

Original research paper

**EVALUATION OF AFLP MARKERS FOR SCREENING RESISTANCE TO
COMMON BEAN ROOT ROT (*Pythium spp.*)**

ABSTRACT

Aims: To investigate potential markers for identification of sources of *Pythium* resistance in bean genotypes preferred by poor small holder farmers. Ten common bean lines generated from a conventional breeding program with differential resistance to the pathogen *Pythium* were subjected to AFLP analysis.

Place and Duration of Study: Department of biological sciences Masinde Muliro University of Science and Technology, Kenya and Bangor University, North Wales, United Kingdom, between September 2010 and December 2012.

Methodology: 45 common bean samples (35 seed samples collected from farmers, markets, seed stockists and 10 breeders seed samples collected from KARI Kakamega Station, Kenya were subjected to genetic variation characterization using AFLP markers. Genetic diversity patterns were determined. Average genetic distances were calculated and similarity coefficients subjected to unweighted pair group method of arithmetic averages.

Results: Two informative AFLP primer combinations yielded 194 polymorphic loci. Genetic distance of bean samples from KARI kakamega had 56 to 414 base pairs with a variability index of 0.63 to 0.90. Combined analysis of bean accessions from KARI breeding program and market class common bean samples revealed a variability index range of 0.62 to 0.90 with 56 to 420 base pairs. Segregation revealed two major groups, with resistant varieties AND1062, R2075, R719, R1946 and SCAM80 being more genetically related to market class of beans. Cluster analysis revealed that resistant bean varieties were genetically different from the susceptible bean varieties.

Conclusion: AFLP marker is an informative, reliable technique for delineating susceptible and resistant *Pythium* root rot dry bean varieties. The information generated will contribute to the propagation of acceptable market class bean lines with resistance to *Pythium* root rot for improved livelihood and increased food security. However, there is need to subject the non-breeders seed samples to SCAR marker PYAA₈₀₀ and pathogenicity testing to correlate the findings.

Keywords: Common beans, *Pythium* root rot, AFLP, Genetic diversity

1. INTRODUCTION

Bean production is an important and widespread farming activity in Kenya. In tropical regions, common bean is characterized by low and unstable grain yields due to various ecological and agronomic parameters. The common bean suffers from several biotic and abiotic production constraints. Among these parameters, bean root rot and a decline in soil fertility are among the major causes leading to bean yield losses [1, 2]. Biotic constraints include diseases such as Angular leaf spot, Anthracnose, root rots, rusts, Halo blight, and Bean common mosaic virus [3, 4, 5, and 6]. These diseases and pests are classified as high to moderate in importance in Kenya highlands [7].

There has been an increase in the importance of *Pythium* bean root rots in several countries of Eastern and Central Africa, such as Burundi, the Democratic Republic of Congo, Kenya and Uganda and Rwanda, due to severe break out of root rots. *Pythium* is caused by a complex of soil borne pathogens that occur singly or in a complex of two or more organisms, with the most important ones being *Fusarium solani* f. sp. *Phaseoli* and *Pythium ultimum* var. *ultimum* that results in considerable losses of about 221,000 million tonnes/year [7, 8, 4 and 9]. Based on spatial distribution damage and effect on yield, *Pythium* species are more frequently associated with severe outbreaks resulting in 70-100% bean loss depending on severity [3, 10]. *Pythium* is known to survive in the soil for several years as oospores that germinate to produce zoospores which infect the root and lower stem [8]. It is very persistent in the soil and when susceptible varieties are used, complete yield losses usually occur when the environmental conditions are favorable for pathogen development [12, 13]. Unfortunately, almost all commercial varieties released in Kenya are susceptible to the fungus [14].

To increase productivity and yield stability on farms, farmers need well adapted market acceptable disease resistant cultivars. The bean breeding program at Kenya Agricultural Research Institute (KARI) Kakamega station has so far relied on conventional breeding characterized by introgression of desirable traits from exotic bean cultivars. *Pythium* root rot resistance bean lines have been developed albeit with low farmers' acceptability of the acceptable varieties. There is need to develop acceptable *Pythium* root rot resistant germplasm of marketing purposes for improved bean productivity [13,14]. This study investigated the potential of AFLP markers to differentiate resistant and susceptible *Pythium* root rot phenotypes and enhance the identification and selection of *Pythium* resistant germplasm for bean improvement programs. Knowledge about identified resistance sources of beans combined with information of the polymorphic levels that exists between the improved resistant bean lines and commercial varieties will reveal the level of diversity and contribute towards development of appropriate protocols with potential to decrease adoption rates and duration taken to release improved bean varieties in Eastern and Central Africa.

2. MATERIAL AND METHODS

Plant material: Ten (10) bean lines developed in a conventional breeding exercise at Kenya Agricultural Research Station – Kakamega Regional Centre were used. The germplasm had 6 resistant, 1 moderately resistant, and 3 susceptible lines (Table 1).

DNA extraction and primer selection: Young fresh leaves about 5 - 6 leaf stage planted in $\frac{3}{4}$ of sterilized soil were harvested for DNA extraction using the Qiagen DNA extraction kit, following the instructions of the manufacturer with minor modifications. 50ng/ul of DNA was used in subsequent AFLP analysis. A total of 7 primers were used for screening (Table 2). Of these, three AFLPs primer combinations were selected due to reproducibility of polymorphic bands. The AFLP adaptors and enzymes were obtained from MWG- Eurofins and New England biolabs. Selective amplification primer pairs E35 M62 and E35 M50 were finally used to screen all the samples due to clarity and reproducibility.

AFLP analysis: The AFLP assay was conducted using a modified version of Vos *et. al.*, (1995) and adapted for use with the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc). Total genomic DNA of the samples was digested with restriction endonucleases (EcoR I and Mse I), producing three species of DNA fragments (i.e. Eco- Eco, Eco- Mse, and Mse-Mse), followed by selective PCR amplification and later electrophoresis using a sequencer. The restriction digestion involved 4µl of NEB₄ buffer, 0.1µl of 5 units EcoR I (50U/µl), 0.5µl of 5 units Mse I/Tru 91 (10 U/µl) and 4µl of PCR water added to a volume containing 500ng DNA and made up to 40µl with PCR grade water. A total of 40 µl of restriction component and DNA component for the 10 samples was incubated for 1 hour at 37°C. The ligation step involved mixing 1µl of 5pMol EcoR I adaptor (Adapter 1: 5'-CTCGTAGACTGCGTACC-3'; Adapter 2: 5'-ATTGGTACGCAGTCTAC-3'), 1µl of 50 pMol MseI/Tru 91adaptors (adapter 1: 5'-GACGATGAGTCCTGAG-3'; adapter 2: 5'-TACTCAGGACTCAT-3'), 1.0µl of T₄ DNA ligase buffer (10x), 0.3µl T₄ DNA ligase, (1 unit) and 6.7µl PCR grade water, to a total volume of 10µl of the ligation component. 10 µl was pipetted to microfuge tubes containing the results of each samples' restriction digest and incubated for 3hours at 37°C. PCR was conducted using primers E00 universal primer for EcoR I and M00 universal primer for Mse I were used for the pre- amplification step. Selective amplification stage involved the use of E33, E35, M50 and M62 primers (Table 2). The pre- amplification primer mix for one sample comprised of universal primer 50ng/µl (E00 for EcoR I) 0.6 µl, universal primer 50ng/µl (M00 for Mse I) 0.6 µl and 3.8 µl of PCR grade water. 5µl of the pre – amplification mix, 10µl Bioline biomix™ PCR master mix containing 2.0mM MgCl₂ and 5µl DNA from the restriction /ligation reaction were put in PCR tubes and placed in a thermal cycler with 30 amplification cycles (Denaturation at 94 °C; annealing at 56 °C for 1 min; polymerization at 72°C for 1 min and further held at 72 °C for 10 min.)

The pre- amplification product was then diluted in PCR grade water in 1: 10, 1:20 and 1: 30 ratios. A PCR mix for selective primers was prepared and it comprised of 5µl of diluted pre- amplification PCR product, 5µl of selective primer mix [0.25 µl of labeled primer (50ng/µl), 0.3 µl of unlabelled primer (50ng/µl) and 4.45 µl of PCR grade water] and 10µl of Bioline Biomix™ containing 2.0mM MgCl₂. Each PCR tube contained a final volume of 20µl. The thermo cycler was programmed to amplify the product in two sets of temperatures. The first set had 13 cycles of amplification while the second had 23 (denaturation at 94 °C for 30 sec; annealing at 65°C; polymerization at 72°C for 1 min; and held at 10°C for 12 hrs) before fragment analysis.

Fragment analysis: The dilution from the selective PCR was optimized for fragment analysis. The PCR product was run on Beckman Coulter CEQ 8000 (Beckman Coulter Inc.) to analyze the fragments generated from the AFLP analysis. A master mix of 40µl of the SLS (de-ionized formamide) and 0.5µl the DNA size standard was loaded in each sample well. 0.5µl of the final PCR product was added to the sample well carefully and covered by a drop of mineral oil to avoid evaporation. The buffer plate was covered using the loading buffer and the plates loaded into the sequencer. The software package for CEQ8000 genetic analysis system (Beckman Coulter, Inc.) was used to input the data detailing well contents, prior to loading the plate into the sequencer and running the analysis. A DNA size standard (PA400) was used to size the fragments. The laser detected fragments present in the spectrum of each fluorophore, producing an electronic profile of relative fluorescence units (RFUs) versus fragment size [15] to detect insertions or a deletion of nucleotides. The traces of samples were compared and visually scored. Peaks were scored if they were between 1000 and 130000 RFUs (vertical axis).

Data scoring and cluster analysis: Data scoring and cluster analysis were performed using simple matching coefficients. The similarity coefficients was subjected to unweighted pair group method of arithmetic averages (UPGMA) and a dendrogram was generated with the help of NTSYS- PC software version 2.1(Exeter software, New York, USA; Rohlf, 2000). The data matrix was subjected to analysis of molecular variance (AMOVA) to partition the genetic variation into within and among the populations' components using GenAlEx software.

3. RESULTS

Polymorphisms

AFLP profiles of the *Pythium* related samples were generated using two AFLP primer combinations (E-TC/M-CAT, E-ACA/M-CTT) and produced a total of 194 polymorphic loci. The size of the AFLP generated fragments by KARI Kakamega samples ranged between 56 and 414 base pairs and 56-420bp for bean market samples.

Population genetic structure analysis

Phenetic structures and principal component analysis

Ten common bean samples from KARI Kakamega using NTSYS-Pc version had the first three eigen values revealing 51.59% similarity (Table 3). R1946 R719, R2075 and AND1062 are closely related genetically compared to Mw001, KK15, Alulu and GLP2.

The degree of similarity within the samples products from a conventional breeding exercise at KARI- Kakamega breeding station generated a dendrogram from un-weighted pair group method with arithmetic averages. Two groups were generated, group A and B (Figure 1). Mw001 was found totally different from the rest of the 9 bean samples. There was a distinct differentiation between the resistant varieties and susceptible varieties in group A. 98% of the bean samples collected from the farmers in Eastern and Western provinces of Kenya clustered together with the common bean varieties in possession of the resistant gene to *Pythium* root rot disease in group I (Figure 2). The susceptible variety Mw001 still was totally different from the rest of the samples analysed. The samples from the breeding program are labeled as resistant (RR), mildly resistant (MR) or susceptible (SS).

Bean Population Screening

For authenticity, the four bean populations used [Eastern (Embu and Meru), Eastern (Mbeere and Machakos), Western and Market centers] were to be screened using a SCAR marker (PYAA 19₈₀₀) developed from previous studies in Colombia (Mahuku, 2007) for comparative analysis. The SCAR marker would have been useful in easier identification of accessions holding the characteristics of resistance to *Pythium* root rot among market class of Kenyan dry bean. Unfortunately, due to lack of publicly accessible primer sequence of the SCAR marker (PYAA 19₈₀₀) for purchase from gene banks, the analysis was not conducted. A similarity analysis was therefore performed on all the samples used in the project including the *Pythium* related germplasm using NTSYS-pc ver.2.1. This was to done to validate the genetic relatedness that existed within the KARI – Kakamega bean samples that had already been evaluated and approved in terms with their reaction to *Pythium* species together with bean samples present in the market and with farmers (Figure 2). The clustering revealed how diversely related market class Kenyan dry beans are in relation to the bean samples developed from the breeding program at KARI- Kakamega. Principal component analysis of the *Pythium* samples (Figure 3) revealed a closer clustering of the resistant varieties AND1062, R1946 and R719.

4. DISCUSSION

A large number of percentages (over 50%) on the component analysis indicate a successful analysis [16]. We observed considerable genetic diversity within the common beans of market class and bean samples conventionally developed from KARI kakamega. Bean root rot caused by several *Pythium* species has been a quandary on beans (*Phaseolus vulgaris* L.) increasing in importance in the recent years in East and Central Africa. Total crop loss when susceptible dry bean varieties are used is evident [3].

An experiment was conducted to monitor *Pythium* root rot disease development in a field setting. Seedlings were planted and watered twice daily for 21 days to favor fungus development [17]. Uprooted seedlings were washed and lesion development scored using the CIAT scale of 1-9 [18]. Conventionally produced *Pythium* root rot resistant dry bean seeds were determined basing on this scaling routine [3]. RWR719, MLB 4989A and AND1062 were used as controls in his experiments and received disease interaction phenotype score of either 1, 2 or 3 denoting resistance to *Pythium ultimum* while GLP 2 was susceptible (8-9 score) under same conditions.

Molecular characterization of 35 different *Pythium* strains affecting the Ugandan common bean was done using RFLP marker, to demonstrate the wide diversity that exists among different bean plants affected by *Pythium* root rot [19]. Difficulties in interpreting the relatedness among the isolates were experienced because of wide intra specific variation that exists within the *Pythium* species. To significantly reduce the effects of this disease on the bean crop, identification of sources of resistance to bean genotypes preferred by resource poor small holder farmers is imperative. Resistant varieties have been identified but they are not of preferred market classes [14].

Subsequent work of characterization has been done of susceptible common bean varieties from Africa [20] in different seed sizes, such as the Kenyan varieties GLP 2 and GLP 585 which are respectively large seeds (Andean gene pool) and small seeds (Mesoamerican gene pool), the Ugandan variety CAL 96 (Calima) with large seeds (Andean gene pool) and the Rwandan variety Urugezi with an intermediate seed size (Mesoamerican gene pool). All the resistant common bean varieties have been characterized into different sizes and are advanced lines from an international breeding nursery run by CIAT (Cali, Colombia). This include the small-seeded variety RWR 719 from the Mesoamerican gene pool, the intermediate-seeded varieties MLB 49-89A and SCAM 80-CM/15, both from the Mesoamerican gene pool, and the large-seeded varieties AND 1055 and AND1062, both from the Andean gene pool [3].

Pythium root rot resistance is controlled by a single dominant gene [21]. His experiments involved investigating the genetic relationship between *Fusarium* and *Pythium* resistance inheritances in RWR 719. There is an association of PYAA19₈₀₀ with *Pythium ultimum* resistance in RWR 719, MLB 49-89A and AND 1062, located 1.5cM from the resistance gene [22]. These common bean varieties are possible sources of resistance to *Pythium* root rot disease. The most effective way of managing bean root rot for small scale farmers is exploiting the host plant resistance [23,24]. KK15 had a distance matrix of 0.71, close to the distance generated from SCAM80 and ALULU of 0.77. There is close genetic relationship between KK15 and the sub cluster of SCAM80 and ALULU compared to the susceptible sub cluster of GLP585 and GLP2 with the distance 0.79. A sub cluster comprising of four resistant varieties (AND1062, R2075, R719 and R1946) had a distance matrix 0.86. The genetic similarity within theses samples is closer compared to the other common bean samples used in the group. Mw001 was most dissimilar to the rest of the samples used in this analysis with a distance matrix of 0.62, indicating a wide genetic dissimilarity with the rest of the bean samples analyzed.

Resistance to *Fusarium solani* is complex and is conditioned by two or more genes [25,26,27], whereas, *Pythium ultimum* resistance is controlled by a single dominant gene, marked by a dominant SCAR marker-PYAA19₈₀₀ [3,14,27]. The presence of joint resistance to both *Fusarium* and *Pythium* root rots have been observed in several resistance sources [4, 9, 28]. Some market class samples are closely related genetically to the *Pythium* related samples developed in a conventional breeding exercise at KARI- Kakamega. In a further breeding exercise, these bean samples can be used as susceptible parents in efforts to obtain crosses for transfer of the resistant genes in improvement programs.

Two major clusters were obtained with majority of the samples belonging to the first cluster, and Mw001 still being totally different from the rest of the samples used (Figure 2). In the first sub cluster four resistant varieties clustered among market classes (AND1062, R2075, R719 and R1946). The second sub cluster had two resistant varieties SCAM 80 and KK15. KK15 is a recent bean line that is resistant to *Pythium* root rot, is high yielding and fast maturing. The gene responsible for resistance against *Pythium* species is tightly linked to the gene responsible for seed colour (KARI report, Kakamega). Most farmers find the colour of the seed coat unacceptable, therefore as much as it is a wonder seed that is being taken up by small scale farmers to counteract poverty and improve on food availability, further crossing should be done to break the linkage between the gene responsible for resistance and seed coat colour, to increase the acceptance rate of this new improved variety among the bean farmers in Kenya.

Efforts to integrate *Pythium* root rot resistant gene into Rwandan susceptible varieties have been successful after carrying out crosses between three local susceptible varieties and two known sources of resistance to *Pythium* root rot, R719 and AND1062 [29]. The Rwandan bean profile of resistance to *Pythium* found in the progenies from back-crosses between (RWR 1668, R617-97A and Urugezi, as recipient parents) and the selected sources of resistance (RWR 719 and AND 1062, as donor parents) is achievable. At each back cross generation A SCAR marker PYAA 19₈₀₀ was used and found to be linked tightly to the gene responsible for resistance in R719 and AND1062. This is a feasible exercise which can be experimented in Kenyan bean breeding institutions to help improve the dry bean of market classes to help improve on their resistance to attacks by root rots.

5. CONCLUSION

Effective bean root rot management calls for the use of integrated approaches with a strong component of resistant varieties. To design resistant gene development in bean varieties ideal by resource- poor small holder farmers, long lasting strategies are dependent on the identification of useful genes and a good understanding of the host – pathogen interaction. The use of resistant cultivars is the most viable option for controlling bean root rot, particularly for small-scale growers to solve this quagmire in the agricultural sector of bean breeding.

To increase the productivity and yield stability of the dry bean of Kenya, there is need to come up with varieties that are well adapted to the consumers' market preferences, acceptable and root rot resistant to be distributed to farmers. Knowledge about identified resistance sources and mode of inheritance of resistant genes is needed to develop more stable resistant cultivars to be adapted to regions of Eastern and Central Africa where bean root rot is an increasing problem.

This study has revealed the genetic relationships between and within bean population from the study areas by use of AFLP marker. It has yielded useful information on the level of polymorphism and genetic relatedness between common bean varieties developed from KARI Kakamega through a convectional breeding program and bean varieties of market classes. Designing our own primers to target the resistant gene could take a long duration of time, which wasn't sufficient within the time frame available to carry out this study. There are primers already designed in similar studies in Rwanda and Colombia, but lack of public access to these primers designed made the complete of the study of developing a protocol to tag resistant and susceptible varieties unfeasible.

7. COMPETING INTERESTS

"Authors have declared that no competing interests exist."

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10. Tables and Figures

Table 1. *Pythium* resistance status, growth habit and seed size characteristics of bean lines used in the study

Table 2. Sequence of AFLP primers used for evaluation of improved bean types from KARI- Kakamega

No. Code Primer sequence (5'→3')

Serial no.	Variety	Reaction to <i>Pythium</i>	Growth habit	Seed size
1.	RWR- 719	Resistant	Determinate	Small
2.	AND 1062	Resistant	Determinate	large
3.	RWR 1946	Resistant	Determinate	medium
4.	RWR 2075	Resistant	Determinate	medium
5.	KK 15	Resistant	Determinate	medium
6.	Scam 80	Resistant	Determinate	Medium
7.	GLP 585	Susceptible	Determinate	Small
8.	GLP 2	Susceptible	Determinate	Medium
9.	Mwitmania	Susceptible	Determinate	Medium
10.	Alulu	Susceptible	Determinate	Large
		1. E24	GACTGCGTACCAATTCTC	
		2. E33	GACTGCGTACCAATTCAAG	
		3. E35	GACTGCGTACCAATTCACA	
		4. M50	GATGAGTCCTGAGTAACAT	
		5. M62	GATGAGTCCTGAGTAACTT	
		6. **E00	GTAGACTGCGTACCATTTC	
		7. **M00	GACGATGAGTCCTGAGTAA	

NB: **E00 **M00 are universal primers

Table 3. Description of genetic variation percentage in the observed relationship among 10 improved bean lines used in the study

Eigen Value	Percent (%)	Cumulative
2.72	27.25	27.25
1.30	13.02	40.27
1.13	11.31	51.59
1.09	10.98	62.58
0.89	8.99	71.57
0.74	7.40	79.04

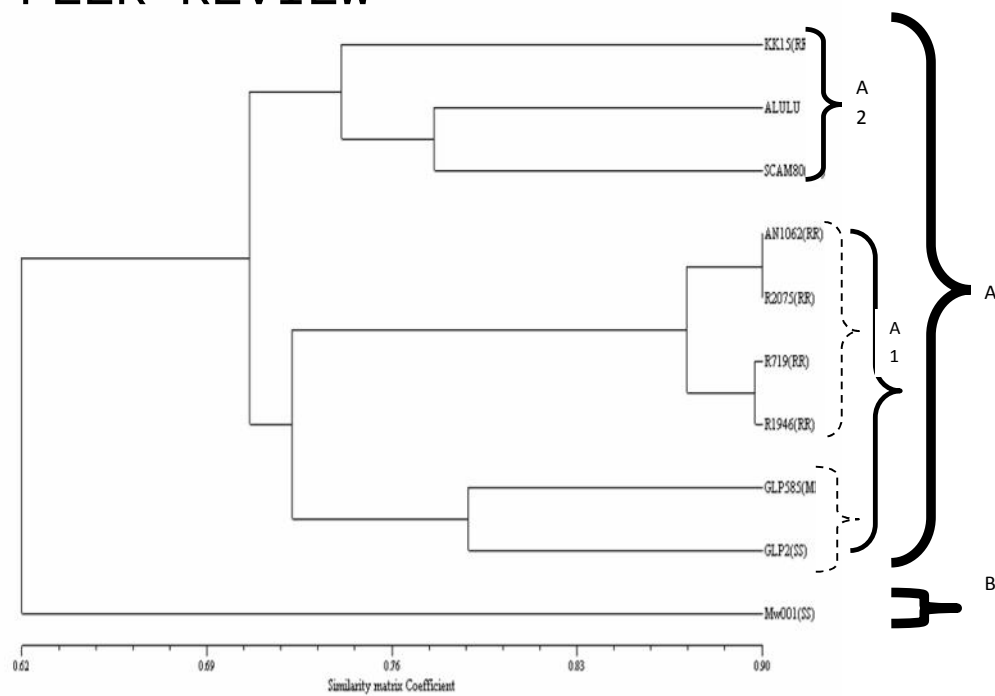


Fig. 1. A Neighbor-joining dendrogram based on Neis' (1978) genetic similarity distances based on AFLP-PCR results for 10 approved bean varieties.

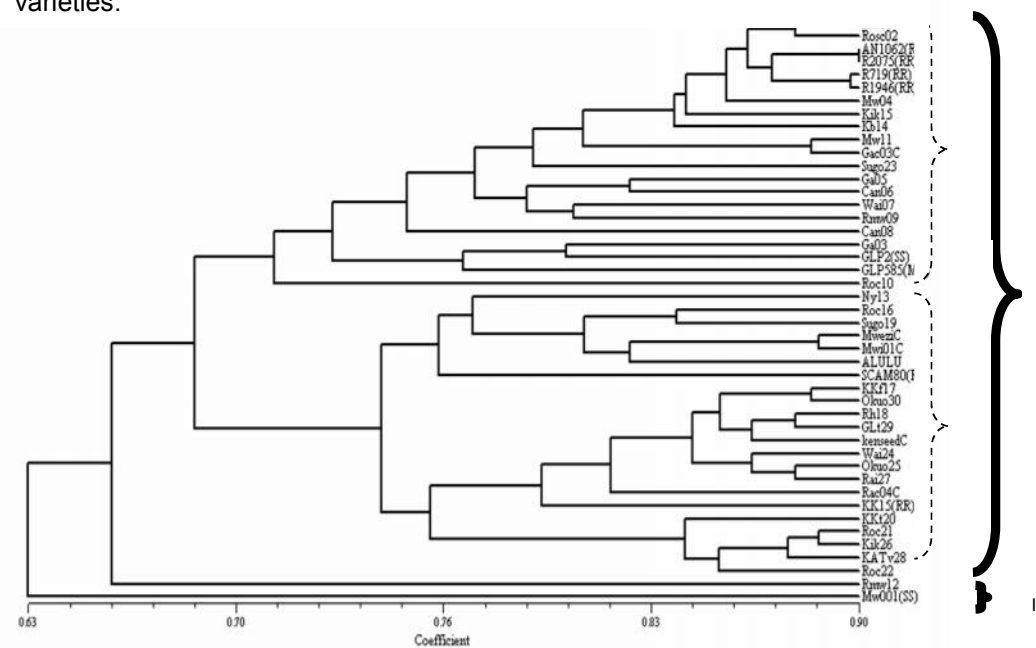


Fig. 2. A dendrogram of 45 bean samples of *P. vulgaris* with *Pythium* related samples from KARI – Kakamega samples loci based on simple matching coefficient similarity matrix.

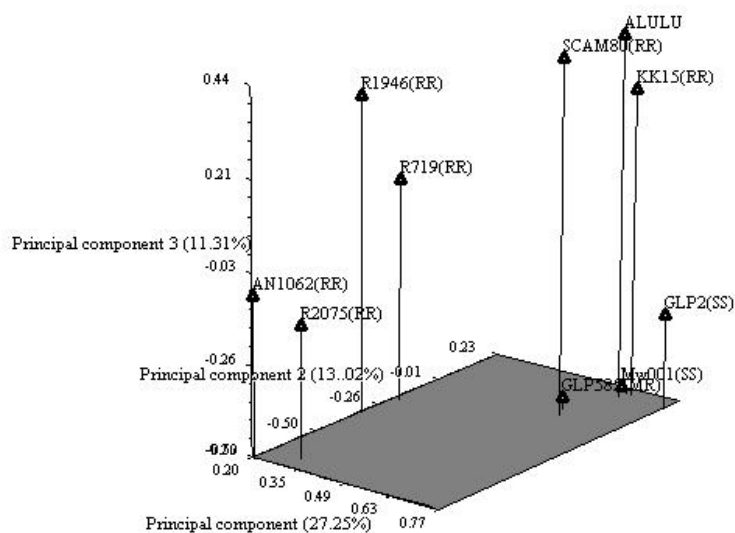


Fig. 3. Principal component analysis describing relationship between 10 bean samples developed from a KARI - Kakamega conventional breeding exercise on *Pythium* root rot resistant varieties.