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Full Length Research Paper

Pathogenic and molecular characterization of *Pythium* species inducing root rot symptoms of common bean in Rwanda

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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;

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Spence, 2003). Infected tissues become spongy, wet, discolored with many cavities. In addition to the previous symptoms, the disease is also characterized by lower leaf yellowing (similar to nitrogen deficiency), stunting, leaf browning and plant death (Pankhust et al., 1995; Ampaire, 2003).

The *Pythium* inducing agents produce several zoospores that enable them to rapidly and continuously re-infect 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 pulsesn(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 $CaCO_3$. 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 TES 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 proteinase K was added and the mixture incubated at 65ºC 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 $MgCl_2$, 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 94ºC 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 Seqmann program (DNASTAR), by performing a nucleotide-nucleotide blast search at the National Center for Biotechnology Information (NCBI) website: http://www.ncbi.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 Tree View 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 × 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, Urugezi, R617-97A, RWR 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. Severity 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 one 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

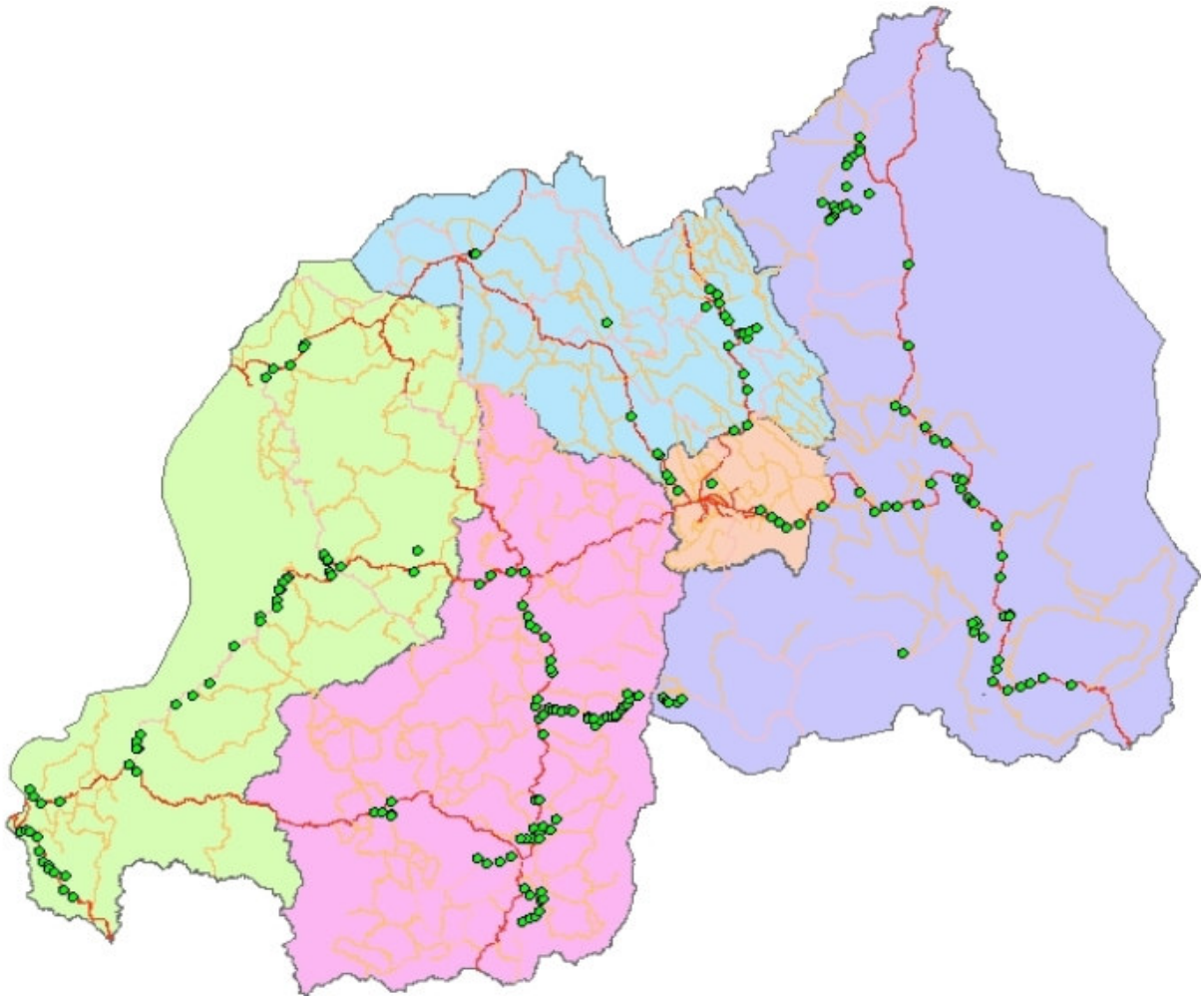

Figure 1. Map of Rwanda showing the places where the samples have been collected during our root rot survey. Different colors shows four provinces (North, South, East, West and Kigali City which has orange color and located in the center of the country). The green spots represent the places where the 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°39'21,1"	029°45'23,4"	1697	18.3	Huye	8HYE a	*Pythium macrosporum*
02°38'59,3"	029°46'38,1"	1697	19.7	Gisagara	12 GIS	*Pythium rostratifingens*
02°39'21,1"	029°45'23,4"	1689	14.2	Gisagara	16 GIS	*P. rostratifingens*
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.

02°12'12,9"	029°15'4,3"	1716	19.4	Karongi	130 KNGI	*Pythium vexans*
02°16'10,2"	029°12'31,5"	1560	20.0	Nyamasheke	37 NSKE	*P. vexans*
02°22'31"	029°05'8"	1598	22.3	Nyamasheke	42 NSKE	*P. spinosum*
02°23'23,6"	029°05'11,1"	1595	23.3	Nyamasheke	43NSKE	*P. diclinum*
02°27'49,2"	028°54'11,6"	1929	22.7	Rusizi	46 RSZb1	*P. spinosum*
02°29'9,1"	028°57'6,9"	1915	22.6	Rusizi	46 RSZb2	*P. spinosum*
02°28'29,2"	028°54'23,8"	1618	24.3	Rusizi	46 RSZ	*P. diclinum*
02°32'14,9"	028°53'41,6"	1659	22.1	Rusizi	49 RSZI a	*Py. rostratifingens*
02°32'19,1"	028°53'13,4"	1657	20.8	Rusizi	49 RSZb	*P. rostratifingens*
02°33'1"	028°54'50,2"	1819	24.0	Rusizi	54 RSZ	*Pythium arrhenomanes*
02°36'4"	028°55'59"	1755	23.0	Rusizi	56 RSZ	*P. diclinum*
02°30'20,6"	029°29'23,2"	2205	20.9	Nyamagabe	64 NGBE	*P. rostratifingens*
02°30'28"	029°31'11,9"	2114	21.2	Nyamagabe	66 NGBE	*Pythium indigoferae*
02°30'35,8"	029°31'8,6"	2112	22.5	Nyamagabe	67 NGBE	*P. conidiophorum*
02°29'5,1"	029°31'6,6"	2121	22.1	Nyamagabe	77 NGBE	*Pythium pachycaule*
02°19'48,1"	029°46'41,9"	1774	23.9	Nyanza	58 NYA	*P. folliculosum*
02°19'6,7"	029°49'29"	1584	24.6	Nyanza	75 NYA	*P. vexans*
02°20'49,1"	029°52'12,5"	1598	23.9	Nyanza	79 NYA	*Pythium folliculosum*
02°19'56,3"	029°53'16,1"	1422	23.4	Nyanza	82 NYA	*P. vexans*
02°19'56"	029°54'8,8"	1395	25.0	Nyanza	84 NYA	*P. folliculosum*
02°18'49,4"	029°54'55,4"	1437	23.6	Nyanza	87NYA	*P. folliculosum*
02°18'35,5"	029°55'25,7"	1435	23.9	Nyanza	88 NYA	*P. vexans*
02°17'59,1"	029°55'30,3"	1456	24.2	Nyanza	89NYA	*Pythium rostratum*
01°18'9"	029°59'20,4"	1452	21.5	Bugesera	92 BGSR	*P. folliculosum*
01°18'9"	029°59'20,7"	1452	24.3	Bugesera	93 BGSR	*P. vexans*
01°18'23,7"	030°00'38"	1522	26.1	Bugesera	94 BGSR	*P. ultimum*
01°18'23,7"	030°00'39"	1522	20.6	Bugesera	95 BGSR	*P. vexans*
01°17'56,6"	030°00'58,5"	1498	22.4	Bugesera	96 BGSR	*P. vexans*
01°57'30,4"	030°09'4,7"	1372	22.1	Gasabo	97 GSB b	*P. vexans*
01°58'7,7"	030°10'6,1"	1366	22.2	Gasabo	97GSBa	*P. vexans*
01°58'40,9"	030°10'54,8"	1354	23.2	Gasabo	98 GSB	*P. vexans*
01°59'17,5"	030°11'39,7"	1345	23.9	Gasabo	98 GSBii	*P. vexans*
01°58'53,5"	030°12'58,5"	1329	23.6	Gasabo	101 GSB	*P. vexans*
01°54'29,6"	030°26'33,8"	1514	25.3	Rwamagana	108 RWM	*P. vexans*
01°54'6,4"	030°29'42,8"	1598	25.4	Kayonza	110 KYNZA	*P. vexans*
01°55'11,1"	030°29'39,1"	1563	26.3	Kayonza	111 KYNZA	*P. rostratifingens*
02°11'14,5"	030°31'49,4"	1636	25.6	Ngoma	117 NGM	*P. vexans*
02°09'52"	030°31'18,4"	1679	25.6	Ngoma	120 NGM	*Pythium chamaehyphon*
02°09'39,2"	030°30'49,3"	1669	25.4	Ngoma	122 NGM	*P. indigoferae*
02°08'40,7"	030°34'30,8"	1684	25.0	Ngoma	124 NGM	*P. vexans*
02°12'57,2"	030°23'35,4"	1330	20.5	Ngoma	126 NGM	*P. vexans*
2°14'39,1"	030°33'17,3"	1373	17.8	Ngoma	128NGM	*P. vexans*
2°15'41,4"	030°38'5,4"	1570	18.1	Kirehe	133 KRHa	*P. vexans*
2°16'23"	030°40'59,4"	1627	20.5	Kirehe	133KRHb	*P. vexans*
1°18'3,7"	030°19'15,1"	1359	28.9	Nyagatare	143 NGTR	*P. conidiophorum*
1°18'20,6"	030°19'10,4"	1359	28.3	Nyagatare	145 NGTR	*P. rostratum*
1°24'22,6"	030°16'28,6"	1370	27.0	Nyagatare	149 NGTR	*P. vexans*
1°24'58,7"	030°16'48,4"	1375	26.4	Nyagatare	151 NGTR	*P. vexans*
1°25'41,9"	030°16'20,7"	1374	27.4	Nyagatare	153 NGTR	*P. ultimum*
1°24'45,3"	030°18'46"	1438	28.3	Nyagatare	158 NGTRb	*P. vexans*
1°44'24,5"	030°07'42,2"	1518	23.7	Gicumbi	162 GCMB	*P. vexans*
1°38'50,5"	030°07'41,3"	2098	25.5	Gicumbi	166 GCMB	*P. diclinum*

Table 1. Contd.

1°36'14,8"	030°05'26,3"	1962	23.7	Gicumbi	171 GCMB	*Pythium chamaehyphon*
1°33'21,6"	030°03'53"	1844	24.0	Gicumbi	173 GCMB	*Pythium cucurbitacearum*
1°55'14,3"	030°00'31,5"	2240	24.9	Nyarugenge	177 NGGEa	*P. vexans*
1°54'32,8"	030°04'0,1"	1743	25.8	Nyarugenge	178 NGGE	*P. torulosum*
1°53'31,8"	029°59'20,6"	1783	26.7	Nyarugenge	180 NGGE	*P. vexans*
1°51'32,3"	029°58'42,7"	1953	25.7	Rulindo	182 NGGE	*P. vexans*
1°51'18,9"	029°58'29,6"	1959	24.8	Rulindo	183 RNDO	*P. dissotocum*
1°47'18,4"	029°55'48,1"	1967	25.7	Rulindo	184 RNDO	*Pythium indigoferae*
1°39'46,1"	029°22'6,4"	2180	27.7	Nyabihu	187 NYAB	*P. vexans*
1°29'34,3"	029°39'41,8"	1715	27.0	Musanze	204 MNZE	*P. diclinum*
1°31'52.6"s	029°35'21.7"	1821	25.1	Burera	188 BRR	*P. ultimum*
1°24'06.9"s	029°44'17.1"	2044	18.4	Burera	189 BRR	*P. rostratum*
02°08'5,5"	029°19'23,2"	1854	21.2	Musanze	207MNZE	*P. vexans*

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.

Pathogenecity

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

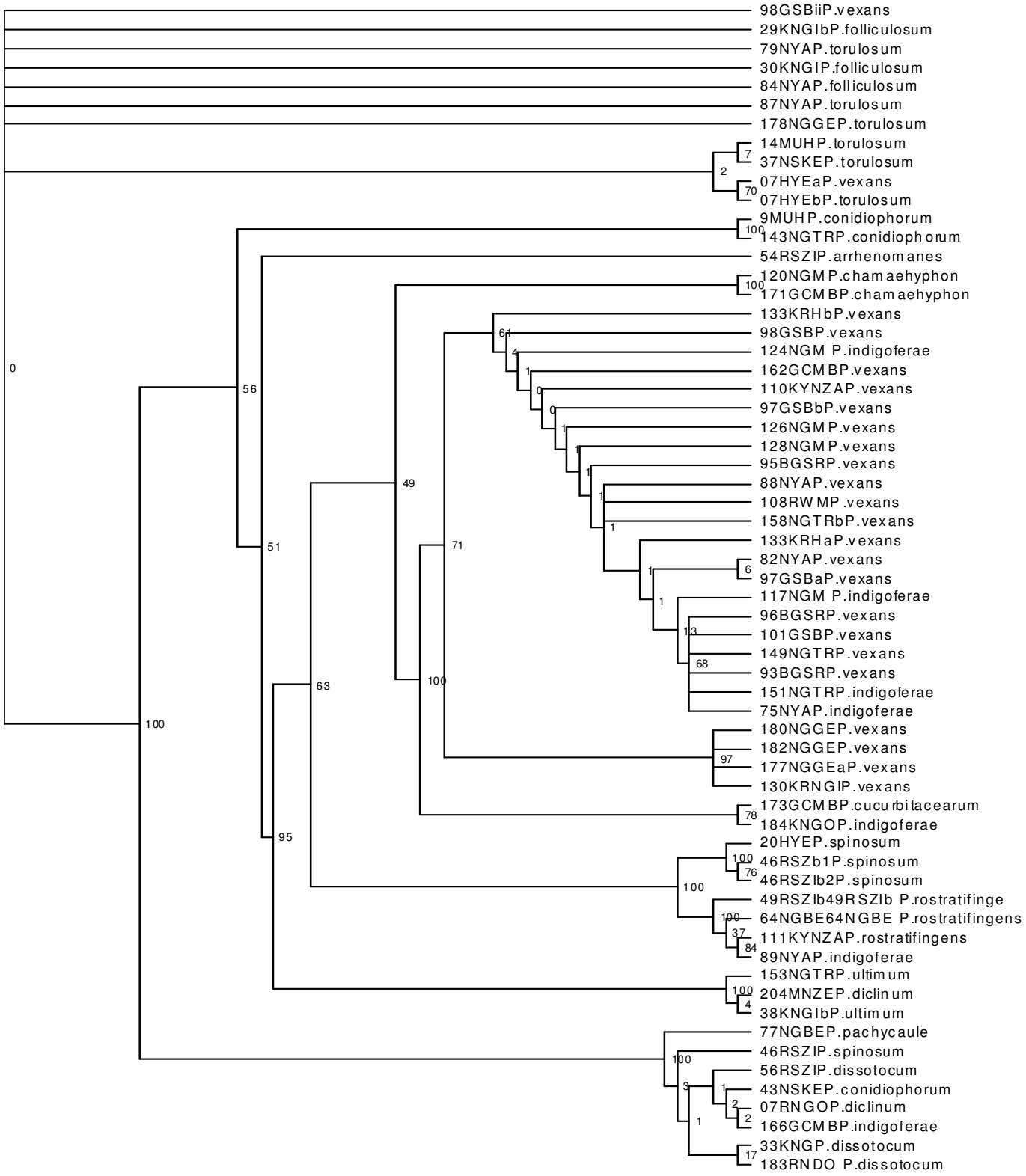

Figure 2. Phylogenetic relationship of *Pythium* spp. from Rwanda based on the ITS ribosomal DNA sequences. The codes following number are relative to district of origin (RNDO: Rulindo; KNG: Karongi, GCMB: Gicumbi, RNGO: Ruhango, RSZI: Rusizi, NSKE: Nyamasheke, NGBE: Nyamagabe, KNGI: Karongi, MNZE: Musanze, NGTR: Nyagatare, NYA: Nyanza, KYNZA: Kayonza, HYE: Huye, NGGE: Nyarugenge, BGSR: Bugesera, GSB: Gasabo, NGM: Ngoma, KRH: Kirehe, MUH: Muhanga, RWM: 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. cucurbitacearum*	*P. diclinum*	*P. conidiophorum*	*P. arrhenomanes*	*P. pachycaule*	*P. ultimum*	*P. vexans*	*P. folliculosum*	*P. macrosporum*	*P. rostratifingens*	*P. spinosum*	*P. dissotocum*	*P. rostratum*	*Total*
Huye			2									1		1		1	5
Gisagara										1			2				3
Nyanza	2		1							3	4						10
Karongi	1								2	1	1				1		6
Muhanga			1			1											2
Ruhango					1												1
Nyamasheke										1				1			2
Rusizi							1						2	2	2		7
Nyamagabe	1					1		1					1				4
Bugesera									1	3	1						5
Gasabo										3							3
Rwamagana																	
Kayonza										1			1				2
Ngoma	4									2							6
Kirehe										1							1
Gatsibo																	
Ngoma	4	1															5
Nyagatare	1					1			1	2							5
Rurindo										1					1		2
Gicumbi		1		1	1					1							4
Nyarugenge			1						1	3							5
Gakenke																	
Nyabihu																	
Rubavu																	
Musanze					1												1
Total	13	2	5	1	3	3	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																Disease expression of cultivars
	P. arrhenomanes	*P. chamaehyphon*	*P. conidio phorum*	*P. cucurbitacearum*	*P. diclinum*	*P. dissotocum*	*P. folliculosum*	*P. indigo ferae*	*P. pachycaule*	*P. rostratifingens*	*P. spinosum*	*P. torulosum*	*P. ultimum*	*P. vexans*	*P. macrosporum*	*P. rostratum*	
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	8.8 A	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	2.1 C	R
RWR617-97A	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	7.9 B	S
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	8.4 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	7.8 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	1.6 DE	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 DC	1.9 DCE	1.7 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	1.3 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	2.2 C	1.9 DC	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 C	1.6 DE	R
SE	0.17	0.19	0.15	0.19	0.16	0.19	0.17	0.23	0.19	0.14	0.17	0.21	0.17	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	< 0.0001	

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 Urugezi 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, Urugezi 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 performent 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 to1400 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 Klemsdal, 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 Otsyula 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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