  |                   | 1             | 3           |                  |                  |                         | 4             |              |                   | 4     |
| Nyabihu        |                  |                   |                |                      |               |                  |                   | 1             | 1           |                  |                  |                         | 2             |              |                   | 2     |
| Rubavu         |                  |                   |                |                      |               |                  |                   | 1             | 1           |                  |                  |                         | 2             |              |                   | 2     |
| Musanze        |                  |                   |                |                      |               |                  |                   | 1             | 1           |                  |                  |                         | 1             |              |                   | 1     |
| **Total**      | 13               | 2                 | 5              | 1                    | 3             | 3                | 3                | 1             | 1           | 1                | 5                | 23           | 6             | 1             | 6     | 4            | 4            | 1            | 79    |
Table 3. Expression of the severity of the *Pythium* species on the bean varieties cultivated in Rwanda.

| Pythium sp. | Beans variety | *Pythium* species severity |
|------------|---------------|---------------------------|
|            |               | **P. arthronomos** | **P. chlamyphomon** | **P. cordito phorumn** | **P. cucurbitaceum** | **P. dicnum** | **P. diselocium** | **P. foliolumosum** | **P. indigo fase** | **P. pachycalce** | **P. rostafirgregens** | **P. spinosum** | **P. tolosum** | **P. ultimum** | **P. vexans** | **P. macroporum** | **P. rostratum** | **Disease expression of cultivars** |
| CAL 96     | 8.7 A         | 7.5 B                    | 8.7 A               | 8.1 A                   | 8.6 A                   | 7.0 C               | 8.2 BA              | 7.3 BA             | 8.0 B              | 8.4 A                   | 7.7 BA              | 8.7 A                   | 8.1 A                   | 8.1 A                   | 8.7 A | S         |
| G 2331     | 2.2 DC        | 2.7 C                    | 1.9 C               | 1.9 DC                  | 1.5 CD                  | 1.4 D               | 1.4 DE              | 1.5 C              | 2.2 D              | 1.6 ED                  | 1.6 C               | 1.9 DC                  | 2.1 B                   | 2.1 C                   | 2.3 C | R         |
| RWR 617-97A| 8.7 A         | 8.5 A                    | 8.6 A               | 7.2 B                   | 8.5 A                   | 7.7 BA              | 8.2 BA              | 7.6 BA             | 8.3 BA             | 8.1 C                   | 7.1 B               | 7.9 BA                  | 8.5 A                   | 8.3 A                   | 7.3 B | R         |
| URUGEZI    | 8.7 A         | 8.4 A                    | 8.9 A               | 8.5 A                   | 8.5 A                   | 8.2 A               | 8.6 A               | 7.7 A              | 8.7 A              | 8.4 BC                  | 8.2 A               | 8.3 A                   | 8.4 A                   | 8.5 A                   | 8.5 A | S         |
| RWR 1668   | 7.5 B         | 7.7 B                    | 8.2 B               | 6.7 B                   | 8.2 A                   | 7.2 BC              | 7.9 B               | 6.9 B              | 6.7 C              | 8.7 BA                  | 8.0 A               | 7.3 B                   | 8.7 A                   | 7.3 B                   | 7.5 B | S         |
| AND 1062   | 1.5 E         | 2.3 DC                   | 1.4 D               | 1.9 DC                  | 1.6 CB                  | 1.4 D               | 1.7 DC              | 1.3 C              | 1.9 ED             | 1.6 ED                  | 1.5 C               | 1.7 DC                  | 1.4 C                   | 1.8 DC                  | 2.1 DC | R         |
| MLB 40-89A | 2.3 C         | 1.9 DE                   | 1.3 D               | 1.6 DE                  | 1.9 CB                  | 1.4 D               | 1.6 DE              | 1.2 C              | 1.4 EF             | 1.7 ED                  | 1.4 C               | 1.9 DC                  | 1.4 C                   | 2.0 DC                  | 1.9 DCE | R         |
| VUNINKINGI | 1.5 E         | 1.4 E                    | 1.3 D               | 1.2 E                   | 1.1 D                   | 1.3 D               | 1.2 E               | 1.5 C              | 1.2 F              | 1.5 E                   | 1.3 C               | 1.4 D                   | 1.1 C                   | 1.5 D                   | 1.5 E | R         |
| AND 1064   | 2.1 DC        | 2.0 D                    | 1.5 DC              | 2.2 C                   | 2.0 B                   | 1.3 D               | 2.2 C               | 1.7 C              | 1.9 ED             | 1.9 D                   | 1.3 C               | 2.3 C                   | 1.5 C                   | 2.3 C                   | 1.9 DCE | R         |
| RWR 719    | 1.8 DE        | 1.9 DE                   | 1.2 D               | 1.7 DE                  | 1.8 CB                  | 1.4 D               | 1.6 DE              | 1.3 C              | 1.5 EF             | 1.8 ED                  | 1.3 C               | 1.6 D                   | 1.2 C                   | 1.5 D                   | 1.6 D | R         |
| SE         | 0.17          | 0.19                     | 0.19                | 0.19                    | 0.19                    | 0.19                | 0.19                | 0.14               | 0.17               | 0.21                    | 0.17               | 0.19                    | 0.19                    | 0.19                    | 0.16 | R         |
| F(9,288)   | < 0.0001      | < 0.0001                 | < 0.0001            | < 0.0001                | < 0.0001                | < 0.0001            | < 0.0001            | < 0.0001          | < 0.0001          | < 0.0001                | < 0.0001          | < 0.0001                | < 0.0001                | < 0.0001                | < 0.0001 | R         |

Means with the same letter within the same column are not significantly different. R: Resistant, S: Susceptible.

observed differences in the severity level recorded on the different bean varieties. As example, for the case of the *P. vexans* used isolate, the symptoms induced on the Uragezi variety were attributed a score of 8.5 while the symptoms induced on the varieties Vuninkingi and AND 1062 were respectively of 1.5 and 1.8. On the same sense, for the case of *P. spinosum*, the symptoms observed on the variety RWR 1668 were scored with 8.0 while the symptoms developing on the variety G2331 were estimated for a severity score of 1.6.

As shown by the data presented in Table 3, two main categories of varieties were differentiated as: (1) Resistant varieties, and (2) Susceptible varieties. In fact, the varieties AND 1062, MLB40-89A, Vuninkingi, AND 1064 and RWR 719 were shown to be highly resistant to root rot disease whatever the *Pythium* species isolate while the varieties CAL96, G2331, RWR617-97A, Uragezi and RWR 1668 exhibited a highly susceptible reaction to the different *Pythium* species inoculated on them. This observation is of great importance as, if a resistance is found there is a chance to have it effective against the different potential *Pythium* species prevailing in the country.

**DISCUSSION**

The isolation protocol used in this experience was perfroment as it allowed isolating several *Pythium* agents from the rotted samples collected areas where the bean root rot disease was prevailing. The PCR reaction was used to achieve molecular characterization of the obtained isolates. It is known that the PCR reaction allows amplifying the fungal ITS fragment with a *Pythium* typical size.
Figure 3. Aspect of root rot symptoms on bean plants grown on soil substrate previously contaminated by *Pythium* inoculum. A: Symptom development induced by inoculation of *P. vexans* on the susceptible reference variety (CAL 96). B: Absence of any root rot symptom on bean plant of the CAL 96 variety sown on a pathogen free substrate.

of 800 bp for the amplified product (Allain-Boule´ et al., 2004; Mahuku et al., 2007). Only 96 isolates over the whole 231 samples allowed generating a product of 800 bp. These results are correlated with observations performed by other authors who found that ITS region varied from 750 to 1050 bp (Allain-Boule´ et al., 2004; Lévesque and De Cock, 2004). To further characterize the isolates suspected to be *Pythium* agents, it was essential to proceed to sequencing the amplified ITS product in order to compare the generated sequences to those of reference *Pythium* species.

In fact, sequencing the ITS sequence constitutes a powerful tool for rapid identification of fungal species. In an investigation relative to identification of *Pythium* species populations affecting common beans in Uganda, Mukalazi et al. (2004) used the same ITS tool to establish the molecular profile of these pathogens (Packer and Clay, 2000; Paul, 2001, 2003).

In the frame of our study, it seemed logical to consider that the root rot symptoms revealed at the field level in Rwanda are induced by a diversity of agents including *Pythium* species. All the *Pythium* species obtained from the diseased samples collected in the various districts in Rwanda induced root rot symptoms when artificially inoculated to different bean varieties. In fact, it is known that major root rot pathogens on beans include other fungal species like *Fusarium*, *Rhizoctonia* and *Thielaviopsis* in addition to *Pythium* as well as the lesion nematode (*Pratylenchus* spp.) (Mazzola et al., 2002; Abawi and Ludwig, 2005; Haas and Défago, 2005). These pathogens may occur in single infections but in some cases, there is a possibility of mixed infections. Isolation protocols from some rotted bean roots did not allow obtaining *Pythium* colonies. This means that the disease symptoms were caused by other factors which could be in relation with other pathogens for example (Nderitu et al., 1997; Masaharu et al., 2006).

The isolated agents identified as belonging to *Pythium* spp. were classified according to their ITS sequences (Wang and White, 1997; Bakkeren et al., 2000). This molecular analysis showed that 16 *Pythium* species were found in the bean samples presenting root rot symptoms in Rwanda. Some of the identified species were previously identified as causing bean root rot disease in
different areas of bean production throughout the world. Similar results were described by Mukalazi (2004) in a study conducted in Uganda. Our results are comparable to those generated by Cilliers et al. (2000) and Harlton et al. (1995). In fact, 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 the *Pythium* species were not necessarily correlated to the host nor restricted in geographical range.

*P. vexans* was shown to be the most widespread *Pythium* species in the country as its presence was revealed with 23 isolates obtained from samples collected in 12 districts. These results are complementary to those published by Rusuku et al. (1997) who concluded that *Pythium* spp. were the most frequently isolated fungi and the widespread in Rwanda. Contrary to our findings, in a similar investigation performed by Green and Dan (2000), it was found that *Pythium ultimum* was the most widespread *Pythium* species that attacks a large number of plant species in Denmark (Mukalazi, 2004).

In our study, it was observed that there was no relationship between the geographic distribution and the *Pythium* species identification. In fact, some species were found under the main different categories of altitudes in Rwanda. This is the case for example of *P. vexans* which was found under three different altitudinal levels: high (1650 to 2300 m), intermediate (1400 to 1650 m) and low (900 to 1400 m). Globally, most of the represented species are found in different zones differing by their respective altitudes (Table 1). In the present situation, it is not yet known if this ubiquity is natural or due to movement of plant and soil by human activities (Opio, 1998; Mukalazi, 2004). For the first time, it was demonstrated through our study that in Rwanda, geographic distribution of *Pythium* spp. by district is variable according to the species. In that frame, *P. vexans* was considered as being the most wide spread in Rwanda as it was found in the highest number of districts where beans are grown.

Based on the data from the pathogenicity tests, it was concluded that all the *Pythium* species isolated in Rwanda were pathogenic on beans. These data confirmed also that the root rot symptoms previously observed on the sampled materials were due to *Pythium* agent. Moreover, there was an important variability of the bean variety reaction following inoculation with the *Pythium* species. In fact, for a given *Pythium* species, it was noticed that there was significant differences in the severity level recorded on the different bean varieties. For a given *Pythium* species, level of symptom severity was varying according to the inoculated variety. In the study carried out by Al-Sa’di et al. (2007), it had been demonstrated that there was an association of three pathogenic *Pythium* spp. inducing damping-off of greenhouse grown cucumber seedlings in Oman. Where the identification of *Pythium* to the species level was based on sequences of the internal transcribed spacer (ITS) of the ribosomal DNA of the 98 *Pythium* isolates collected during the survey (Allain-Boulé et al., 2004; Paul, 2003; Herrero and Klemasd, 1998).

According to our results, each of the tested bean varieties showed similar reactions to all the *Pythium* species used in this study. In other words, if a given bean variety was susceptible to one *Pythium* species, the same variety was susceptible to all the other *Pythium* species used in the present study.

The same observation have been recorded with the resistant varieties as if a given variety was resistant to one *Pythium* species, it was also resistant to all the other *Pythium* species. This is very important because after identifying a resistant variety, this one can be integrated into the strategy of controlling *Pythium* bean root rot whatever the region where beans are grown in Rwanda.

In a work aiming to characterize the inheritance of resistance to *Pythium* root rot in common beans, Buruchara et al. (2007) and Otusula et al. (2003) observed that resistance against *P. ultimum*, the most predominant species in their conditions, was of a dominant nature. If this property is confirmed in the case of our study, it should be easily undertaken a global breeding program to improve the level of resistance found in the most popular bean varieties in Rwanda. As the resistance to *Pythium* seems to be effective to various species of this genus, identification of some resistant varieties should constitute a preliminary and fundamental step prior to undertaking breeding strategies aiming at introgressing the resistance genes in the popular bean varieties.

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