COMMUNAUTE FRANCAISE DE BELGIQUE
ACADEMIE UNIVERSITAIRE WALLONIE-EUROPE
UNIVERSITE DE LIEGE GEMBLOUX AGRO-BIO TECH

Identification of *Pythium* species inducing common bean (*Phaseolus vulgaris* L.) root rot symptoms and development of backcrosses to improve the level of varietal resistance to this disease

NZUNGIZE RUSAGARA John

Dissertation originale présentée en vue de l'obtention du grade
de Docteur en sciences agronomiques et ingénierie biologique

Promoteur : Pr. Jean Pierre BAUDOIN
Co-Promoteur : Dr. Jean Pierre BUSOGORO

2012

RESUME

NZUNGIZE R. J. (2012). Identification des espèces de *Pythium* induisant les symptômes de la maladie racinaire du haricot commun (*Phaseolus vulgaris*) et le développement des rétrocroisements pour améliorer le niveau de résistance variétale à cette maladie. **Thèse de Doctorat. Belgique : Université de Liège, Gembloux Agro-Bio Tech, 119p., 6 fig., 7 tab.**

Le haricot commun (*Phaseolus vulgaris* L.) est la légumineuse comestible la plus cultivée dans le monde. Il constitue une culture prioritaire dans divers pays d'Afrique de l'Est et est cultivé principalement par des petits fermiers pour leur consommation domestique, l'excédent étant vendu sur les marchés.

D'importantes pertes de rendement du haricot commun provoquées par des maladies racinaires ont été relevées dans plusieurs pays de l'Afrique de l'Est y compris le Rwanda. Cette situation s'explique en partie par l'intensification de la culture des haricots, l'absence de rotations, la pratique de la culture continue de cette légumineuse et la très forte diminution de la fertilité des sols. Bien que les maladies racinaires du haricot soient causées par divers pathogènes qui se développent dans le sol en fonction des conditions environnementales, les espèces de *Pythium* sont les pathogènes fongiques les plus fréquemment liés aux épidémies graves dans les divers pays de l'Afrique de l'Est. Les études sur les maladies racinaires ont indiqué que la culture continue des haricots, une pratique courante en Afrique orientale, aggrave le problème.

Le présent travail a été entrepris afin d'améliorer la résistance variétale du haricot commun pour limiter les dégâts causés par *Pythium* au Rwanda. Une analyse de la diversité des espèces de *Pythium* liées aux pourritures racinaires a été réalisée grâce à des collectes d'échantillons de plantes du haricot commun dans tout le pays et grâce à la caractérisation des espèces de *Pythium* provoquant ces pourritures racinaires. A partir des échantillons racinaires montrant des symptômes de pourritures racinaires 96 isolats de *Pythium* ont été isolés. Leur caractérisation moléculaire sur base de séquences moléculaires de la zone ITS de l'ADN ribosomique a permis de les classer en 16 espèces de *Pythium*. L'étude de la distribution de chacune des espèces identifiées au sein des échantillons ainsi analysés a montré que *Pythium vexans* est le taxon le plus répandu dans les diverses zones produisant le haricot commun au Rwanda.

Des tests de pathogénicité des 16 espèces de *Pythium* identifiées ont été effectués sur un ensemble de 10 variétés de haricot commun. Les résultats ont révélé que toutes les espèces de

Pythium identifiées étaient pathogènes sur le haricot commun : elles induisaient toutes des symptômes de pourritures racinaires en conditions contrôlées. Cependant, les variétés de haricot utilisées dans cette étude ont montré des différences en termes de sévérité des symptômes suite à l'inoculation avec chacune des 16 espèces de *Pythium*.

A l'issue de ces travaux de caractérisation des espèces de *Pythium* isolés au Rwanda, un schéma d'amélioration de la résistance variétale a été mis en œuvre. Il est basé sur un protocole de rétrocroisements et l'assistance d'un marqueur moléculaire (PYAA 19_{800}). Les parents récurrents sont composés de trois variétés de haricot traditionnellement cultivées au Rwanda tandis que les deux parents donneurs sont une variété résistante du pool génique mésoaméricain et une variété résistante du pool andin. La descendance obtenue à l'issue de ce programme de rétrocroisements a été soumise à des essais de pathogénicité par inoculation avec une souche de *Pythium ultimum* en conditions contrôlées afin de vérifier l'efficacité de ce protocole d'amélioration. Ces essais ont montré que dans la descendance tous les individus manifestant la présence du gène marqueur étaient également résistants à *Pythium* avec des niveaux de sévérité très faibles à l'issue des tests d'inoculation.

Mots clés: Haricot, *Phaseolus vulgaris*, *Pythium,* Rwanda, Caractérisation et Introgression.

SUMMARY

NZUNGIZE R. J. (2012). Identification of *Pythium* species inducing common bean (*Phaseolus vulgaris* L.) root rot symptoms and development of backcrosses to improve the level of varietal resistance to this disease. **Philosophiae Doctor (PhD) Thesis. Belgium: Liege University Gembloux Agro-Bio Tech, 119p., 6 fig., 7 tab.**

The common bean (*Phaseolus vulgaris* L.) is the most important food legume grown worldwide. It is a prioritary crop in various countries of East Africa and is grown mainly by small farmers for home consumption, the excess being sold in markets.

Important yield losses of common bean induced by root rot diseases have been identified in several countries in East Africa including Rwanda. This is partly explained by the intensification of the cultivation of beans, the absence of rotations, the practice of continuous cultivation of this legume, and decline in soil fertility. Although bean root rot symptoms are caused by a number of soil borne pathogens depending on environmental conditions, *Pythium* spp. are the fungal pathogens most frequently associated with severe epidemics in eastern Africa. Studies on root rot have indicated that continuous cropping of beans, a common practice in eastern Africa, exacerbates the problem.

This work was undertaken to improve the varietal resistance of common bean to limit the damages caused by *Pythium* in Rwanda. An analysis of the diversity of *Pythium* species associated with root rot was conducted through collect of samples of common bean plants throughout the country and through the characterization of the *Pythium* species causing root rot.

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. Molecular characterization using the ITS-DNA sequences was also carried out on samples isolated on infected beans roots. The study of the distribution of each species identified within the samples analyzed, revealed that *Pythium vexans* is the most widespread taxon in the different common bean producing areas in Rwanda.

Pathogenicity tests of the 16 identified *Pythium* species were performed on a set of 10 common bean varieties. The results showed that all identified *Pythium* species were pathogenic to common bean: they all induce symptoms of root rot under controlled conditions. However, the common bean varieties used in this investigation showed differences in their reaction to inoculation with the 16 *Pythium* species.

At the end of this work of the characterization of *Pythium* species isolated in Rwanda, a scheme for improving varietal resistance has been implemented. It is based on a backcross protocol assisted by molecular marker (PYAA 19_{800}). Recurrent parents are composed of three common bean varieties traditionally grown in Rwanda while the two donor parents are a resistant variety of the Mesoamerican gene pool and a resistant variety of Andean pool.

The progeny obtained after the backcrossing program was subjected to the pathogenicity trials by inoculating with a strain of *Pythium ultimum* in controlled conditions in order to verify the effectiveness of this improvement protocol. These trials have shown that in the offspring all the individuals showing the presence of the marker gene were also resistant to *Pythium* with very low levels of severity at the end of inoculation tests.

Key world: **Beans, *Phaseolus, vulgaris*, *Pythium,* Rwanda, Characterization and Introgression.**

Dédicace

A la mémoire de mes regrettés parents HABIYAMBERE Euphrasie et GATWAZA Mathias, et de mon frère Niyomugabo Rusimbi Damien.

A mes frères et sœurs qui ont assuré mon éducation après la mort de mes parents qui nous ont quitté lorsque j'étais trop jeune.

A mon épouse Mutajogire Nyinawase Marie Jeanne et à nos enfants, Michael, Gabriel et Belinda Nzungize.

REMERCIEMENTS

Ce travail n'aurait pas été réalisé si je n'avais pas bénéficié des soutiens financiers des Institutions suivantes: Coopération Technique Belge (CTB), Institut des Sciences Agronomiques du Rwanda (ISAR) et Kirkhouse Trust (KT). Qu'elles reçoivent ici le témoignage de ma profonde gratitude.

Je tiens à remercier le professeur Jean-Pierre Baudoin, promoteur, pour m'avoir donné la possibilité de réaliser cette recherche au sein de son unité, m'avoir guidé avec rigueur et un esprit critique pendant la réalisation de ce travail de recherche. Ses qualités scientifiques et humaines ont largement contribué à l'aboutissement de cette thèse.

Je tiens aussi à exprimer ma profonde reconnaissance au Docteur Jean-Pierre Busogoro, co-promoteur, pour m'avoir accordé sa confiance et m'avoir guidé dans mes réflexions scientifiques tout au long de ce travail. Que cette thèse soit le témoignage de toute ma gratitude pour ses précieux conseils et surtout pour sa disponibilité.

Je tiens à remercier vivement Docteur Robin Buruchara, pour sa confiance, sa disponibilité, ses conseils judicieux et surtout pour m'avoir accueilli dans son laboratoire.

Mes remerciements sont également adressés aux Professeurs André Toussaint et Pierre Bertin pour m'avoir fait l'honneur d'accepter d'être rapporteurs de ce travail. Je remercie également les Professeurs Guy Mergeai et Hugo Magein pour leur participation à mon jury de thèse. Qu'ils trouvent ici l'expression de ma plus haute considération.

Je voudrais vivement remercier tous les membres du département de « CIAT, Pan Africa Bean Research Alliance » basé à Kampala pour leur accueil si chaleureux, pour leur disponibilité et leur immense gentillesse. Je garderai un merveilleux souvenir de ces années passées parmi eux.

Je remercie également les membres du Programme haricot de l'ISAR qui m'ont aidé pendant l'échantillonnage des racines de haricot au Rwanda et qui ont mis en ma disposition les semences de haricot qui ont été utilisées dans ce travail.

Je voudrais témoigner ma gratitude à tous les membres du Centre d'Information et de Communication Agricoles (CICA) du Rwanda pour l'hospitalité et les encouragements qu'ils m'ont réservé pendant mon séjour à Kigali.

Mes remerciements s'adressent également à tout le personnel de l'Unité de Phytotechnie Tropicale et Horticulture pour leur sympathie et leur disponibilité lors de mes nombreuses sollicitations.

Les familles de mes cousines installées en Belgique m'ont été d'une très grande utilité pendant le temps que j'ai passé en Belgique, qu'elles reçoivent le témoignage de ma gratitude. Mon séjour à Kampala a été confortable grâce à l'accueil et la compagnie que m'ont réservé Mr et Mme Munyakazi, qu'ils reçoivent le témoignage de ma profonde gratitude.

Je remercie également ma belle famille et ma famille pour avoir pris soin de ma femme et de mes enfants pendant mes longs moments d'absence.

A toutes les personnes qui de près ou de loin qui ont contribué à l'aboutissement de ce travail, j'adresse mes sincères remerciements.

Enfin, je réserve ma plus grande et profonde gratitude à mon épouse Mutajogire Nyinawase Marie Jeanne et nos enfants, Michael, Gabriel et Belinda Nzungize, pour leur soutien moral et la chaleur humaine qui ont contribué à me réconforter pendant les moments difficiles de ce travail.

Conférences scientifiques internationales ayant accepté l'exposé de résultats obtenus dans le cadre de cette Thèse

African Bea Consortium (ABC) annual meeting and training course of the Kirkhouse Trust (KT) funded ABC, "Marker **Use-A step by step process**" held on the 17th and 18th May 2010 at Sokoine University of Agriculture (SUA), Morogoro, Tanzania (**Poster**).

The 3rd National University of Rwanda International Conference on Food and Nutrition Security and Integrated Pest Management, November 2-4, 2010, Huye, Rwanda (**Oral presentation**).

World Academy of Science, Engineering and Technology, NH Naarden, July 13-15, 2011 Amsterdam, the Netherlands (**Oral presentation**).

4th Annual International Symposium on Agricultural Research to be held in Athens, Greece on 18-21 July 2011(**Oral presentation**).

Participation aux conférences scientifiques internationales

Modern Breeding Techniques for Improvement of Leguminous Plants organized by the Institute Plant Biotechnology for Developing Countries, August 18th -28th 2009, University of Gent, Gent, Belgium.

Second International Symposium on Multi-strata Agroforestry Systems with Perennial Crops: Making ecosystem services count for farmers, consumers and the environment, September 17th - 21st, 2007 - CATIE - COSTA RICA (**Poster**).

Sustainable Agriculture productivity for improved food security and livelihoods: Proceedings of National Conference on Agricultural Research Outputs, 26th-27th March 2007, Serena Hotel, Kigali, Rwanda, ISAR, 673 P, E20 (**Oral presentation**).

Abbreviations

BLAST: Basic Local Alignment Search Tool

CIAT: Centro Internacional de Agricultura Tropical

CMA: Corn Meal Agar

CRBD: Completely Randomized Block Design

DNA: Deoxyribonucleic acid

dNTPS: deoxyribonucleotide triphosphate

ITS: internal transcribed spacers

MAB: Marker assisted backcrossing

MAS: Marker assisted selection

NCBI: National Center for Biotechnology Information

PCR: Polymerase Chain Reaction

PDA: Potato Dextrose Agar

rDNA: Ribosomal Deoxyribonucleic acid

RNA: Ribonucleic acid

rRNA: Ribosomal ribonucleic acid

SCAR: Sequence Characterized Amplified Region

TABLE OF CONTENTS

CHAPTER I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is an important legume grown worldwide for its high nutritional and economic value (Sarikamis *et al*., 2009). It is cultivated in the tropical, subtropical and temperate zones. Among the legume species, common bean is the most widely distributed species in the different production areas throughout the world. In fact, it occupies more than 90% of production areas sown with *Phaseolus* species (Baudoin *et al*., 2001).

In most production areas, common beans are considered to be a very important food as they are nutrient dense with high contents of protein, micronutrients, vitamins, dietary fibers, and also have a low glycemic index. Legume seeds contain ω-3 which helps preventing cancer (Scarmeas *et al*., 2006; Scarafoni *et al*., 2007; Zhang, 2007; Tang *et al*., 2008; Villegas *et al*., 2008). The crop is also an important source of income throughout sub-Saharan Africa, especially for women who grow it both for subsistence and sale to urban populations (CIAT, 1995).

At the agro-ecological level, this food legume is cultivated in various environments ranging from sea level to more than 3,000 meters. The production is mainly ensured by small farmers with average land holdings of less than one hectare and using poor technologies like the lack of irrigation and limited use of fertilizers or pesticides. Additionally, most of the bean production in Latin America and sub-Saharan Africa, where three- quarters of the crop is grown, takes place on steep, erosion-prone slopes with low soil fertility (CIAT, 2006).

In terms of productivity, it is observed that in developing countries, the average grain yield of common bean varieties is still very low. It is due to combination of various factors that this level of production remains restricted. In fact, it is generally considered that the use of poor management practices of crop husbandry, restricted improved cultivar adoption and

susceptibility to biotic and abiotic stresses contribute to limiting the level of production reached in legume production (Sharma and Lavanya, 2002; Bayuelo-Jiménez *et al*., 2003; Rainey and Griffiths, 2005).

Among the biotic constraints hampering the productivity of common bean, the common bean root rot disease caused by *Pythium* spp is considered as a main constraint. This disease is considered as being the most damaging disease for common bean production in Eastern and Central Africa including Rwanda. More specifically, the bean production system in Rwanda is characterized by a series of different specific conditions where common bean varieties are grown under high demographic pressure and with a very limited use of crop rotation under lower soil fertility (Wortmann *et al*., 1998; Graham *et al*., 2003).

Although several measures have been used to control root rot, none has allowed solving definitively the constraint. Common bean root rot disease management has been possible to some extent only through the use of a combination of control options (cultural, chemical, and biological) which utilize the concept of Integrated Pest Management (Otsyula *et al*., 2005; Abawi *et al*., 2006).

Among the different control strategies to be taken into consideration for the use of the Integrated Pest Management option, the use of resistant or tolerant bean varieties has to be part of the global control scheme to increase the success of the IPM control. It is thus important to make sure that there is an access and adoption, especially for the resource poor farmers, of common bean varieties that are resistant to the most common soil-borne pathogen(s) occurring in the production region (Abawi *et al*., 2006). Unfortunately, popular commercial bean varieties currently grown in Rwanda are susceptible to the prevailing root rot pathogens, while known resistant varieties are associated with undesirable characteristics such as late maturity, black seed colour, and small seed size (Otsyula *et al*., 1998).

To design and assess management practices and to develop host resistance to the important pathogens of common bean root rot, studying the biological structure and spatial distribution of these pathogens is a prerequisite condition for a successful implementation of the control strategy including deployment of resistant varieties.

The present work was undertaken to reduce the damage caused by *Pythium* root rot in Rwanda by increasing resistance level to the disease in the most popular common bean varieties grown in this country. To contribute to this general objective, a preliminary characterization of the *Pythium* agents responsible for common bean root rot symptoms prevailing in Rwanda was performed prior to setting up a breeding strategy aiming at increasing the level of resistance to *Pythium* root rot. This preliminary investigation of *Pythium* characterization is essential prior to development of a breeding strategy as it is crucial to take into consideration the pathogen population structure if resistant varieties are to be developed and diffused in view of controlling bean root rot. On this basis, the specific objectives of the present study were as follows:

1. to describe the genetic diversity of *Pythium* species associated with *Pythium* root rot of common beans in Rwanda.
2. to test the pathogenicity of the characterized *Pythium* species on different popular local common beans varieties (R617-17A, RWR 1668 and Urugezi) and on introduced common beans genotypes (RWR 719 and AND 1062).
3. to develop and evaluate the efficiency of a breeding strategy based on introgression of resistance genes into different common bean popular varieties grown in Rwanda.

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CHAPTER II

PYTHIUM ROOT ROT OF COMMON BEAN: BIOLOGY AND CONTROL METHODS

Inpress, submitted for publication in “Biotechnologie, Agronomie, Société et Environnement”

PYTHIUM ROOT ROT OF COMMON BEAN: BIOLOGY AND CONTROL METHODS
POURRITURE RACINAIRE DU HARICOT COMMUN CAUSEE PAR *PYTHIUM*: BIOLOGIE ET METHODES DE LUTTE

J. NZUNGIZE[1*], F. LYUMUGABE[2], J. P. BUSOGORO[3] and J. P. BAUDOIN[1].

Authors' addresses: [1] University of Liege, Gembloux Agro-Bio Tech, Belgium; [2] National University of Rwanda, Butare, Rwanda; [3] Belgium Technical Cooperation, IPM project, Kigali, Rwanda.

Acknowledgment

The authors would like to express their warmest thanks to the Belgian Technical Cooperation, the Kirkhouse Trust and the Rwanda Agricultural Research Institute for financial support.

*Corresponding author:

1. NZUNGIZE RUSAGARA John

Tropical crop husbandry and Horticulture

2, passage des déportés, 5030 Gembloux - Belgique

Telephone : +3281622410

Fax : +32 81 61 45 44

E-mail: nzungizej@gmail.com

2. National University of Rwanda, Biotechnology Unit.

P. O. Box: 117 Butare, Rwanda. E.mail: flyumugabe@nur.ac.rw

3. Belgium Technical Cooperation, IPM project, Kigali, Rwanda. jpbusogoro@yahoo.fr

PYTHIUM ROOT ROT OF COMMON BEAN: BIOLOGY AND CONTROL METHODS

POURRITURE RACINAIRE DU HARICOT COMMUN CAUSEE PAR *PYTHIUM*: BIOLOGIE ET METHODES DE LUTTE

Abstract

Pythium root rot constitutes a highly damaging constraint on the common bean, *Phaseolus vulgaris* L., grown in several areas of Eastern and Central Africa where this food legume is cultivated intensively under poor conditions of crop rotation due to the land exiguity in the region. Yield losses of up to 70% in traditional local bean cultivars have been reported in Kenya and Rwanda. In this study, a detailed analysis on the biology and diversity of *Pythium* genus has been carried out to understand the mechanisms leading to disease development. To reduce the damages provoked by this disease, various control methods were analysed.

Key words: *Phaseolus, vulgaris*, *Pythium*, Root rot, Bean, Resistance

Résumé

La pourriture racinaire causée par *Pythium* constitue une importante contrainte pour la production du haricot commun (*Phaseolus vulgaris* L.) dans plusieurs régions de l'Afrique Centrale et Orientale où la production du haricot est intense dans des conditions de non respect des schémas de rotation à cause de l'exiguïté des terres. Des pertes de rendement allant jusqu'à 70% au sein des cultivars locaux traditionnels de haricot ont été rapportées au Kenya et au Rwanda. Dans ce travail, une analyse détaillée de la biologie du genre *Pythium* et de sa diversité a été conduite pour comprendre les mécanismes qui mènent au développement de la maladie. Pour réduire les dégâts provoqués par ce pathogène, diverses méthodes de contrôle ont été analysées.

Mots clés: *Phaseolus, vulgaris*, *Pythium*, Pourriture, racinaire, Haricot, Resistance.

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most widely cultivated food legume species all over the world (Baudoin et al., 2001). The crop is exploited in a wide range of areas extending from around 52^{o} North latitude to 32^{o} South latitude and from sea level to 3000 m above sea level, displaying great variation in growth habits and length of vegetation (FAO, 2005).

Practically, common bean is a major source of low cost calories, proteins, dietary fibers, minerals and vitamins for poor populations (Hillocks et al., 2006). In Rwanda, common beans provide up to 25% of the total calories and 45% of the total dietary proteins which is considered as being the highest percent in the world (Pachico, 1993).

Pythium species are spread worldwide (Paul, 2004).The importance of *Pythium* bean root rots has increased in several countries of East and Central Africa like Burundi, Democratic Republic of Congo, Kenya and Uganda (Otsyula et al., 2003). For example in Western Kenya and in Rwanda, many farmers stopped growing beans between 1991 and 1993 due to a severe outbreak of root rots that caused serious food shortages and price increases beyond the reach of many resource poor households (Nekesa et al., 1998).

In tropical regions, common bean is characterized by low and unstable grain yields due to various ecological and agronomic parameters. Among them, bean root rot and decline of soil fertility have been cited as being among the major causes leading to bean yield losses (Miklas et al., 2005).

In Rwanda, western Kenya and south western Uganda, *Pythium* spp. are the fungal pathogens most frequently associated with severe root rot epidemics (Rusuku et al., 1997). Yield losses of up to 70% in commercial bean cultivars have been reported in Rwanda and Kenya (Otsyula et al., 2003). In view of understanding the biology of root rot diseases, a study was conducted in Kenya, Rwanda and Uganda (Mukalazi, 2004) to identify the causal agents. In these countries the following species were isolated from bean samples affected by root rot symptoms: *P. nodosum* Bhatn, *P. echinulatum* Matthews, *P. pachycaule* Shtayeh, *P. oligandrum* Drechsler, *P. acanthicum* Drechsler, *P. chamaehyphon* Sideris, *P. folliculosum* Paul, *P. indigoferae* Butler, *P. irregulare* Buisman, *P. lutarium* Shtayeh, *P. macrosporum* Vaartaja, *P. myriotylum* Drechsler, *P. paroecandrum* Drechsler, *P. torulosum* Coker, *P. vexans* de Bary, *P. zingiberis* Takah, *P. graminicola* Subraman, *P. spinosum*

Sawada, *P. ultimum* Trow, *P. arrhenomanes* Drechsler, *P. catenulatum* Matthews, *P. diclinum* Tokun, *P. dissotocum* Drechsler, *P. rostratum* Butler, *P. salpingophorum* Drechsler and *P. deliense* Meurs.

Root rot diseases are widespread in the world and often considered as a major constraint to bean production, reducing both yield and quality (Abawi and Widmer, 2000). Depending on the pathogen(s) involved in the development of the disease, general root rot symptoms might include any combination of various traits like poor seedling establishment, damping-off, uneven growth, leaf chlorosis, premature defoliation, death of severely infected plants, and lower yield (Schwartz et al., 2007; Abawi et al., 2006).

Pythium is a complex genus containing over 200 described species with a broad host range and occupying a variety of terrestrial and aquatic ecological habitats (Dick, 2001). The presence of the pathogens and the severity of the disease are associated with intensification of land use, inappropriate crop rotations and/or reduced fallow periods, leading to a decline in soil fertility and build up of soil pathogen inoculum (Abawi et al., 2006).

In a study conducted in South Western Uganda, seven *Pythium* species from various crops associated with beans were obtained: *Pythium macrosporum, Pythium oligandrum, Pythium spinosum* Sawada isolated from sorghum, *Pythium glomeratum* B. Paul isolated from potato, *Pythium arrhenomanes* isolated from maize, *Pythium ultimum* isolated from peas and *Pythium heterothallicum* W.A. Campb. & F.F. Hendrix isolated from sweet potato (Gichuru, 2008). If these various *Pythium* species are identified on plants species intercropped with beans, it is likely that controlling bean *Pythium* root rot with crops rotation practices could be of a limited efficiency. In the present work, a study of various biological characteristics of the *Pythium* agents of Rwanda is undertaken. Such investigation was conducted for a better understanding of the conditions leading to disease development in view of setting up appropriate control methods to reduce the yield losses caused by *Pythium* root rot.

2. Taxonomy and biological characteristics of *Pythium* spp.

The genus *Pythium* belongs to the family Pythiaceae, order Pythiales, class Oomycetes, Phylum Oomycota and Kingdom Chromista (Kirk et al., 2008).

Pythium species are fungal micro-organisms with a filamentous vegetative body called mycelium. The mycelium of *Pythium* species is colourless, sometimes lustrous, occasionally slightly yellowish or greyish lilac (Owen-Going, 2008). The mycelia branch out apically at right angles to form structures known as hyphae. The hyphae are hyaline, the main hyphae are mostly 5-7, occasionally up to 10 µm wide. Cross septa are lacking except in old, mostly empty hyphae or where they delimit reproductive organs. (Plaats-Niterink, 1981). Protoplasmatic streaming is often clearly visible in young hyphae. According to Postma et al., (2009), hyphal walls are composed of 80-90% polysaccharide, mainly ß1-6 linked glucans and ß1-3 and ß1-4 (cellulose). It should be noted that *Pythium* spp. do not contain chitin or chitosan in hyphal walls, whereas protein and lipid contents vary between 3-8% and 1-3%, respectively (Postma et al., 2009). Pathogenic *Pythium* spp. may produce hyphae with swollen digitate regions called appressoria which enable the fungus to attach and penetrate to host cells (Lévesque and de Cock, 2004).

Pythium spp. can reproduce both asexually and sexually. The asexual reproduction takes place by means of zoosporangia and zoospores. In *Pythium* the zoospores are not formed in the sporangium itself but in a vesicle outside it (Stanghellini and Hancock, 1971). The sporangium is separated from the rest of the mycelium by a cross wall. On the sporangium, one observes a development of a tube. The undifferentiated content of the sporangium moves through this tube and forms a vesicle at its end. At this level of development, the zoospores are delimited and start moving (Stanghellini and Hancock, 1971). After 10 to 20 minutes, the wall of the vesicle disappears and zoospores swim away in divergent directions. Zoospores are only liberated under wet conditions. Production of sporangia or hyphal swellings can be stimulated by Mg, K, and Ca ions (Postma et al., 2009). Exudates of roots and germinating seeds have a stimulatory effect on the germination of sporangia and mycelial growth (Stanghellini and Hancock, 1971).

The sexual reproduction takes place by means of oogonia and antheridia. The female organs, the oogonia, are spherical to limoniform and are intercalary or terminal. The oogonial wall can be smooth or ornamented with projections. The antheridia, the male organs, consist of an antheridial cell which can be sessile on a hypha, intercalary, or formed terminally on an antheridial stalk (Postma et al., 2009). The antheridial cell touches the oogonium and forms a fertilization tube which penetrates the

oogonium. (Plaats-Niterink, 1981). The antheridia are termed monoclinous if they originate from the oogonial stalk and diclinous if they originate from a different hypha not closely connected with the one subtending the oogonium (Hendrix and Campbell, 1969). After fertilization the oogonial content forms a zygote, evolving into the oospore. Only in rare cases more than one oospore is produced inside an oogonium. The oospore wall is smooth except in *P. dictyosporum* where oospores are reticulate (Plaats-Niterink, 1981).

Some stimulatory effects of Ca, Mg, K, Zn and Mn ions on growth and reproduction by oospores have been mentioned (Hsu and Hendrix, 1972). Important factors for sexual propagation are sterols (chlolestorol, ß-sitosterol, etc.). Sterols stimulate growth and reproduction by oospores and permit survival to high temperatures which disrupt the cell membranes permeability to the antifungal constituents (Pystina, 1974). After maturation of the oospore, a dormant phase is usually necessary, before germination takes place. At germination, the oospore is converted into a thin-walled structure, which produces a germ tube (Lumsden et al., 1987). Oospore germination is made of two stages: first, the absorption of the endospore, depending on an exogenous calcium supply (Lévesque and de Cock, 2004), and secondly germ tube formation depending on the presence of exogenous carbohydrate sources (Stanghellini and Russell, 1973).

3. Morphological characteristics to identify *Pythium* genus

Morphological characteristics and size of each structure have been taken in the past as criteria to identify species within *Pythium*. These include as more important: (1) the presence of sexual reproductive structures - homothallic or heterothallic; (2) the sporangial morphology - spherical, filamentous or lobulate; (3) the oogonial wall character - smooth or ornamental; (4) the oospore character - plerotic or aplerotic; and (5) the antheridial characteristics (Matsumoto et al., 1999). The major morphological criteria for *Pythium* species identification are based on qualitative characteristics that may vary depending on the culture conditions and the isolate tested (Dick, 2001). Differences in the value attributed to each character have resulted in a confusing taxonomic system for the *Pythium* species (Uzuhashi et al., 2010). As a consequence, a more relevant approach to identify *Pythium*

species will be to combine traditional morphological characterization with molecular analyses (Kageyama et al., 2005).

4. Molecular characterization and phylogeny of *Pythium* genus

The ITS (Internal Transcribed Spacer) region of the rDNA has become a useful tool in fungal taxonomy and can be used to identify or detect different *Pythium* species (Belbahri et al., 2008; Matsumoto et al., 1999). In a study conducted on 116 *Pythium* species by Lévesque and de Cock (2004), the ITS region including the 5.8S gene was shown to have a size varying between 750 and 1050 bp. The results from Lévesque and de Cock (2004) but also from Kageyama et al. (2005) revealed that sequences of the rDNA ITS region were very different among *Pythium* species. Thus, sequence data of this region had been frequently used to identify and classify *Pythium* species (Matsumoto et al., 1999; Vasseur et al., 2005).

Molecular data have also been used for phylogenetic analyses of *Pythium* and related genera based on the rDNA large subunit (LSU) D1/D2 and ITS, b-tubulin, or mitochondrial cytochrome oxidase II (coxII) gene (Belbahri et al., 2008).

The phylogeny of *Pythium*, as based on ITS sequences, reveals a divergence between *Pythium* species. *P. aphanidermatum* and *P. deliense* represent one of the best demonstrations of speciation between two very closely related species. The spacers differ by only 3% and yet the species exhibit differences in several morphological characters that are slightly but consistently different between the two species (Herrero & Klemsdal, 1998).

P. attrantheridium is described on isolates from cavity spot lesions of carrots as well as apple and cherry seedlings from various locations widely distributed in Canada and the USA. It had only 5% ITS divergence with *P. intermedium*, but was morphologically distinct and could not be mated with *P. intermedium* (Allain-Boulé et al., 2004). The ITS region of the nuclear ribosomal DNA of *P. longisporangium* is comprised of 890 bases and a BLAST search gives the closest resemblance of this oomycete with the following species: *P. bifurcatum* (AY083935), *P. longandrum* (AY039713), *P. terrestris* (AY039714) and *P. hypogynum* (AY455804), with the respective similarity percents: 99.6, 96.2, 84.6 and 84.6. Other species are also relatively closed to *P. longisporangium*, on the basis of a

morphological trait (hypogynous type of antheridia) and also on some resemblances between their ITS sequences: *P. segnitium* (AY149173) with 74.8% similarities, *P. canariense* (AY06561) with 60.7% similarity and *P. rostratum* (AJ233456) with only 57.6% similarity with *P. longisporangium* ITS sequence (Paul et al., 2005).

5. Ecology of *Pythium* spp.

Pythium species can be found in various ecological areas like soils from arable land, pastures, forests, nurseries, marshes, and from water (Plaats-Nterink, 1981). In general soil temperature can affect spore germination, germ tube growth and zoospore discharge (Tedla and Stanghellini, 1992). However, each *Pythium* species has its specific optimal development conditions. For example, *P. ultimum* and *P. dissotocum* inhabit cool (10-15°C) and wet soil as saprophytes on crop residues. Other *Pythium* root rots, such as those caused by *P. aphanidermatum* Edson, *P. irregulare* Buisman, *P. sylvaticum* Campbell and *P. myriotylum* Drechsler occur in warm (25-36°C) and wet soil (Owen-Going, 2008). *Pythium* species have been recovered in soils with pH ranging from 3.6 to 7.2 (Martin and Loper, 1999). However, *Pythium* spp. populations were higher in soils with pH ranging from 6.8 to 7.2 and lower in soils with pH ranging from 3.6 to 5.5 (Martin and Loper, 1999). Soil's pH influences some phases of *Pythium* species life cycle such as formation of oospores and sporangia. Alkaline soils (above a pH of 7) favor the growth of *Pythium* species (Lumsden et al., 1987). *Pythium* species are more abundant in cultivated than in uncultivated soils: cultivation and incorporation of plant residues into the soil tend to create favorable conditions for faster decomposition of organic matter, thus the fungal food availability in that environment (Hendrix et al., 1971). However, some *Pythium* species are mycoparasites. For example, *P. oligandrum* is nonpathogenic on 12 species of crops from six families, including sugar beet, cucumber, wheat, peas, nephrolepis and common beans (Dušková E., 1995; Wulff et al., 1998). It does not attack their tissues but occurs on the root surface, predominantly in the regions of hypocotyl – taproot, together with plant pathogenic fungi. It utilises the root exudates and fungus hyphae on the root surface, including those of the plant pathogens, for its own nutrition (Brožová, 2002).

6. *Pythium* root rot control methods

6.1. Chemical control of *Pythium* spp.

Once introduced in the soil, *Pythium* spp. may persist for many years through resistance structures such as oospores, zoospores and sporangia (Onokpise et al., 1999). In these conditions, applying chemical treatments to kill the pathogen could be an efficient method. There are many specific pesticides like Benomyl, Captafol, Captan, Carboxin, Metalaxyl, Propamocarb hydrochloride and etridiazole which were already proven to be efficient in controlling *Pythium* root rot diseases on beans. However, some like Benomyl are only active on growing mycelium, but not on the resting stage. In the same context, soil fumigants such as methyl bromide, chloropicrin and vorlex are highly effective biocides that kill *Pythium* agents (Abawi et al., 2006). In Latin America and Africa, one of the safest and most economical uses of chemicals to control *Pythium* pathogens consists of coating the seeds. This usually results in an effective protection of seeds and young seedlings for about 2 to 3 weeks after sowing (Schwartz et al., 2007; Abawi et al., 2006). However, given the conditions prevailing in diverse developing countries like in Eastern and Central Africa, poor farmers cannot easily afford applying chemical control. Moreover, the large scale use of chemical treatment could constitute a source of soil and water contamination while at the same moment the lowly educated farmers could face some health risks related to handling chemical pesticides. Therefore, the only use of chemical control can be considered as not sustainable for bean production by poor farmers of most developing countries.

6.2. Biological control of *Pythium* spp.

Biological control of soil-borne diseases is particularly complex because the pathogens occur in a dynamic environment at the interface of rhizosphere. Rhizosphere is typified by intense microbial activity involving a high population of microorganisms, rapid change of pH, salt concentrations, osmotic and water potential (Handelsman and Stabb, 1996). Microorganisms indigenous to the rhizosphere are ideal for biological control, since the rhizosphere provides a first-line defense for roots against attacks by plant pathogens (Weller, 1988). Microorganisms can protect the plant from fungal

attacks by the production of antifungal metabolites, competition with the pathogen for nutrients, niche exclusion, parasitism or lysis of the pathogen, or induction of plant resistance mechanisms (Whipps, 2001).

Beneficial microorganisms of interest for biological control of plant pathogenic *Pythium* spp. have been identified among fungi and bacteria. Isolates of *Trichoderma* spp. and *Gliocladium* spp. are antagonists of *Pythium* induced soil-borne diseases (Howell et al., 1993) and several strains are already commercially available for biological control of *Pythium* root rots (Fravel, 2005). Various actinomycete species including *Streptomyces*, *Actinoplanes,* and *Micromonospora* (El-Tarabily et al., 1997) could inhibit *Pythium coloratum* cavity-spot on carrots. Other bacteria effective against *Pythium* are found in various genera including *Enterobacter*, *Erwinia*, *Bacillus*, *Burkholderia*, *Stenotrophomonas*, and *Rhizobium* (Bardin et al., 2004), but the most extensively studied group of bacterial biological control agents are *Pseudomonas* spp. (Chin-A-Woeng et al., 2003).

Competition for organic carbon and iron is one of the mechanisms through which some biocontrol agents suppress *Pythium* spp. (Hoitink and Boehm, 1999). Sensitivity of *Pythium* spp. to competition and antagonism during its saprophytic phase of growth is one of the key factors to manage *Pythium* diseases through biological control (Martin and Loper, 1999).

In contrast to this view, it is commonly known that *Pythium* spp. propagules germinate rapidly in response to seed or root exudates and quickly infect seeds or roots, which complicates biological control (Whipps and Lumsden, 1991). Therefore it is of great importance that the activity of the biological control agent must coincide with the period of host susceptibility and should persist as long as the plant remains susceptible. Insufficient survival of the antagonists may lead to inadequate or partial control. From a field experience conducted in Western Kenya, it was concluded that one approach to address this limitation is the introduction of a food base such as compost that supports the activity of antagonists but does not stimulate the activity of the pathogen (Hoitink and Boehm, 1999; Otsyula et al., 1998). However, the compost must be free of *Pythium* root rot pathogens to increase the chance of having the *Pythium* root rots effectively controlled (Martin et al., 1985).

6.3. Control by genetic resistance

The use of resistant common bean cultivars is the most efficient management strategy against root rot diseases. It is especially appropriate for small farmers with low inputs. However, this strategy requires the development of adapted common bean cultivars with resistance to all major root rot pathogens that prevail in a given bean growing region (Abawi et al., 2006).

Plant resistance to diseases is defined as the ability of the host plant to hinder the growth and/or development of the pathogen (Parlevliet, 1979). Resistance was classified by van der Plank (1963) in two main categories as vertical and horizontal resistance. Vertical resistance is race-specific and characterized by specific interactions between the plant genotypes (or varieties) and the pathogen races (or strains). On the other side, horizontal resistance is race-nonspecific and characterized by the absence of any specific interaction between host and pathogen genotypes (Robinson, 1987).

In order to identify resistance to *Pythium ultimum* root rot within *Phaseolus vulgaris*, screenhouse evaluation with artificial inoculation was carried out by Otsyula et al., 1998 and Buruchara et al., 2001. For the artificial inoculation, an isolate from *P. ultimum* was chosen due to its large distribution and severity in East and Central Africa as reported by Mukalazi, (2004). The tested common bean materials belonged to the two major genepool of the food legume, i.e. the Mesoamerican and Andean genepool (Singh et al., 1991).The severity of lesions on roots was scored using the CIAT 1-9 scale (van Schoonhoven and Pastor-Corrales, 1987).

Susceptible common bean varieties from Africa were characterized by different seed sizes, such as Kenyan varieties GLP 2 and GLP 585 with respectively large seeds (Andean genepool) and small seeds (Mesoamerica genepool), Ugandan variety CAL 96 (Calima) with large seeds (Andean genepool) and Rwandan variety Urugezi with intermediate seeds size (Mesoamerica genepool). All the resistant common bean varieties were advanced lines from an international breeding nursery conducted by CIAT (Cali, Colombia) and were also characterized by different seed sizes, such as the small-seeded variety RWR 719 from Mesoamerican genepool, the intermediate-seeded varieties MLB 49-89A and SCAM 80-CM/15 both from Mesoamerica genepool and the large-seeded varieties AND 1055 and AND 1062 both from Andean genepool (Otsyula et al., 2003).

The genepool origin of the evaluated common beans genotypes is helpful to predict combining ability and to set up crossing programmes likely to display wide segregations and ecological adaptation (Singh et al., 1991; Díaz & Blair, 2006). Due to its worldwide distribution, the Mesoamerican genepool is the most widely grown and have smaller seeds than those from the Andean gene pool (Singh et al., 1991; Beebe

et al., 2000). Cultivars of the Mesoamerican genepool are adapted to a range of hot, humid to moderate climates in the tropics and subtropics but are also grown in high latitudes in the United States and Argentina. As Mesoamerican genotypes are predominant in Rwanda, any breeding programme should take this reality in consideration before choosing common bean varieties to be crossed (Díaz & Blair, 2006; Buruchara et al., 2001).

Crosses between susceptible varieties (GLP 2, GLP 585, CAL 96 and Urugezi) used as female parents and resistant varieties (RWR 719, MLB 49-89A, SCAM 80-CM/15, AND 1055 and AND 1062) were undertaken in the research station of CIAT-Kawanda and the F1 hybrids were backcrossed with the recurrent susceptible parents (Ostyula et al., 2003).

Resistance to *Pythium ultimum* was expressed in all F1 plants using the resistant genotypes as male parents. This shows that resistance to *Pythium ultimum* is inherited as a dominant character (Ostyula et al., 2003, Mahuku et al., 2007).

To determine the number of genes necessary for *Pythium* root rot resistance, the segregation of F2 and backcross plants was analyzed. Chi square value reveal that goodness of fit was obtained for segregation ratios of 3:1(resistant: susceptible) in F2, 1:1 when the F1 were backcrossed to GLP 2, GLP 585, CAL 96 and Urugezi, and 1:0 when the F1 were backcrossed to RWR 719, SCAM 80-CM/15, MLB 4889A, AND 1055 and AND 1062 (resistant varieties). From these results, it is assumed that resistance to *P. ultimum* is controlled by one dominant gene. Whatever the genepool origin and the parental genotypes used in the combinations (Otsyula et al., 2003; Buruchara et al., 2001).

Molecular assisted selection could be applied to speed up the selection process in a breeding program. A SCAR marker named PYAA 19_{800} was characterized as being associated with *Pythium* root rot resistance gene in RWR 719 and AND 1062 and successfully used in selection for resistance to common bean *Pythium ultimum* root rot (Mahuku et al., 2007). In a backcrossing programme, all

individuals devoid of the SCAR marker can be identified early and eliminated during the breeding process.

Additional sources of resistance among the bean genepools should be looked for with the aim to detect new genes of resistance to different *Pythium* species and to develop varieties adapted to the various agro ecological zones (Buruchara et al., 2001). This process would contribute to the sustainability of the released varieties and thus ensure longer control of the disease in common bean production systems (Otsyula et al., 2003).

7. Conclusions

Root rot diseases are widespread and considered as a major constraint to common bean production as they reduce significantly the bean yield worldwide. Various disease control options are available including different methods such as chemical control, biological control, genetic resistance methods and cropping practices. However, there is a crucial issue of resistant varieties seed availability for users which has to be addressed in view of reaching a majority of farmers. In these conditions, improved varieties with disease resistance represent one of the most practical, economical, and easily adopted disease management strategies for the majority of bean producers in developing countries who have small land and limited economic resources (Beebe and Pastor-Corrales, 1991; Otsyula et al., 1998).

As previous studies have revealed that resistance to *Pythium* root rot is controlled by a single dominant gene (Mahuku et al., 2007; Otsyula et al., 2003), released varieties which are deficient for resistance to *Pythium* root rot disease, can be improved by backcross programme. To speed up such breeding programme, the use of molecular markers associated with the resistance to *Pythium* root rot is helpful in carrying out a rapid screening of the large progeny populations. However, the fact that resistance to *Pythium* root rot is under control of a single gene constitutes a risk factor which can lead to resistance breakdown. This danger is amplified by the fact that there is a high diversity of the pathogen populations. To improve the sustainability of this control method, it is therefore more relevant to diversify sources of resistance and to integrate other control methods, mainly appropriate cropping practices in view of reducing the risk to buildup rapidly *Pythium* inoculums. For example, ridging and

deep tillage increase aeration and drainage, creating less favourable conditions for disease development (Beebe and Pastor-Corrales, 1991; Abawi and Widmer, 2000; CIAT, 1992; Voland and Epstein, 1994).

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CHAPTER III

PATHOGENIC AND MOLECULAR CHARACTERIZATION OF *PYTHIUM* SPECIES INDUCING ROOT ROT SYMPTOMS OF COMMON BEAN IN RWANDA

Published in African Journal of Microbiology Research Vol. 5(10), pp. 1169-1181, 18 May, 2011.

PATHOGENIC AND MOLECULAR CHARACTERIZATION OF *PYTHIUM* SPECIES INDUCING ROOT ROT SYMPTOMS OF COMMON BEAN IN RWANDA

J. NZUNGIZE[1], P. GEPTS[2], R. BURUCHARA[3], S. BUAH[3], P. RAGAMA[4], J. P. BUSOGORO[5] and J. P. BAUDOIN[1].

Authors' addresses : [1] University of Liege, Gembloux Agro-Bio Tech, Belgium; [2] University of California, Davis; [3] CIAT, Pan Africa Bean Research Alliance, Kampala, Uganda ; [4] National Agriculture Research Organization, Kampala, Uganda ; [5] Belgium Technical Cooperation, IPM project, Kigali, Rwanda.

*Corresponding author: nzungizej@gmail.com

Abstract

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, R 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 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 in breeding programmes 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 in Eastern and Southern Africa region (Pachico, 1993; Wortmann et al., 1998). 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 (Melotto et al., 2005).

The crop production is hampered by several constraints among which 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 (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).

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). 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).

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 (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 percent control of the disease in the first 2-3 months of crop growth, substantial yield increases were observed in cereals (5-20%), canola (5-30%) and pulses (5-50%) (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 rot as well as yield losses (Buruchara and Scheidegger, 1993).

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). 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 that fact 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 of 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: (i) collecting *Pythium* isolates, (ii) mapping the geographical distribution of collected isolates, (iii) characterizing the collected isolates by molecular profiles and (iv) 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 (900-1400 m), intermediate (1400 - 1650 m) and high (1650 - 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 symptoms characteristics of *Pythium* infection such as wilting, stunting and chlorosis.

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 during 20 minutes. The antibiotic preparation

[Rifamycin (0.03g/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 0.5-2 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 (20-25°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 50µl final reaction volume containing 5µl of 10X PCR buffer,

8µl of 25mM $MgCl_2$, 2.5 µl of 1.25mM dNTP, 0.2 µl of each primer (20µM) [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 0.2µl Taq DNA polymerase (5U/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 5mg/ml of ethidium bromide in a TBE (1 time concentrated) as the running solution. The electrophoretic migration was carried out during 2 hours under a 100V 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 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 programme (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 programme (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 three times 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 meters above sea level (masl), average rainfall is 1224 mm per annum and average daily temperatures are 15.3C (minimum) and 27.3C (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, sterilized soil was mixed with the infested millet at a ratio of 1:10 v/v in wooden trays of 42 cm 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, Urugezi, R 617-97A, RWR 1668, Vuninkingi, RWR 719), plus a set of three varieties provided by CIAT and already known as being resistant to *Pythium* in other regions (MLB-40-89A, AND 1064 and AND 1062) and one variety (CAL 96) known as being susceptible to *Pythium*.

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 1-2 were considered as being no pathogenic while those with an average score of 3-5 were considered moderately pathogenic and those with an average score of 6-9 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

We collected 231 samples which were used to isolate the *Pythium* spp. The 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 hours 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 rot 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. The 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 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.

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 were collected.

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).

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

Latitude	Longitude	Altitude(m)	Temperature (˚C)	District	Isolates codes	*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	*Pythium 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	*Pythium 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	*Pythium 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	*Pythium 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	*Pythium ultimum*
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	*Pythium vexans*
02°22'31"	029°05'8"	1598	22.3	Nyamasheke	42 NSKE	*Pythium spinosum*
02°23'23,6"	029°05'11,1"	1595	23.3	Nyamasheke	43NSKE	*Pythium diclinum*
02°27'49,2"	028°54'11,6"	1929	22.7	Rusizi	46 RSZb1	*Pythium spinosum*
02°29'9,1"	028°57'6,9"	1915	22.6	Rusizi	46 RSZb2	*Pythium spinosum*
02°28'29,2"	028°54'23,8"	1618	24.3	Rusizi	46 RSZ	*Pythium diclinum*
02°32'14,9"	028°53'41,6"	1659	22.1	Rusizi	49 RSZI a	*Pythium rostratifingens*

Latitude	Longitude	Altitude(m)	Temperature (˚C)	District	Isolates codes	*Pythium* species
02°32'19,1"	028°53'13,4"	1657	20.8	Rusizi	49 RSZb	*Pythium 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	*Pythium diclinum*
02°30'20,6"	029°29'23,2"	2205	20.9	Nyamagabe	64 NGBE	*Pythium 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	*Pythium 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	*Pythium folliculosum*
02°19'6,7"	029°49'29"	1584	24.6	Nyanza	75 NYA	*Pythium 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	*Pythium vexans*
02°19'56"	029°54'8,8"	1395	25.0	Nyanza	84 NYA	*Pythium folliculosum*
02°18'49,4"	029°54'55,4"	1437	23.6	Nyanza	87NYA	*Pythium folliculosum*
02°18'35,5"	029°55'25,7"	1435	23.9	Nyanza	88 NYA	*Pythium 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	*Pythium folliculosum*
01°18'9"	029°59'20,7"	1452	24.3	Bugesera	93 BGSR	*Pythium vexans*
01°18'23,7"	030°00'38"	1522	26.1	Bugesera	94 BGSR	*Pythium ultimum*
01°18'23,7"	030°00'39"	1522	20.6	Bugesera	95 BGSR	*Pythium vexans*
01°17'56,6"	030°00'58,5"	1498	22.4	Bugesera	96 BGSR	*Pythium vexans*
01°57'30,4"	030°09'4,7"	1372	22.1	Gasabo	97 GSB b	*Pythium vexans*
01°58'7,7"	030°10'6,1"	1366	22.2	Gasabo	97GSBa	*Pythium vexans*
01°58'40,9"	030°10'54,8"	1354	23.2	Gasabo	98 GSB	*Pythium vexans*
01°59'17,5"	030°11'39,7"	1345	23.9	Gasabo	98 GSBii	*Pythium vexans*
01°58'53,5"	030°12'58,5"	1329	23.6	Gasabo	101 GSB	*Pythium vexans*
01°54'29,6"	030°26'33,8"	1514	25.3	Rwamagana	108 RWM	*Pythium vexans*
01°54'6,4"	030°29'42,8"	1598	25.4	Kayonza	110 KYNZA	*Pythium vexans*
01°55'11,1"	030°29'39,1"	1563	26.3	Kayonza	111 KYNZA	*Pythium rostratifingens*
02°11'14,5"	030°31'49,4"	1636	25.6	Ngoma	117 NGM	*Pythium 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	*Pythium indigoferae*

Latitude	Longitude	Altitude(m)	Temperature (˚C)	District	Isolates codes	*Pythium* species
02°08'40,7"	030°34'30,8"	1684	25.0	Ngoma	124 NGM	*Pythium vexans*
02°12'57,2"	030°23'35,4"	1330	20.5	Ngoma	126 NGM	*Pythium vexans*
2°14'39,1"	030°33'17,3"	1373	17.8	Ngoma	128NGM	*Pythium vexans*
2°15'41,4"	030°38'5,4"	1570	18.1	Kirehe	133 KRHa	*Pythium vexans*
2°16'23"	030°40'59,4"	1627	20.5	Kirehe	133KRHb	*Pythium vexans*
1°18'3,7"	030°19'15,1"	1359	28.9	Nyagatare	143 NGTR	*Pythium conidiophorum*
1°18'20,6"	030°19'10,4"	1359	28.3	Nyagatare	145 NGTR	*Pythium rostratum*
1°24'22,6"	030°16'28,6"	1370	27.0	Nyagatare	149 NGTR	*Pythium vexans*
1°24'58,7"	030°16'48,4"	1375	26.4	Nyagatare	151 NGTR	*Pythium vexans*
1°25'41,9"	030°16'20,7"	1374	27.4	Nyagatare	153 NGTR	*Pythium ultimum*
1°24'45,3"	030°18'46"	1438	28.3	Nyagatare	158 NGTRb	*Pythium vexans*
1°44'24,5"	030°07'42,2"	1518	23.7	Gicumbi	162 GCMB	*Pythium vexans*
1°38'50,5"	030°07'41,3"	2098	25.5	Gicumbi	166 GCMB	*Pythium diclinum*
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	*Pythium vexans*
1°54'32,8"	030°04'0,1"	1743	25.8	Nyarugenge	178 NGGE	*Pythium torulosum*
1°53'31,8"	029°59'20,6"	1783	26.7	Nyarugenge	180 NGGE	*Pythium vexans*
1°51'32,3"	029°58'42,7"	1953	25.7	Rulindo	182 NGGE	*Pythium vexans*
1°51'18,9"	029°58'29,6"	1959	24.8	Rulindo	183 RNDO	*Pythium 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	*Pythium vexans*
1°29'34,3"	029°39'41,8"	1715	27.0	Musanze	204 MNZE	*Pythium diclinum*
1°31'52.6"s	029°35'21.7"	1821	25.1	Burera	188 BRR	*Pythium ultimum*
1°24'06.9"s	029°44'17.1"	2044	18.4	Burera	189 BRR	*Pythium rostratum*
02°08'5,5"	029°19'23,2"	1854	21.2	Musanze	207MNZE	*Pythium vexans*

In summary, only 96 isolates of the 231 samples had the *Pythium* expected specific size of ITS fragment (800 bp), these isolates were submitted to the 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. The table 2 contains the number of isolates classified in each *Pythium* species per district in Rwanda.

Considering the *Pythium* species geographical distribution, *P.vexans* was shown to be the most widespread species in the country as its presence was revealed with 23 isolates distributed in 13 districts (Table 2). The species *P.indigoferae* was found in samples from 6 districts, while the species *P. torulosum, P. ultimum* and *P. 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 *P. cucurbitacearum*, *P. arrhenomanes*, *P. pachycaule* and *P. 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 sowing 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.

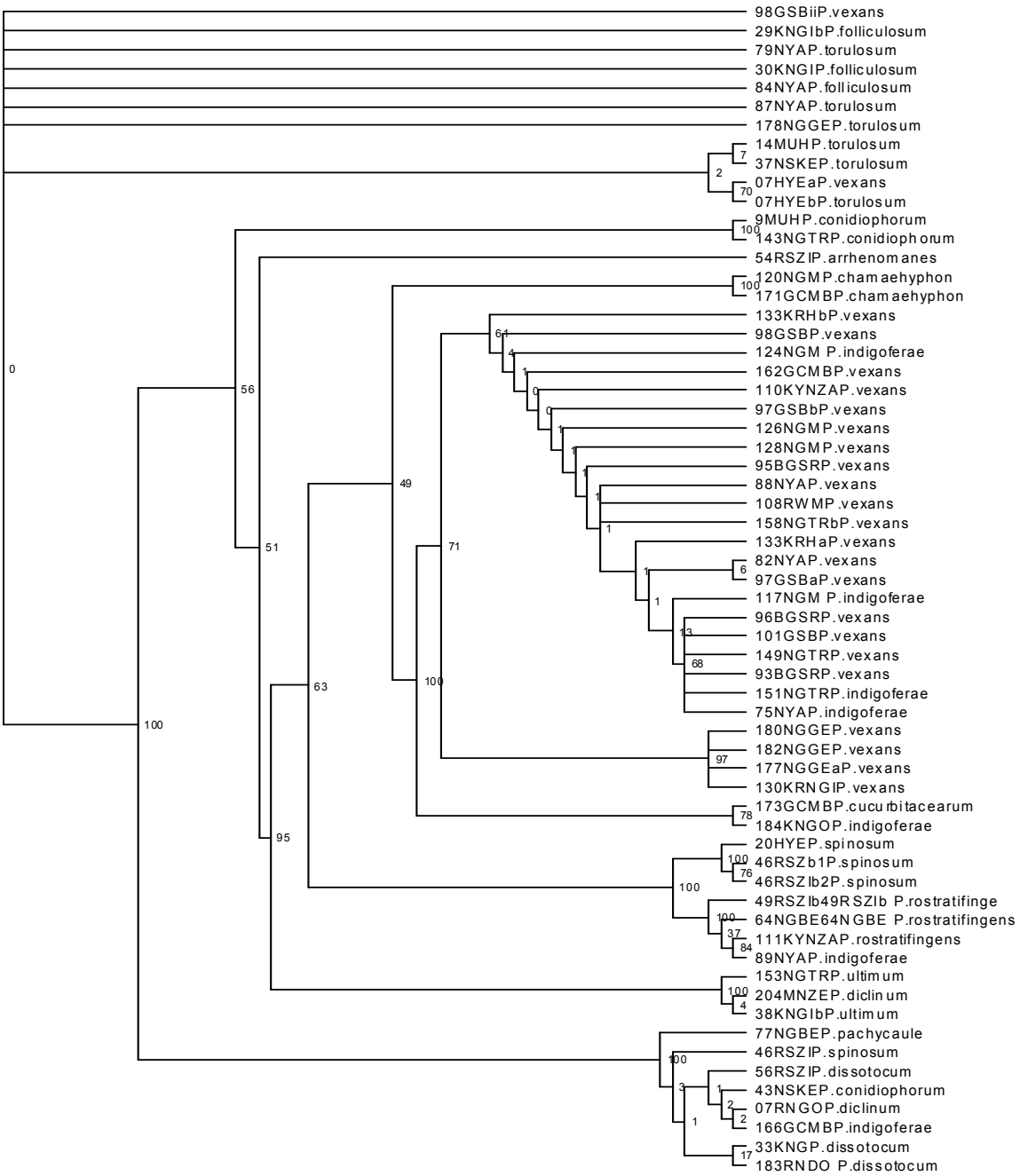

Figure 2. Phylogenetic relationship of *Pythium* spp. from Rwanda based on the ITS ribosomal DNA sequences. The codes following number are relative to district of origin (RNDO: Rulindo; KNG: Karongi, GCMB: Gicumbi, RNGO: Ruhango, RSZI: Rusizi, NSKE: Nyamasheke, NGBE: Nyamagabe, KNGI: Karongi, MNZE: Musanze, 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 programme.

Table 2. Distribution of the *Pythium* species isolated from the different bean samples affected by root rot symptoms per district in Rwanda

District	*P. indigofera e*	*P. chamaehy phon*	*P. torulos um*	*P. cucurbitace arum*	*P. diclin um*	*P. conidioph orum*	*P. arrhenom anes*	*P. pachyc aule*	*P. ultim um*	*P. vexa ns*	*P. folliculo sum*	*P. macrospo rum*	*P. rostratifin gens*	*P. spinos um*	*P. dissoto cum*	*P. rostrat um*	*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**

District	*P. indigofera*	*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*
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**

The morphological aspect of the root rot symptoms development on the bean plants is illustrated by the pictures presented in the figure 3.

Figure3. Aspects 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.

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.

As the artificial inoculation with the different *Pythium* species always resulted in the 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 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 corresponded to a severity score of 1.6.

As shown by the data presented in the table 3, two main categories of varieties were differentiated as following: (i) resistant varieties and (ii) susceptible varieties. In fact, the varieties AND 1062, G2331, MLB 40-89A, 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, R 617-97A, Urugezi and RWR 1668 exhibited a highly susceptible reaction to the different *Pythium* species inoculated on them. This observation is of the greatest 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.

Table 3. Expression of the severity of the *Pythium* species on the common bean varieties cultivated in Rwanda

	***Pythium* species severity**																Disease
P. species	*P.*	*P.*	*P.*	*P.*	*P.*	*P. disso*	*P.*	*P.*	*P. pachy*	*P.*	*P.*	*P.*	*P.*	*P.*	*P.*	*P.*	expression
	arrheno	*chamae*	*conidio*	*cucurbita*	*diclinum*	*tocum*	*follicu*	*indigo*	*caule*	*rostrati*	*spinosum*	*torulosum*	*ultimum*	*vexans*	*macrosporum*	*rostratum*	of cultivars
Beans	*manes*	*hyphon*	*phorum*	*cearum*			*losum*	*Ferae*		*fingens*							(average/variety)
Varieties																	
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	8,2 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	1.9 R
R 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	7.9 B	8.0 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	8.4 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	7.6 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	1.7 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	1.7 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	1.3 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	1.9 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	1.6 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	
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. Isolates that had an average disease score of 1-2 were considered as being no pathogenic while those with an average score of 3-5 were considered moderately pathogenic and those with an average score of 6-9 were considered to be highly pathogenic. Evaluation of the disease symptom importance was performed on 30 plants per each variety.

DISCUSSION

The isolation protocol used in this experience was successful 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 of 800 bp for the amplified product (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 -1050 bp (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 is helpful for the identification of species that cannot be distinguished using only morphological characteristics, for testing interbreeding potential or for rapid identification of fungal species (Matsumoto et al., 1999). 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. However, it was observed that in the generated dendrogram, multiple ITS sequences occurred in the same *Pythium* species. This explains why the different isolates of the same *Pythium* species were classified in different clusters of the phylogenetic tree. The presence of different ITS copies in the

same individual has also been reported for vertebrates, fungi and protozoa (Ko & Jung, 2002; Hui et al., 2007; Belbahri et al., 2008).

The same situation has been observed in our study for the characterization of *Pythium* species; thus the concept of species based only on ITS sequence to characterize the *Pythium* agents should be evaluated carefully. In order to improve the use of the ITS region for diagnostic in fungi, it is recommended to analyse the secondary structure of ITS 2, and the unique sequence of loop 2, in order to create species-specific probes in a micro array format, and consequently reduce the effect of multiple ITS copies (Landis & Gargas 2007; Brasier et al., 1999).

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.) (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.

The isolated agents identified as belonging to *Pythium* spp. were classified according to their ITS sequences. 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 most widespread in Rwanda. In a similar investigation performed by Green and Dan (2000) but in a different agroecological condition (Denmark), it was found that another species, *P. ultimum*, was the most widespread *Pythium* species that attacks a large number of plant species in this country.

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 (1650-2300 m), intermediate (1400-1650 m) and low (900-1400 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). 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 widespread 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 common 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 were significant differences in the severity level recorded on the different bean varieties. For a given *Pythium* species, level of symptom severity varied according to the inoculated variety.

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. However, as evaluation of population structure has important implications for genetic improvement of common bean in terms of predicting

combining ability, it could be better to evaluate these different common bean varieties for their classification in different genepools. This classification would facilitate designing the crossing programmes (Singh *et al.,* 1991; Díaz & Blair, 2006).

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 *Pythium 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 will facilitate to undertake a global breeding programme 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.

Acknowledgment

The authors would like to express their warmest thanks to CIAT for having given technical assistance and laboratory facilities to Mr Nzungize during his investigation. Moreover, the project was supported by the Belgian Technical Cooperation and the Kirkhouse Trust which are thanked through this work.

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CHAPTER IV

INTROGRESSION OF *PYTHIUM* ROOT ROT RESISTANCE GENE INTO RWANDAN SUSCEPTIBLE COMMON BEAN CULTIVARS

Published in the African Journal of Plant Science Vol. 5(3), pp. 193-200, March 2011.

INTROGRESSION OF *PYTHIUM* ROOT ROT RESISTANCE GENE INTO RWANDAN SUSCEPTIBLE COMMON BEAN CULTIVARS

J. NZUNGIZE[1], P. GEPTS[2], R.BURUCHARA[3], A.MALE[3], P.RAGAMA[4], J. P. BUSOGORO[5] and J. P. BAUDOIN[1].

Authors' addresses : [1] University of Liege, Gembloux Agro-Bio Tech, Belgium ; [2] University of California, Davis; [3] CIAT, Pan Africa Bean Research Alliance, Kampala, Uganda ; [4] National Agriculture Research Organization, Kampala, Uganda ; [5] Belgium Technical Cooperation, IPM project, Kigali, Rwanda.

*Corresponding author: nzungizej@gmail.com

Abstract

A breeding scheme was carried out to introgress resistance gene to bean *Pythium* root rot in various commercial varieties grown in Rwanda. The achieved crosses were performed between three selected susceptible varieties (R617-97A, RWR 1668 and Urugezi) which are adapted to the various ecological production zones of Rwanda and two known sources of resistance to *Pythium* root rot (RWR719 and AND1062). Following each inter varietal combination, a series of 4 successive backcrosses was achieved by using the susceptible parents as the recurrent lines to be improved for their respective behavior to *Pythium* root rot disease. At each backcross generation, the PYAA 19_{800} SCAR marker linked to *Pythium* root rot resistance was used to achieve an early selection for resistance

to the disease among the different progenies. The target materials serving for the molecular analyses were prepared from young trifoliate leaves of 2-weeks bean plantlets.

It was observed that at each backcross generation, there were variable proportions of plants exhibiting presence of the resistance gene according to the SCAR marker profiles.

Finally, to assess if the individual plants exhibiting the SCAR marker are effectively resistant to the *Pythium* root rot disease, inoculation tests were carried out with a *Pythium ultimum* strain on each of the self pollinated BC4 progenies of different parental combinations. None of the plants showing the SCAR marker exhibited development of *Pythium* root rot symptoms after inoculation, confirming thus real introgression of the resistance characteristics through the breeding scheme adopted in our work.

Key words: ***Phaseolus, vulgaris*, *Pythium,* Molecular, Root rot, Bean, genotype, Resistance.**

Introduction

The common bean (*Phaseolus vulgaris* L.) is the most important food legume crop grown worldwide (Wortmann and Allen, 1994; Wortmann et al., 1998; Buruchara, 2006). Beans are considered to be an excellent food as they are nutrient dense with high contents of protein, micronutrients, vitamins, dietary fiber, and also have a low glycemic index (wortmann and Allen, 1994; Bennink, 2005; Widers, 2006). Diverse forms of bean consumption including fresh or dry grains, green leaves and green pods (Kimani et al., 2006) are common in Rwanda. World annual global production of dry beans is estimated to reach 19.5 million t with Brazil being the highest producer with an estimated annual production of 4 million t (FAOSTAT, 2007).

Production of common beans throughout the different production areas in the world is hampered by various biotic and abiotic factors leading to a continuous decline of productivity. In Rwanda where the bean production is estimated to reach a level of 163,865 T (MINAGRI, 2009), the bean root rot disease has been found to constitute one of the major biotic constraints for the crop production.

Bean root rot caused by *Pythium* spp. is one of the most damaging diseases affecting common bean (*Phaseolus vulgaris*) in East and Central Africa sub-region where beans are grown in intensive agricultural production systems (Wortmann et al., 1998). A complete yield loss usually occurs when susceptible varieties are grown under environmental conditions which are favorable for the pathogen development like high level of humidity and low temperature varying between 14 and 17ºC (Buruchara and Rusuku, 1992). To reduce the level of production damages caused by this disease, several control strategies including the use of resistant varieties and performing soil amendments by using organic fertilizers can be performed. Moreover, exploiting the biological control

based on a competition effect between bean, pathogenic and non pathogenic isolates of *Pythium* can be used to limit the damages due to the disease (Spence, 2003). Finally the use of chemical applications through seed treatment has been shown to be efficient by ensuring a significant limitation of *Pythium* damages in beans (Bhardwaj et al., 1994).

The use of resistant varieties is considered to be the most viable option for controlling *Pythium* bean root rot particularly for small-scale growers (Otsyula et al., 1998). Previous screen house and field evaluations carried out in Kenya, Rwanda and Uganda allowed identifying a few bean lines with resistance properties to *Pythium* root rot disease. Among those lines, the genotypes RWR 719 and AND1062 with resistance to this disease (Buruchara and Kimani, 1999) have been characterized as having a molecular marker associated with the resistance property. Various investigations relative to inheritance of the bean resistance to *Pythium* in different varieties including RWR 719 and AND1062 have been achieved and revealed that resistance to *Pythium* root rot disease is controlled by a single dominant gene (Otsyula et al., 2003; Mahuku et al., 2007).

Once potential sources of resistance are identified, they can be used to introduce the trait of resistance into some popular commercial varieties. For that purpose, recurrent backcrossing following preliminary intervarietal hybridization is a traditional breeding method commonly employed to transfer alleles at one or more loci from a donor to an elite variety (Reyes-Valdes, 2000).

Molecular markers are tools that can be used as chromosome landmarks to facilitate assessing the effective introgression of chromosome segments (genes) associated with economically important traits (Semagn, 2006).

During the past two decades, the tendency to increase ability of transferring target genomic regions using molecular markers resulted in an important achievement of genetic mapping experiments. The aim is to develop molecular markers to be used for marker assisted backcrossing (MAB), also called marker assisted selection (MAS), marker assisted introgression or molecular breeding (Faleiro et al., 2000).

The use of MAB markers can constitute a very useful tool to speed up the breeding programme as only individuals exhibiting the markers constitute the materials of interest to be analyzed in more details (Faleiro et al., 2004).

That type of markers have been identified and/or used for MAB in several plant species, including maize, rice, wheat, barley, tomato, potato, sunflower, pea, bean, rye, millet, cotton, soybean, sorghum, cowpea, tobacco, turnip rape, cauliflower, sunflower, alfalfa, carrot, sugarcane, sugar beet, and grape (Gupta et al., 1999; Babu et al., 2004). Molecular markers do not require genetic engineering and cultivars to be developed by MAB are not transgenic and therefore do not face the public resistance against transgenic crops. The success of MAB depends upon several factors like the distance between the closest markers and the target gene, the number of target genes to be transferred, the genetic base of the trait, the number of individuals that can be analyzed and the genetic background in which the target gene has to be transferred, the type of molecular marker(s) used, and available technical facilities (Francia et al., 2005). The most favorable case for MAB is when the molecular marker is located directly within the gene of interest (Dekkers, 2003).

Several important genes in breeding for disease resistance and quality traits are inherited recessively (Frisch and Melchinger, 2001b; Semagn et al., 2006). In conventional backcross programmes for introgression of a recessive target gene, presence or absence

of that gene in a backcross individual is determined by a phenotypic assay of progeny generated either by selfing or by crossing with the donor parent (Semagn et al., 2006). In the case of bean resistance to *Pythium* root rot, a SCAR marker named PYAA 19_{800} was characterized as being associated with *Pythium* root rot resistance gene in RWR 719 and AND 1062 (Mahuku et al., 2007). This marker was already validated and successfully used in selection for resistance to bean root rot.

The objective of this work was to transfer the previously identified gene of resistance to *Pythium* species into susceptible commercial varieties adapted to the various ecological production zones of Rwanda. In that context, a breeding scheme based on a series of backcrosses between two resistant varieties (RWR 719 and AND1062) and the commercial susceptible varieties grown in Rwanda (R 617-97A, RWR 1668 and Urugezi) was adopted.

Materials and Methods

Genetic materials and study site

Seeds of three susceptible commercial cultivars were provided by the Rwanda national bean programme (R 617-97A, RWR 1668 and Urugezi), while seeds of the resistant varieties (RWR 719 and AND 1062) were provided by the CIAT Regional Office in Uganda. The genotype RWR 719 is a small seeded variety of Mesoamerican genepool that is resistant to all species of *Pythium* while AND1062 of the Andean genepool is the only large seeded variety resistant to *Pythium* (Mukalazi et al., 2001). These cultivars were released in Rwanda based on various characteristics presented in Table1.

Table 1. Type of reaction to *Pythium* and characteristics of genotypes used in the study

Cultivars	Reaction to *Pythium*	Growth habit	Seed size	Seed color	Maturity (days)	Yield (ton/ha)
Urugezi	Susceptible	Determinate	Medium	Red mottled	Medium	2
RWR 1668	Susceptible	Determinate	Large	Brown cream	Early	1,5- 2
R 617-97A	Susceptible	Determinate	Large	White	late	1.7 – 2.5
RWR 719	Resistant	Determinate	Small	Red	late	0,9
AND 1062	Resistant	Determinate	Large	Kidney Red	Medium	0,9

Source: CIAT 2004.

The experimental investigations were carried out at both laboratory and screen house levels in the CIAT regional centre of Kawanda-Uganda. This site is located at 0°25′05″ N and 32°31′54″ E at 1190 meters above sea level (masl) with an average rainfall of 1224 mm per annum and average daily temperatures of 15.3C (minimum) and 27.3C (maximum).

Planting conditions

The recipient cultivars R 617-97A, RWR 1668 and Urugezi were grown alongside the donor parent RWR 719 and AND 1062 in the screen house. The loam soil mixed with sand and organic manure (in ratios of 3:1:1) was sterilized by steam sterilization. Plastic pots (3kg) were filled at 3/4 with soil and three seeds were sowed in each pot. The so treated pots were watered every day around 8:00 am. NPK fertilizer was applied at flowering stage to improve plant vegetative growth and general vigor. For the crossing block establishment, recipient as well as donor parents were planted at different times (3 to 4 days interval) to ensure that there were constant flowers for both the donor and recipient plants.

Hybridization protocol

The female recipient plants were crossed with the male donor plants to develop an F_1 generation. During the following backcrossing cycles, F_1 plants were used as females while the recurrent parents were used as male parents. Male flowers were collected using tweezers when they were just opened. Flowers were collected the day of pollination and kept in paper bags for the shortest possible time until they were used. Buds selected on the female plants had increased in size and had lost the green color of immature buds but had not yet started to split. Such buds were considered as not yet self-pollinated and were ready to open a day later. Female flowers were opened and anthers were carefully removed to reduce or avoid self-pollination to take place since the bean flower was complete and therefore capable of self-pollination.

To achieve pollination, the stigma of male flower coated with pollen was removed using tweezers. The stigma of female flower was exposed and dusted with pollen from the male plant. The crossing was done early in the morning or later in the evening to avoid any damage which would be caused by the sun heat. Instruments used were washed thoroughly in alcohol to eliminate any pollen grain from previous flowers. The pollinated flower was then closed back with its petals to reduce possible natural crossing and also to preserve humidity around the stigma. Fertilized flowers were marked with a tag (Oscar and Luz, 1987) with primary information concerning the cross for easy further management of the crossing programme.

In the present work, the breeding scheme included a F1 generation and a series of 4 backcrossing generations as presented in the Table 2.

Table2. Scheme of crossing between susceptible and resistant varieties to *Pythium* spp.

Crossing	Type of crossing
Original cross	Cultivar A x Resistant rr RR
1 st Backcross	F1 x Cultivar A Rr rr 50% genes from A
2 nd Backcross	BC1 x Cultivar A Rr : rr rr 75% genes from A
3 rd Backcross	BC2 x Cultivar A Rr : rr rr 87,5% genes from A
4 th Backcross	BC3 x Cultivar A Rr : rr rr 93,75% genes from A
	BC4 Rr:rr 96,875% genes from A
	Self Rr plants from BC4 to obtain plants homozygous for RR

Source: (Poelman and Sleper, 1995).

Leaf samples preparation and DNA extraction

Total genomic DNA was extracted from young trifoliate leaves collected from 2-week-old plants in the screen house according to the procedure described by Mahuku (2004).

The samples were formed by 20 BC1 plants and 40 BC2, BC3, BC4 plants of each parental combination. The leaves were plucked from the plants and put in plastic bags labeled with the right identification number. The bags were then put on ice and transferred to the laboratory for DNA extraction using the FTA card procedure and subsequent analysis by the polymerase chain reaction using molecular marker ($PYAA19_{800}$). The collected leaves were spotted on the FTA plant saver cards following Whatman technologies. The samples were overlaid with parafilm and crushed using a porcelain pestle and mortar, followed by the following successive steps:

- The crushed leaf tissue was left to dry at room temperature for 1 hour;
- A 2 mm leaf disc was excised using a Harris unicore borer;
- The disc was washed two times using FTA purification reagent (100µl);
- The material was incubated for 3 minutes at room temperature;
- The disc was also washed again two times by using isopropanol (100µl);
- The material was incubated for 3 minutes at room temperature;
- The disc was dried in PCR tube;
- Sample discs were thus ready for addition of PCR master mix.

Polymerase Chain Reaction analyses

The PCR master mix consisted of 0,2 mM of dNTPs, 2mM $MgCl_2$, 1µ/25µl of Taq Polymerase,1X PCR Buffer and 0,4 µM of each primer. Sequences of the used primers were 5' - TTA GGC ATG TTA ATT CAC GTT GG-3' for primer 1 and

5'- TGA GGC GTG TAA GGT CAG AG-3' for primer 2 (Mahuku, 2004).

The 25µl-reaction PCR reaction volume was subjected to 34 amplification cycles in a BIO RAD MyCycler thermal cycler consisting of 1 cycle 94°C for 5 min, and 34 cycles including each the steps of denaturation at 94ºC for 40 seconds, annealing at 63°C for 40 seconds, and extension at 72°C for 40 seconds. These cycles were followed by a final extension for 7 min at 72°C and a holding temperature of 4°C.

Amplification products were separated through electrophoresis migration in a 1.2% agarose gel covered by a 0.5X TBE buffer under a voltage of 100V for 45 minutes. For the visualization, the gel containing ethidium bromide (0.5/ml) was lighted with ultraviolet light and photographed for scoring (Mahuku et al., 2007).

Pathogenicity tests on the backcross progenies

Inoculum of one *Pythium ultimum* strain was multiplied by plating mycelia on autoclaved millet grains (100 g) mixed with 200 ml of water in 500 ml bottles. One isolate of *Pythium ultimum* preserved at CIAT-Kawanda was used in this study based on its distribution and severity in East and Central Africa region as previously characterized by Mukalazi, (2001).

After two weeks of incubation under darkness and 25°C, pre sterilized soil was mixed with the infested millet at a ratio of 1:10 v/v in wooden trays of 42 cm x 72 cm. Each tray contained 10 plants of each bean cultivar and each line from self pollinated BC4 of the different parental combinations used in this evaluation analysis. The trays were set up in a Completely Randomized Block Design (CRBD) with three replications.

After germination, the seedlings were watered two times per day to provide a favorable 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). Cultivars that had an average disease score of 1-2 were considered as being resistant while those with an average score of 3-5 were considered tolerant and those with an average score of 6-9 were considered to be susceptible (Abawi and Pastor- Corrales, 1990).

Results

Genotypic results

All the individual plants of the F_1 generation were supposed to be uniform and heterozygous for the targeted resistance characteristics. Based on the morphological aspect of either seeds as well as of their resulting plantlets, the uniformity within F1 generation was confirmed. Moreover, whatever the combination of bean genotypes, all the seeds of the F1 generation were of the same morphological aspect as that of their female parent line.

At the first backcross (BC_1), progenies were analyzed, by using the molecular marker PYAA 19_{800}, for presence of the gene of resistance to *Pythium*.

At the different backcross generations, molecular analyses using the PCR technology were undertaken to assess the presence of the targeted gene conferring resistance to *Pythium* root rot disease.

Globally, it was observed that the molecular pattern was variable at the level of each backcross generation as there was a mixture of individuals showing the targeted marker and other individuals without the marker of interest in this study (fig 1).

The evolution of proportion of individuals showing the presence of the targeted zone is showed in the Table 3 using the PYA19_{800} SCAR marker.

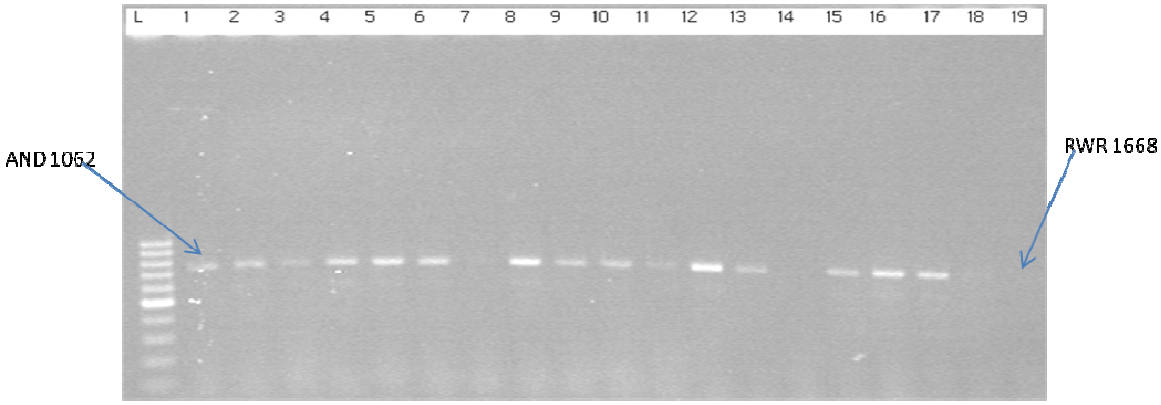

Fig1. Amplification of BC_4 plants samples from [(BC_4 RWR 1668 X AND 1062) X RWR 1668] plus two parental cultivars (AND 1062 & RWR 1668). L= DNA ladder. Sample 1 = the resistant parent, 19= the recurrent (RWR 1668) parent, from 1 to 18= BC_4 plants samples from [(BC_4 RWR 1668 X AND 1062) X RWR 1668].

The results based on the different proportions of individuals with the different molecular patterns allowed performing chi square analysis for each type of hybridization and at each backcross generation. The backcross populations BC1 [(Urugezi x AND 1062) x Urugezi], [(R 617-97 x RWR 719) x R 617-97] and [(R 617-97 x AND 1062) x R 617-97] fitted 1:1 ratios at 5% and 1%, while the remaining BC1 crosses were not significant (Table 3).

The resistant progenies were crossed to the recurrent parents to generate BC_2 progenies. The BC2 populations were analyzed as for the BC1 and results showed that χ^2 goodness-of-fit to the 1:1 ratio of the parental combinations [(RWR 1668 x RWR 719) x RWR 1668], [(RWR 1668 x AND 1062) x RWR 1668], [(Urugezi x AND 1062) x Urugezi] and [(R 617-97 x RWR 719) x R 617-97] were highly significant (Table 3). The parental combination [(Urugezi x RWR 719) x Urugezi] was significant at 1:1 ratio and only the χ^2 goodness-of-fit to the 1:1 ratio of the parental combination [(R 617-97 x AND 1062) x R 617-97] was not significant. As shown in table 3, this parental combination had the highest number of resistant progenies, followed by [(Urugezi x RWR 719) x Urugezi].

In BC3 generation the χ^2 goodness-of-fit to the 1:1 ratio was highly significant in only one parental combination [(R 617-97 x AND 1062) x R 617-97)], while all the other parental combinations did not show significance.

At the BC_4 generation, the parental combination [(RWR 1668 x RWR 719) x RWR 1668)] fitted the 1:1 ratio significantly and [(RWR 1668 x AND 1062) x RWR 1668)] fitted the 1:1 ratio at highly significant level. The same parental combination [(RWR 1668 x AND 1062) x RWR 1668)] had the lowest number of the plants showing presence of the marker of interest compared to the other parental combinations (Table 3).The other parental combinations of this backcross generation were not significant. At the BC1 and BC2 generations, 32.5% and 24% of progenies showed the presence of marker ($PYAA19_{800}$) linked to the resistance gene. At the BC3 and BC4 generations, about 42% of plants in the two generations showed the presence of the molecular marker. At the BC4 generation, the resistant plants were self-pollinated and the harvested seeds were used to perform a pathogenicity test carried out in a screen house. The figure 2 shows the evolution of seed color at the different generations of the backcross programme, taking the example of URUGEZI X AND 1062 parental combination.

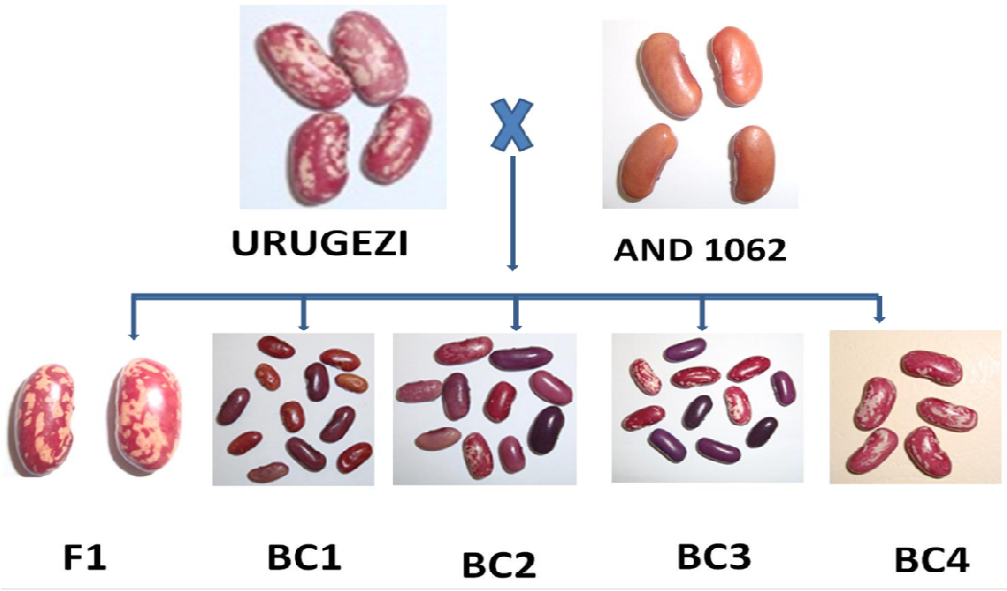

Fig2. Evolution of seed color at the different generations of the breeding scheme: case of the parental combination [(URUGEZI X AND 1062) X URUGEZI]

Table 3. Test of χ^2 for a goodness-of-fit to the 1:1 ratio of different crosses of BC_1, BC_2, BC_3 and BC_4

Crosses	No. Plants		χ^2 BC1	No. Plants		χ^2 BC2	No. Plants		χ^2 BC3	No. Plants		χ^2 BC4
	P	A	1:1	P	A	1:1	P	A	1:1	P	A	1:1
(RWR 1668 x RWR 719) x RWR 1668	6	14	3.2 ns	5	35	22.5 **	18	22	0.4 ns	13	27	4,9*
(RWR 1668 x AND 1062) x RWR 1668	11	9	0.2 ns	10	30	10 **	24	16	1.6 ns	11	29	8.1**
(URUGEZI x RWR 719) x URUGEZI	11	9	0.2 ns	12	28	6.4 *	19	21	0.1 ns	19	21	0.1ns
(URUGEZI x AND 1062) x URUGEZI	4	16	7.2 **	7	33	16.9 **	14	26	3.6 ns	26	14	3.6 ns
(R 617-97 x RWR 719) x R 617-97	3	17	9.8 **	6	34	19.6 **	15	25	2.5 ns	16	24	1.6 ns
(R 617-97 x AND 1062) x R 617-97	4	16	7.2**	17	23	0.9 ns	10	30	10**	16	24	1.6 ns

Progeny size analyzed: n = 20 for BC_1 and n=40 for BC_2, BC_3 and BC_4.

P: plants showing the presence of the marker of resistance on the agarose gel, A: plants which did not show the presence of marker of resistance on agarose gel, ns: not significant,

*, **: significant at 5% and 1% probability level, respectively.

Pathogenicity tests

Results of the pathogenicity tests are presented in the Table 4 where the data were collected and expressed as scores of the disease severity. As shown in the table, two references were used in the inoculation tests; the susceptible reference was the commercial variety CAL 96 for which the observed symptoms were scored at an average disease severity of 8.47 while the resistant reference was the variety RWR 719 with an average disease severity score of 1.33. All the plants resulting from the breeding scheme carried out in our study showed a level of root rot symptoms varying between the two reference varieties. In fact, the disease severity varied between 2.23 and 3.73 for the plants resulting successively from [(RWR 1668 X RWR 719) x RWR 1668] and [(URUGEZI X RWR 719) x URUGEZI] parental combinations.

According to the adopted scoring system, all these plants were estimated to be resistant except progenies of the parental combinations [(URUGEZI X RWR 719) x URUGEZI] and [(R 617-97A X RWR 719) x R 617-97 A] which were scored as tolerant.

Table 4. Bean root rot severity in 6 selfed BC4 progenies and 2 references (30 plants per class of materials)

Variety and hybrid	Extreme values	Mean	t Grouping	Disease expression of bean cultivars
CAL 96	6-9	8.47	A	Susceptible
(URUGEZI X RWR 719)x URUGEZI	2-9	3.73	B	Tolerant
(R 617-97A X RWR 719) X R 617-97A	2-9	3.20	BC	Tolerant
(R 617-97A X AND 1062) X R617-97A	2-9	2.90	DC	Resistant
(URUGEZI X AND 1062) X URUGEZI	2-9	2.73	DC	Resistant
(RWR 1668 X AND 1062) X RWR 1668	2-7	2.67	DC	Resistant
(RWR 1668 X RWR 719) X RWR 1668	1-5	2.23	D	Resistant
RWR 719	1-2	1.33	E	Resistant

Means with the same letter are not significantly different, α= 0.05, LSD= 0.76, SE=0.27, Pr> |t|<.0001.

To complement the data presented in this previous table, a further analysis based on the frequency distribution of the total plants evaluated for *Pythium* root rot severity was undertaken with all the parental combinations from the selfed backcross 4. The figure 3 presents the results obtained for the parental combination (URUGEZI X RWR 719) X URUGEZI.

To complement the data presented in this previous table, a further analysis based on the frequency distribution according to the CIAT visual scale whose scores vary from 1 to 9 was undertaken. The figure 3 presents the results obtained for the parental combination of BC4 (URUGEZI X AND 1062) X URUGEZI by classifying the various tested plants in severity levels. The remaining parental combinations are presented in the annex1.

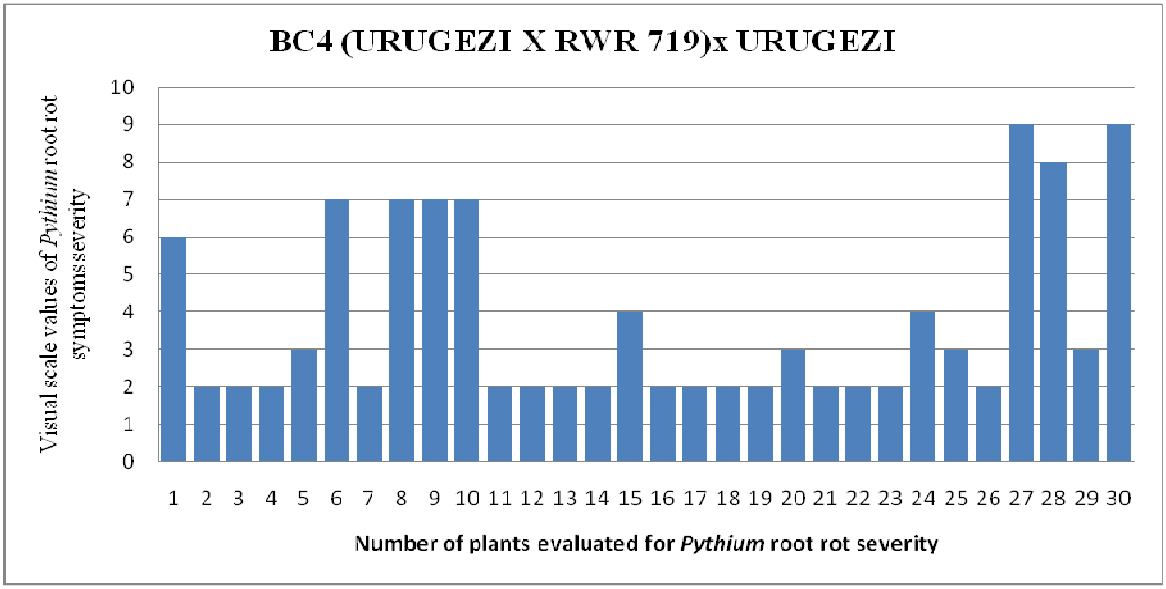

Figure 3. Evaluation of *Pythium* root rot symptoms on parental combination of Backcross 4 (URUGEZI x RWR 719) x URUGEZI

On this figure, severity of *Pythium* root rot symptoms was assessed using the CIAT visual scale whose scores vary from 1 to 9. It is observed that based on the recorded data, 8 plants were susceptible with scores varying between 6 to 9, 6 plants were scored as tolerant, having a score varying between 3 to 4 and 16 plants were resistant to *Pythium* root rot disease. It can be noticed the absence of a continuous distribution of the reaction behavior in the different 9 scores of severity symptoms.

Discussion

In the present study, we evaluated the feasibility of improving the resistance level to *Pythium* root rot disease in different common bean varieties used in Rwanda. The work was carried out by performing a series of intervarietal hybridizations followed by backcrosses using the parental lines to be improved as the male parent.

To speed up the selection process, a molecular marker named $PYAA19_{800}$ (Mahuku et al., 2007) was used for an early identification of individuals holding the characteristics of resistance to *Pythium* root rot disease. Molecular analyses performed with the backcross

progenies facilitated identification of individuals holding the resistance marker revealing the presence of resistance gene. However, the analysis based on χ^2 test which was carried out at the different backcross generations has shown various signification levels of our null hypothesis which was of 1:1 inheritance of the resistance marker gene. If we take the cases relative to the BC4 generation, it appeared that for most of the considered crosses, there was no signification for the χ^2 values except for the crosses (RWR 1668 x RWR 719) x RWR1668 and (RWR 1668 x AND 1062) x RWR 1668 for which the obtained values for χ^2 were significant. This means that in the majority of our cases, the null hypothesis of 1:1 is confirmed at the level of BC4 generation. For the few cases where this hypothesis is not confirmed, it became interesting to take into consideration the situation at the other BC generations; the obtained values were not significant at the BC1 and BC3 generations while the same values were significant at the BC2 and BC4 generations. That situation could be due different factors like (1) the sampling precision and (2) the accuracy of the PCR reaction used to amplify the targeted marker.

The pathogenicity tests based on biological tests involving inoculation assays constituted the ultimate assessment of the effective introgression of the resistance properties. The severity of disease symptoms recorded on individual plants issued from the self pollinated BC4 lines was lower than the one recorded in susceptible varieties. This observation allowed classifying these plant materials resulting from the breeding process in the category of tolerant or resistant genotypes whatever the parental combination involved.

To assess if the resistance/susceptibility behavior is controlled by a qualitative or a quantitative trait, frequencies distribution of plant individuals for *Pythium* root rot evaluation were considered according to the recorded reaction following the inoculation phenomenon. It was revealed that only a limited number of severity levels were represented for each tested progeny. In these conditions, it should be considered that the reaction to *Pythium* inoculation

is under control of a qualitative factor as there is a lack of continuous phenotypic variation ranging from high susceptibility to high resistance (Nandy et al., 2008).

Given that profile of resistance to *Pythium* found in the progenies from backcrosses between (RWR 1668, R 617-97A and Urugezi, as recipient parents) and the selected sources of resistance (RWR 719 and AND 1062, as donor parents), it can be concluded that the introgression of the resistance to *Pythium* root rot disease from the two donor parents was successful. Other authors (Otsyula et al., 2003; Mahuku et al., 2007) have found similar results in the genetic improvement of common beans for the resistance to *Pythium* root rot.

Mahuku et al. (2007) reported the identification of molecular markers (SCAR: sequence characterized amplified region) linked to the resistance genes in RWR 719, AND 1062 and MLB 49-89A. The mechanism of inheritance of gene conferring resistance to *Pythium* root rot in beans was studied by Otsyula et al. (2003). In their investigations, seeds of susceptible commercial cultivars were provided by bean national programs of Rwanda (Urugezi), Uganda (CAL96) and Kenya (GLP2 and GLP585) while resistant varieties RWR 719, SCAM-80-CM/15, MLB 49-89A, AND1055, and AND1062 were provided by the station of Kawanda belonging to CIAT. These authors concluded that the inheritance of resistance to *Pythium* root rot with the tested cultivars was monogenic.

In our own results, some parental BC combinations confirm the monogenic resistance to *Pythium* root rot. The introduction of the gene controlling the resistance to *Pythium* root rot through the backcross programme can be obtained both from the small seeded RWR 719 (Mesoamerican genepool) genotype and the large seeded AND 1062 genotype (Andean genepool).This means that the gene of resistance can be transferred from varieties belonging to both genepools (Andean and Mesoamerican).

In the BC4 generation, we self-pollinated the plants showing the presence of the molecular marker and a sample of 10 self-pollinated plants per parental combination was used to carry

out the pathogenicity test. The low scores observed in progenies of the different parental combinations showed a strong correlation between the presence of the marker and the resistance of plants.

In conclusion, a global strategy of controlling *Pythium* root rot disease including the use of genetic resistance in combination with other appropriate control methods could be efficiently applied.

On the basis of the results obtained during this study, genotypes RWR 719 and AND1062 are recommended as donors to transfer *Pythium* root rot resistance into commercial varieties which will increase yield stability in farmer's fields in Rwanda.

There are various disease control methods of bean root rot such as chemical control, biological control, genetic resistance methods and cropping practices (Abawi and Pastor Corrales, 1990; Buruchara, 1991: Otsyula et al., 1998).

Many specific pesticides like Benomyl, Captafol, Captan, Carboxin, Metalaxyl, Propamocarb hydrochloride and Etridiazole were already proven to be efficient in controlling *Pythium* root rot diseases on beans. In the same context, soil fumigants such as methyl bromide, chloropicrin and vorlex are highly effective biocides that kill *Pythium* agents (Abawi et al., 2006). In Latin America and Africa, one of the safest and most economical uses of chemicals to control *Pythium* pathogens consists of treating the seeds. This usually results in an effective protection of seeds and young seedlings for about 2 to 3 weeks after sowing (Schwartz et al., 2007; Abawi et al., 2006).

Beneficial microorganisms of interest for biological control of plant pathogenic *Pythium* spp. have been identified among fungi and bacteria. Isolates of *Trichoderma* spp. and *Gliocladium* spp. are antagonists of *Pythium* induced soil-borne diseases (Howell et al., 1993) and several strains are already commercially available for biological control of *Pythium* root rot (Fravel, 2005).

Host resistance offers the cheapest but most effective strategy for farmers, particularly small landholders for the control of bean root rot (Otsyula and Ajanga, 1994; Wortmann et al., 1998).

Cultural control methods involve crop rotation, planting on ridges, use of organic manures and inorganic fertilizers. Crop rotation including resistant varieties of beans keeps the soil inoculum level of *Pythium* oospores low in systems (Rosado et al., 1985; Hall and Phillips, 1992).

Planting beans on ridges to increase aeration and reduce soil moisture has been reported to be effective in reducing *Pythium* oospores levels. Hilling up soil around the stem of bean seedlings encourages the growth of adventitious roots, allowing the plant to recover from *Pythium* root rot attack (Averre, 1999; CIAT, 2004).

Application of fertilizers greatly enhances crop ability to withstand *Pythium* root rot attack through availability of plant nutrients readily taken up by weakened plant roots (Buruchara, 1991; Voland and Epstein, 1994). Buruchara (1991) and Buruchara and Cheidegger (1993), showed that incorporating *Leucaena spp.* leaves and twigs of *Calliandra calothyrsus* Meisn and *Sesbania sesban* (L.) Merr. as green manure two weeks before planting reduces plant mortality and increases bean grain yield. These amendments enhance soil microbial activity and plant nutrition thus enhancing tolerance to root rot (CIAT, 2004).

On the other hand, the risk of the breakdown of resistance to *Pythium* root rot on beans should be studied. There is always the risk of losing the resistance to *Pythium* root rot in backcrossing programmes. To reduce this danger, it is necessary not only to diversify the sources of resistant materials from the *Phaseolus* genepool but also to initiate population improvement programmes in order to develop common bean varieties having horizontal resistance to bean root rot (Miklas et al., 2006).

Acknowledgment

The authors would like to express their warmest thanks to CIAT for having given technical assistance and laboratory facilities to Mr. Nzungize during his investigations. Moreover, the project was supported by the Belgian Technical Cooperation, the Kirkhouse Trust and the Rwanda Agricultural Research Institute which are thanked through this work.

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CHAPTER V

GENERAL CONCLUSION AND PROSPECTS

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Common bean is an important crop, especially for developing countries where it provides proteins in vegetarian or meat-poor diets. In addition, bean seeds are easily stored in a dry form and plant interactions with symbiotic nitrogen-fixing bacteria make the crop valuable for sustainable agriculture through maintenance of soil fertility (Olivera *et al*., 2004; Slatni *et al*., 2008).

The common bean, *Phaseolus vulgaris* L., is one of the principal crops in Rwanda. It is grown throughout the country by 95% of the farmers and provides 65% of the protein and 32% of the calories in the Rwandan diet (Sperling, 2001). However, production of common bean in Rwanda is greatly affected by many key factors such as diseases, pests and lack of resistant varieties. Root rot disease is one of the major biotic constraints that affect common bean production in Rwanda. The disease is caused by multiple soil-borne pathogens and it is considered as the most damaging in East and Central Africa including Rwanda. *Pythium* spp. is one of a complex of soil-borne pathogens causing root rot diseases on bean (Rusuku *et al.,* 1997; Otsyula *et al*., 1998; Wortmann *et al*., 1998).

The disease can lead to total yield losses in susceptible varieties. It is controlled by a number of methods, one being the use of metalaxyl-based fungicides that are usually applied as seed dressings. However, this treatment has been reported to offer a bare minimum protection (Salih and Agreeb, 1997; Louise and Paul, 2006). Another approach that has been proved to be more effective than a single control measure is the combination of organic amendments, raised beds and resistant varieties (Buruchara and Scheidegger, 1993; Voland and Epstein, 1994).

On the other hand, the most sustainable and viable means of controlling the disease is the use of resistant varieties. (Otsyula and Ajanga, 1994; Garret *et al*., 2001).

However, selection and sustainable use of resistant varieties have to take into account diversity of causal agents (Falconi *et al*., 2007; Louise and Paul, 2006). It is for this reason that in the current study, an attempt was made to characterize *Pythium* agents inducing root rot diseases on common bean in Rwanda. This was considered of paramount importance in developing a breeding strategy aimed at improving the resistance to that disease and meeting the conditions of a sustainable management of the resistant varieties. Indeed, a study of the bean *Pythium* populations in Rwanda would assist in identifying and exploiting the sources of resistance to a maximum of *Pythium* pathotypes found in the country.

The main objective of this study was to improve resistance to *Pythium* root rot in major local common bean varieties, which are the most preferred by local farmers and consumers. The study was organized into different components including: (1) collection, isolation, characterization and taxonomical identification of *Pythium* isolates found in various regions of Rwanda, (2) analysis of pathogenicity profile of different *Pythium* species, (3) reaction of common bean varieties against *Pythium* root rot and (4) setting up a breeding process to introgress the resistance genes in traditional common bean varieties cultivated in Rwanda.
The main results of our investigations are as follows.

A survey to establish the status of the root rot diseases in Rwanda revealed that the symptoms are caused by a diversity of soil-born agents including *Pythium* species. In some of the diseased plant samples, we were unable to isolate any *Pythium* species.

In order to identify the *Pythium* taxa, 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') (Mahuku, 2004).

During the present study, 16 *Pythium* species were characterized and their locations in Rwanda identified. *P vexans* was found to be the most prevalent species in the country as its presence was revealed in 23 isolates obtained from samples collected in 12 districts (table 2). Some of the identified *Pythium* species were previously identified as causing bean root rot disease in different areas of bean production throughout the world, especially in some countries like Burundi, Democratic Republic of Congo, Kenya, Rwanda, Uganda, USA,… (Rusuku *et al.,* 1997; Mukalazi, 2004).

The species *P.indigoferae* was found in samples from 6 districts, while *P. torulosum, P .ultimum* and *P. rostratifingens* were found in only 4 districts. The remaining *Pythium* species identified among the samples collected in Rwanda were distributed in a lower number of districts with the species *P.cucurbitacearum*, *P. arrhenomanes*, *P. pachycaule* and *P. rostratum* being the less widespread as having been found in only one district for each species.

No relation was observed between altitudes and prevalence of some particular *Pythium* species. This is illustrated by various examples like the case of *P. vexans* which was found under high altitudes (2240 m), intermediate altitudes (1560 m) and low altitudes (1372 m).
An attempt to inoculate the Rwandan common bean varieties with the *Pythium* species isolated in our collected samples showed that there were no differences in the reaction regardless of the pathogen species used (table 3, chap.3).

In addition, it was revealed that all the different *Pythium* species isolated from diseased bean tissues collected in Rwanda induced root rot symptoms when inoculated to the susceptible

common bean varieties (CAL96, RWR617-97A, Urugezi and RWR 1668) under screen house conditions. This suggested that, if a given bean variety was susceptible to one *Pythium* species, the probability is high that the same variety would be susceptible to all the other *Pythium* species used in the present study (Table 3, chap. 3). This observation was considered noteworthy because once a resistant genotype is identified in presence of one *Pythium* species, it can be integrated into a breeding programme aimed at improving *Pythium* bean root rot resistance whatever the region where beans are grown in Rwanda.

Various investigations relative to inheritance of the bean resistance to *Pythium* root rot in different varieties including RWR 719 and AND1062 have been achieved and revealed that resistance to *Pythium* root rot disease is controlled by a single dominant gene (Otsyula *et al*., 2003; Mahuku *et al.,* 2007).

In our research work, a backcross programme undertaken to introgress genes of resistance to *Pythium* root rot in various local susceptible varieties grown in Rwanda resulted in new genotypes showing resistance to this disease. In fact, these breeding lines were obtained from backcrosses using as recurrent parents three traditional cultivated varieties in Rwanda: RWR 1668, R 617-97 and Urugezi and as donor resistant parents two varieties: AND 1062 from the Andean genepool and RWR 719 from Mesoamerican genepool. This was a major achievement due to the fact that all the common beans evaluated in this study were found to be susceptible to root rot disease. Introgression of resistance genes through backcrossing protocols was easy and rapid to achieve. Segregation of resistant and susceptible plants in BC generations from some parental combinations suggests also the monogenic resistance to *Pythium* root rot, with the gene of resistance being dominant.

As a diversity of *Pythium* species was identified, it should be better to diversify also the sources of resistance and to integrate them in a breeding programme, with the aim to develop

varieties with durable resistance. On the basis of some morphological characteristics, most of varieties grown in Rwanda belong to the Mesoamerican genepool. In fact, the majority of Rwandan common beans possessed smaller seed size, larger cordate bracteole size and type II or III growth habit that corresponded to the race Mesoamerica description of Singh *et al.* (1991b). The type II corresponds to indeterminate bush growth habit, and type III indicates indeterminate prostrate growth habit (Fernandez *et al*., 1986).

By trying to diversify the sources of resistance to *Pythium*, breeders should focus their efforts to Mesoamerican genotypes for improving Rwandan common beans against *Pythium* root rot. Even if the first choice looking for resistance to *Pythium* is Mesoamerican genepool, *Pythium* root rot resistance gene can be also obtained from the Andean genepool. Indeed this genepool of Andean origin may contain other mechanisms of resistance or tolerance to *Pythium* root rot and have also additional capacities of adaptation to the various traditional cropping systems.

On another hand, the occurrence of breakdown of resistance to *Pythium* root rot on beans should be studied. There is always the risk of losing the resistance to *Pythium* root rot if this trait is due to one single gene. To reduce this danger, it is necessary not only to diversify the sources of resistant materials from the *Phaseolus* genepool but also to initiate population improvement programmes in order to develop bean varieties combining both horizontal and vertical resistance to bean root rot (Miklas *et al*., 2006).

Isolation protocols from some rotten bean roots did not show any *Pythium* colonies. This means that the disease symptoms were caused by other pathogens. As it was revealed in our study, root rot can be caused by a complex of soil born diseases. To cope with this problem, we recommend the following steps: (1) sampling in the different agroecological regions of Rwanda a large collection of common bean roots having root rot symptoms, (2) applying isolation protocols to identify the different pathogens (*Fusarium* spp, *Rhizoctonia solanii* and

Pythium spp), (3) identification of sources of resistance among the common bean genepool to these different pathogens and (4) using genes pyramiding methods in order to have one variety combining resistance genes to root rot pathogens (CIAT, 2005).

The largest ex-situ collection of *Phaseolus* is located at the International Centre for Tropical Agriculture (CIAT) near Cali (Colombia). It contains over 40,000 entries, of which 35 000 are *Phaseolus vulgaris* (Brink and Belay, 2006). According to estimates, this collection represents 50-75% of the variability present in the centers of diversification for domesticated types, but only less than 30% of the wild types diversity (Wortmann *et al*., 1998; Brink and Belay, 2006). Among the bean germplasm collections held in Africa are: Bunda Agricultural College in Lilongwe, Malawi (6000 entries), National Genebank of Kenya, KARI in Kikuyu (3000 entries) and the Rwanda Agricultural Research Institute in Butare (3000 entries) (Wortmann, 2006). Taking into account such large variability of genetic resources of the common bean, we recommend setting up pathogenicity test of *Pythium* species on a large sample of common bean varieties in an effort to diversify the sources of resistance to *Pythium* root rot and to integrate them in a breeding programme.

Some cultural practices are known to reduce severity and incidence of root rot diseases. For example, the incidence of *Fusarium spp, Pythium spp.* and *Rhizoctonia solani* on beans was reduced when maize was included in rotations (Rosado *et al.,*1985). Rotations of three to four years are effective in reducing the buildup of *Fusarium solani* f. sp. *phaseoli*, *Rhizoctonia solani* and various *Pythium* sp on common beans. Other crops such as sunflower, potato and soybean which are susceptible to these fungi should not be used in rotation system. Maize and wheat are good rotation choices (Rusuku *et al.,* 1997; Harvey, 2004).

Also, sowing dates in relation to rains affect the incidence and severity of *Pythium* because bean plants are most susceptible to root rot diseases during the first 2 to 3 weeks of crop

growth (Salih and Agreeb, 1997). Therefore, adjusting the planting time to avoid excess rainfall during that period can help the plants escaping disease attack at the most vulnerable stage.

Deep ploughing and use of raised ridges to grow beans has been found to reduce root rot favoured by high moisture, such as *Rhizoctonia solani* J.G. Kühn, *Sclerotium rolfsii* Sacc*, Fusarium oxysporum* Schlecht and *Pythium* spp root rot. This is because ridging and deep tillage increase aeration and drainage, creating less favourable conditions for disease development (CIAT, 2004).

Application of organic soil amendments is also known to reduce root rot diseases (Voland and Epstein, 1994). Buruchara (1991) and Buruchara and Cheidegger (1993) showed that incorporating *Leucaena spp.* leaves and twigs of *Calliandra calothyrsus* Meisn and *Sesbania sesban* (L.) Merr. as green manure two weeks before planting reduces plant mortality and increases bean grain yield. These amendments enhance soil microbial activity and plant nutrition thus enhancing tolerance to root rot (CIAT, 2004).

Finally, as the improved varieties are to be used by farmers, it is recommended to evaluate their performance under various production conditions based on factors like: (1) soil fertilization; (2) types of rotation; (3) intercropping regime and (4) integration in varietal mixtures. Evaluation of the improved common bean varieties under a wide array of field conditions will guide us to identify the best combination of cultural practices with the target of reaching higher bean productivity under efficient and sustainable control of *Pythium* root rot in Rwanda.

Once the resistant improved varieties are stabilized, the national bean research programme should work with local NGO/farmers cooperatives to support local seed production of improved varieties.

The bean research programme of Rwanda should be involved in distribution of improved seeds to farmers on a seed credit basis, where supplied seed is planted and some of the seed harvested by farmers is recovered by the Institution. The purpose of this approach is to show to the farmers the benefits of new bean varieties (Rubyogo *et al.,* 2007).

This programme must use small packs of seeds of new bean varieties which farmers can buy. The idea is to sell small quantities of seed through local shops, extension agencies, NGOs, or other local outlets (Phiri *et al*., 2000).

Finally, the research team should work with small and large seed producers in partnership.

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ANNEX

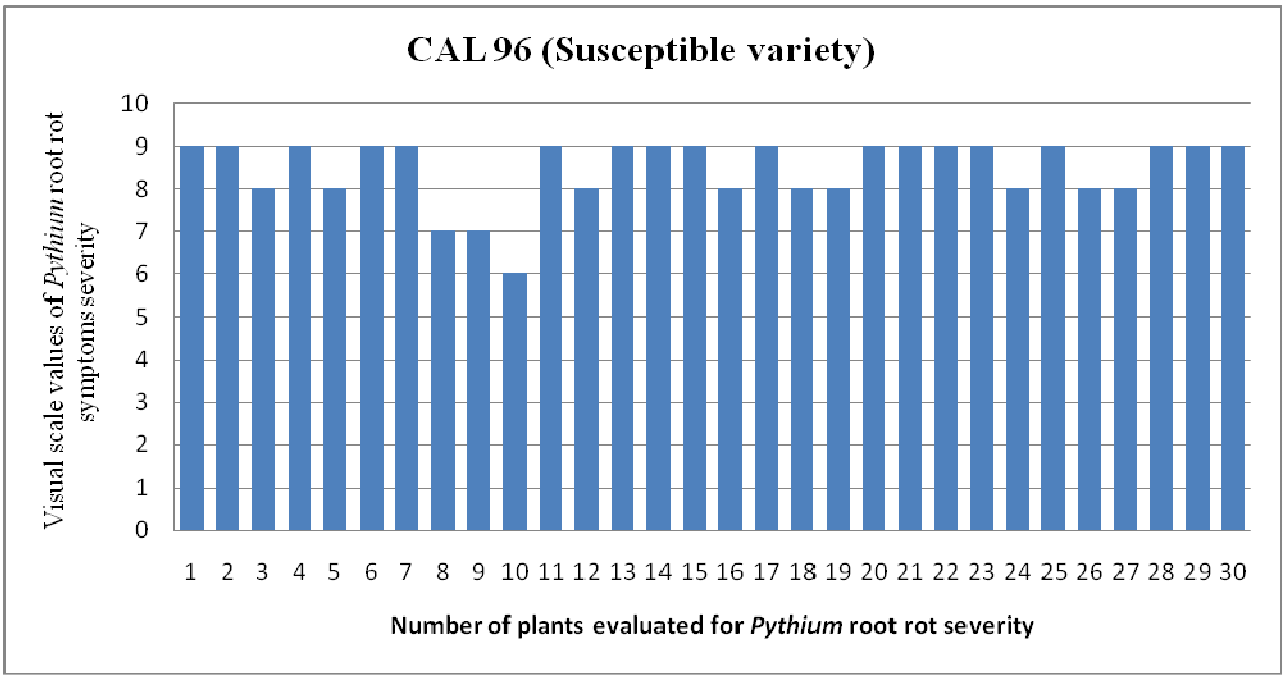

Figure 1. Evaluation of *Pythium* root rot symptoms on variety CAL 96

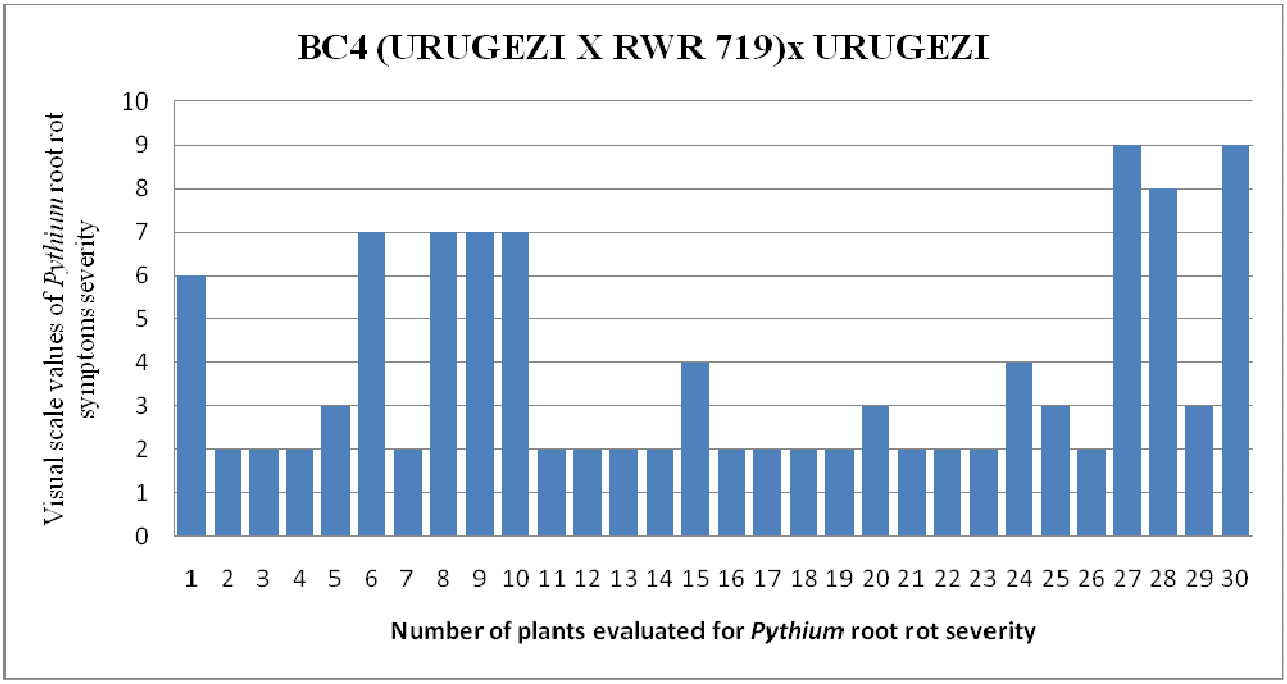

Figure 2. Evaluation of *Pythium* root rot symptoms on parental combination of **Backcross 4 (URUGEZI x RWR 719) x URUGEZI**

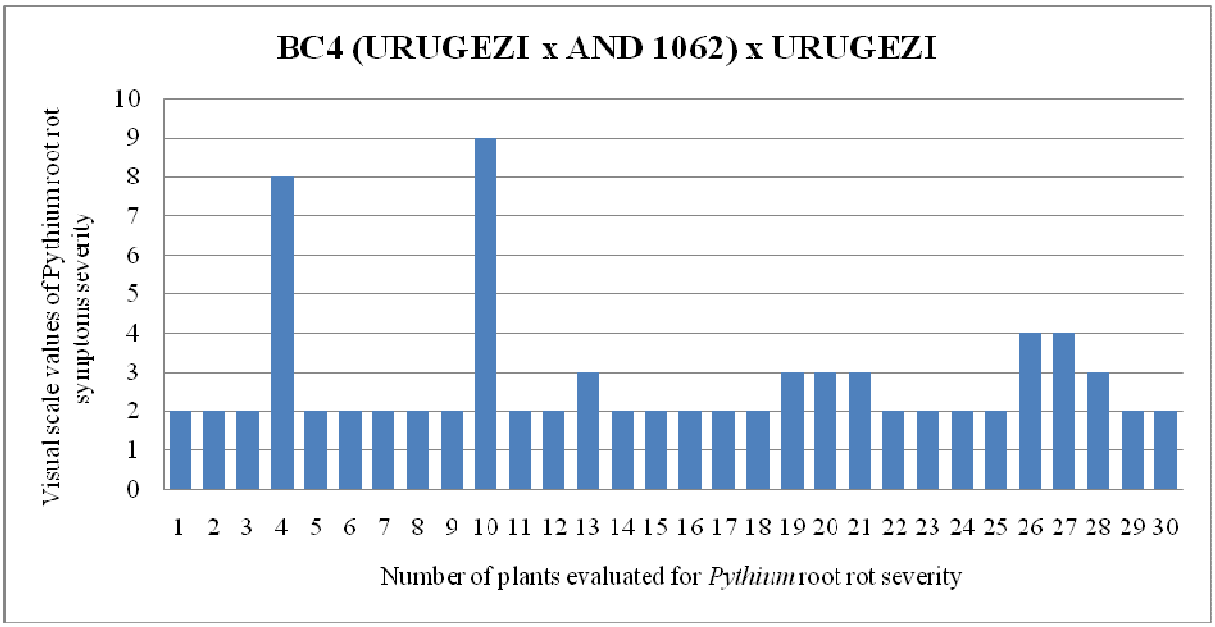

Figure 3. Evaluation of *Pythium* root rot symptoms on parental combination of Backcross 4 (URUGEZI x AND 1062) x URUGEZI

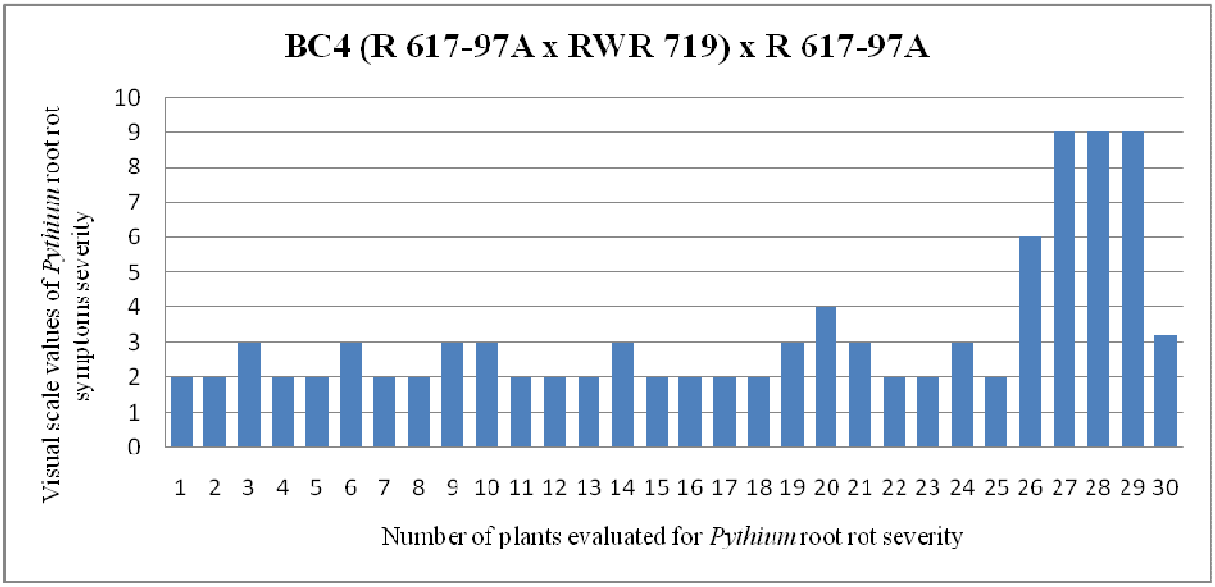

Figure 4. Evaluation of *Pythium* root rot symptoms on parental combination of Backcross 4 (R 617-97A x RWR 719) x R 617-97A

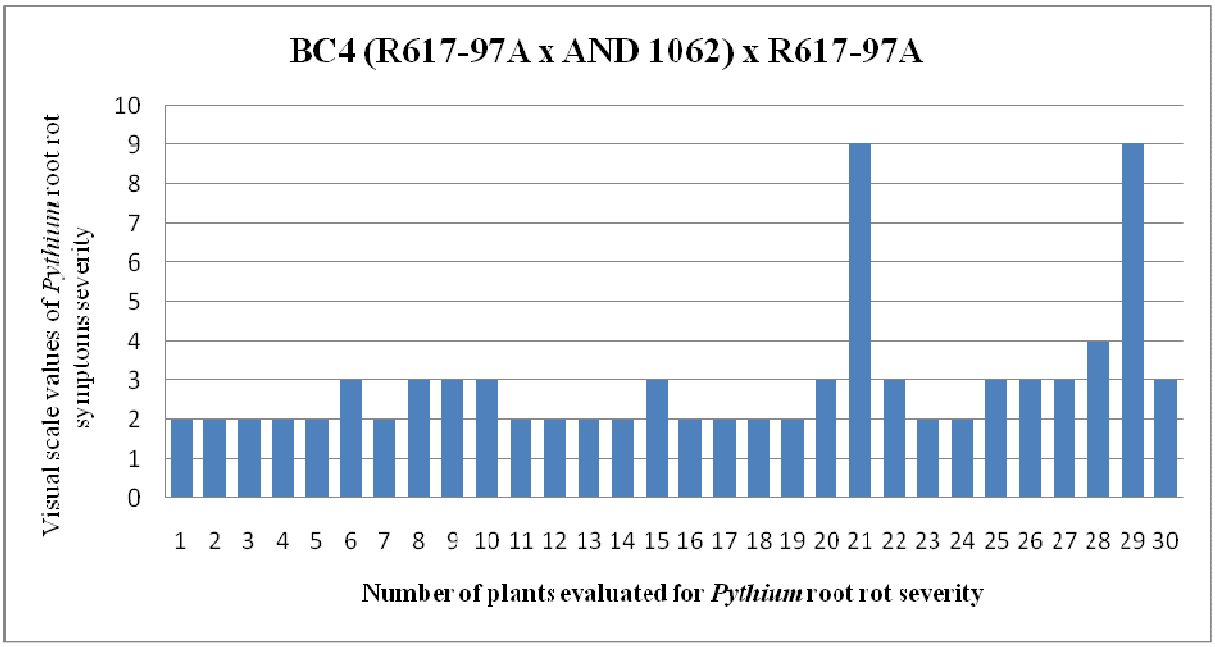

Figure 5. Evaluation of *Pythium* root rot symptoms on parental combination of Backcross 4 (R 617-97A x AND 1062) x R 617-97A

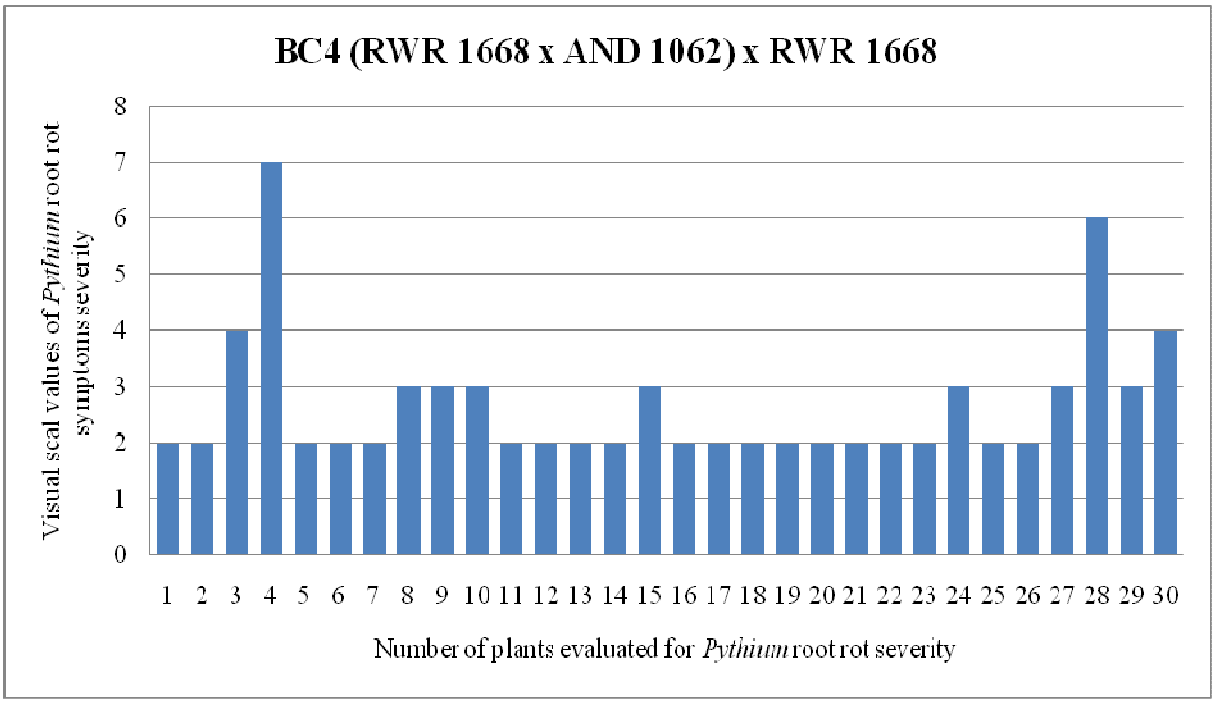

Figure 6. Evaluation of *Pythium* root rot symptoms on parental combination of Backcross 4 (R WR 1668 x AND 1062) x RWR 1668

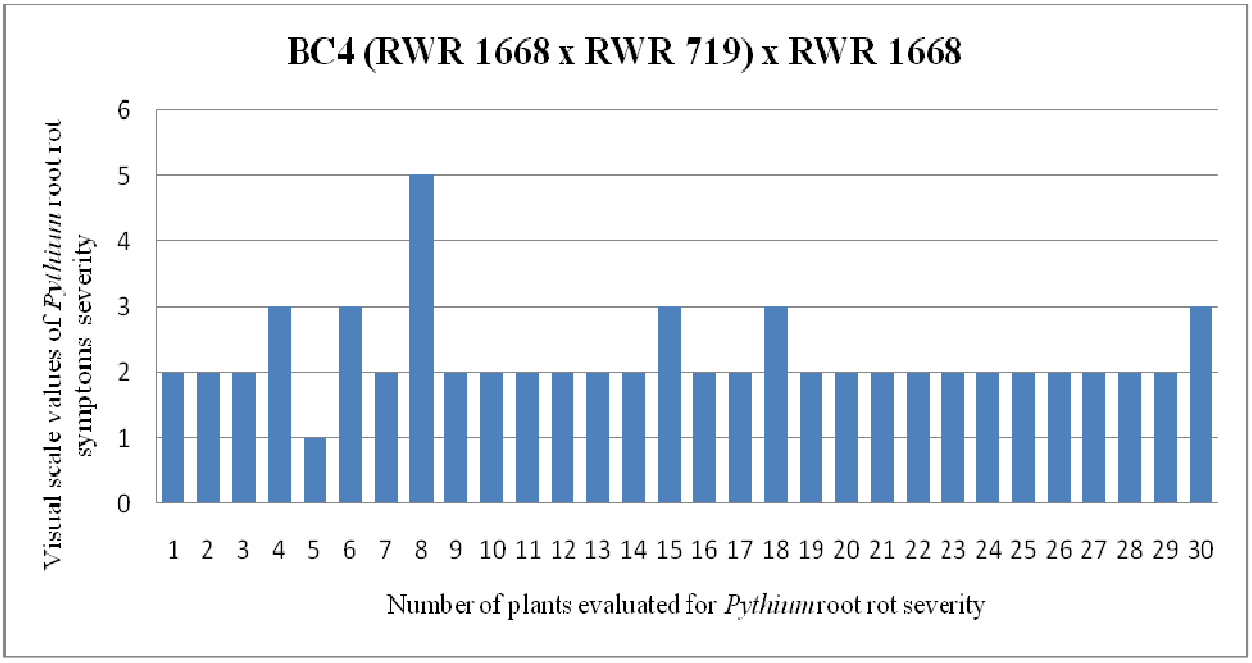

Figure 7. Evaluation of *Pythium* root rot symptoms on parental combination of Backcross 4 (R WR 1668 x RWR 719) x RWR 1668

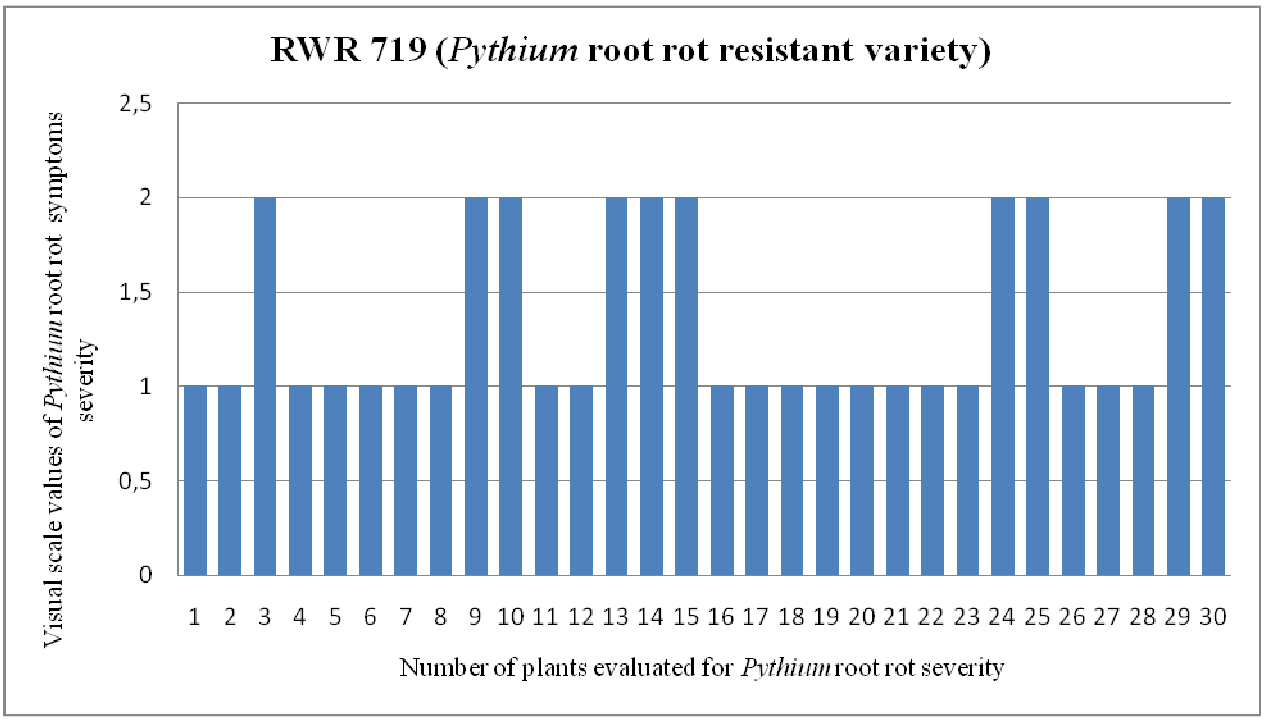

Figure 8. Evaluation of *Pythium* root rot symptoms on variety RWR 719