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Molecular Characterization of *Pythium*(Py) Species Affecting Tobacco in the Float Tray Seedling Production System

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Abstract:*The genetic diversity of 9 isolates of Pythium (Py) species associated with damping- off, stem and root rot of tobacco seedlings in the float tray seedling production system in Zimbabwe was characterized using Universal rice primers URP primers derived from the repetitive sequence of the Korean weedy rice. Characterization of Pythium species in Zimbabwe has been based on morphological and physiological characteristics, which are time consuming and require high expertise. In this study,out of the 9 Universal Rice Primers (URP)used 4 primers were effective in producing polymorphic DNA profile patterns from DNA of Pythium species used. The isolates included Py 14, Py 15, Py 19, Py 20, Py 21, Py 25, Py 25, Py 29, Py 30 and Py 64 of which Py 19 was the known isolate which is Pythiummyriotylum. A useful detection method was obtained and the PCR approach using UR Primers was also useful in studying the diversity of Pythium at inter- and intra-species level. The PCR method has allowed for the monitoring for the presence of Pythium species as a diagnostic method for water and soil samples and even for disease diagnosis.*

Keywords:*Pythium, molecular characterization, PCR assay, Universal Rice Primers, Intra-species*

1. INTRODUCTION

Pythium is a soil-borne fungus that causes damping-off, root and stem rot of younger plants and feeder root necrosis of field plants in many crops including cucumber, ginger, cabbage, maize [8] and tobacco [3]. Members of the genus Pythium occupy several ecological niches while most of these species are saprophytes in various types of soils and environments [1].

In Zimbabwe, tobacco seedling production is mostly done from June to September and Pythiumhadnot been a problem with the conventional or traditional seedbed seedling production system. However, the introduction of the float tray seedling production system has seen the emergence of the pathogen. This has seen Pythiumdiseases becoming one of the most vital limiting factors in seedling production as the pathogen is resident in irrigation water. Field trials done at Tobacco Research Board (TRB) by the Pathology section showed that, better yields were obtained in fields from trays produced with metalaxyl as a preventive measure against Pythium. Fields with seedlings grown in float trays without metalaxyl as a preventive measure were greatly affected and had very low yields [10].

Pythiumis well adapted to living in water, where it will produce sporangia (the fruiting body). The sporangia form zoospores which swim around and infect the roots and are the main means of spread in crops raised hydroponically especially the float tray system used in tobacco seedling production [3]. Pythium produces oospores (as resting spores) and chlamydospores which are overwintering structures but does not produce the resting spores in low pH soils [7]. In this overwintering stage of the Pythium, the oospores are resistant to low temperature and other adverse factors and they also require a resting period before they germinate [13]. Exudates from roots of germinating seeds or actively growing plants stimulate the sporangia and oospores to produce germ tubes [7].

Pythium diseases have become very important for tobacco seedlings as the mechanism of dissemination for Pythiumis well adapted to the float tray system being used in tobacco seedling production. The zoospores can easily move throughout the seedling bed in the water and reach epidemic levels in a very short time. In the event that infected plants live, their vigor is reduced, resulting in poor quality transplants [3].

Symptoms are usually observed when plants are 25 days or older (when roots start growing to the bottom of the tray) and begin as yellow areas that cover an entire greenhouse section or an entire outdoor float tray seedbed in a few days. The lower leaves turn yellow, plants wilt and the roots become light brown to grey in colour in the early stages of infection and become brown to grey with a slimy texture in a few days. The infected roots later fall off, leaving plants without a root system [3].

The detection methods are a major step for understanding and preventing diseases caused by Pythium. Currently, Pythiumspecies identification in Zimbabwe, at TRB, is based on morphological and biological characteristics that areisolation of Pythium species on selective media, microscopic methods or baiting [12]. Morphological identification is difficult, time consuming and requires high expertise. Identification using sexual structures is difficult for species without sexual structures [9]. Pythiumspecies are a challenge to characterise using conventional or traditional methods, as some of their symptoms overlap with those of other pathogens like P. nicotianae which causes Black Shank, hence making identification difficult [13].

DNA- based techniques have become an effective means of identifying pathogens [8].The use of molecular techniques using the polymerase chain reaction (PCR) has been used for identification and characterization, of pathogens for example, Rhizoctoniasolani[5], Verticullium, GaeumannomycetesandPhoma[13]. Wang and White [14] developed specific primers from the ribosomal DNA ITS regions for identification of Pythiumviolae. However, the ITS sequences were not variable making the design of primers for detection and identification of closely related taxa difficult. The PCR technique offers the advantages of speed, sensitivity, flexibility and specificity.

In this study, the PCR technique was employed using primers designed from the repetitive sequence pKRDof the Korean weedy rice, termed Universal Rice Primers (UR Primers). The primers are long and hence they have greater reproducibility and this is aided by the high annealing temperatures employed in the PCR reaction [5]. The primers have been used in the fingerprinting of diverse genomes of plants, animals and microbes (ibid). The use of primers derived from plant genomes as molecular markers, i.e UR Primers, has not been used in the fingerprinting of Pythium species. The objectivesof this study were to develop a detection method for Pythium species using the URP-PCR technique and also distinguish between the Pythium species isolates.

2. MATERIALS AND METHODS

2.1 Fungal isolates

Pythium isolates used in this study were obtained from Plant Pathology, Tobacco Research Board and are listed in Table 1. A total of 9 isolates were Isolated from different hosts and sites. The isolates were stored under oil and were revived by streaking on Potato Dextrose Agar (PDA)[4]slopes which were incubated at 28 °C for 3 days. The isolates were inoculated on PDA in Petri dishes and cultured for 3 days in an incubator at 28 °C. An active culture of each isolate was obtained and inoculated onto Potato Dextrose Broth (PDB) in 375 ml culture bottles and cultured for 10 days at room temperature (24-28 °C) under dark conditions. The mycelia were harvested by filtration, dried in an oven at 60°C overnight, ground to a fine powder and stored in a -20^0Cfreezer for DNA extraction.

2.2 DNA Extraction

DNA from mycelium was extracted using the modified CetylTrimethyl Ammonium

Bromide (CTAB) procedure [2].Dichloromethane was substituted with chloroform in the extraction procedures. The procedure included preheating 3% CTAB isolation buffer which was added to 0.1 g of ground isolate in a 2ml eppendorftube and also adding 10 µL of Proteinase K and incubating for 1 hour at 65 °C with occasional mixing every 10 minutes. 10 µL RNase A was added and incubation continued for another 10 minutes. Extraction with chloroform was done twice mixing thoroughly and spun in a centrifuge at 12752 x gfor 10 minutes to separate phases. Precipitation of DNA was done using 1% CTAB and isopropanol and a final wash with 70% ethanol before drying and re-dissolving in TE buffer.

2.3 URP-PCR

Optimal PCR conditions were established in the preliminary experiments to establish the reproducibility of the PCR amplification reaction. According to the experiments, an annealing temperature of 55 °C was not conducive for ensuring amplification.

The URP- PCR was initiated in a 50 µl PCR reaction which contained 10 µL of 5X Green GoTaq Flexi Buffer (Promega,USA), 0,2 mMdNTPs, 1,5 mMMgCl, 1,2 µM UR Primer and 2,5 unitsTaq Polymerase (Promega, USA). To this was added 2 µL of diluted DNA (~12 ng μL^{-1}). Amplification was initiated by denaturation at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 30 seconds, 53 °C for 1 minute and 72 °C for 1 minute and a 5 minute final extension at 72 °C in a Gene Amp® PCR System 9700 (Applied Biosystems) [5].

The URP-PCR amplified products were analyzed by electrophoresing 13,5 µL on a 1,5% agarose gel in 0.5 TAE buffer stained with ethidium bromide and visualized in a UV trans illuminator gel documentation system.

2.4 Analysis of results

Relatedness among the 9 isolates of Pythium species was estimated by means of scorable DNA bands amplified from different URP and UP markers. Each band was considered as a character and was scored as either present (coded as 1) or absent (coded as 0)

Cluster analysis with the Unweighted Pair Group method with arithmetic average (UPGMA) algorithm was performed using the Jaccard's similarity coefficient by the GENSTAT 9th Edition software to produce dendograms.

3. RESULTS AND DISCUSSION

URP 2F

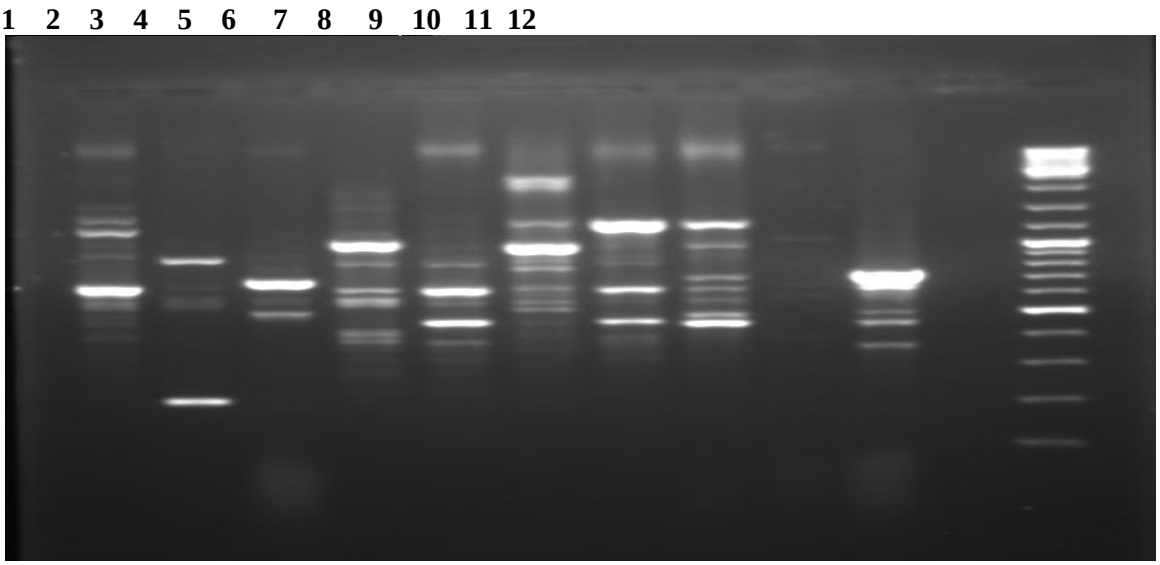

Fig 1.0 *Amplification products of the 9 Pythium isolates using primer URP 2F. Clear URP 2F bands range from 200-10 000 bp in size. Lane 1-9: Py 14, Py 15, Py 20, Py 21, Py 25, Py 29, Py 30, Py 64, Py 19 (+ve control), Lane 10: RhizoctoniaSolani (-ve control), Lane 11: Water (-ve control), Lane 12: DNA Ladder Mix*

Genomic DNA was extracted by the modified CTAB protocol and 4 UR Primers were used to amplify the isolates and at an annealing temperature of 53 °C they produced DNA profiles for all the isolates, as shown in **Fig 1.0 - 4.0**. Clear detectable bands were obtained with all the primers and dendograms were constructed for all the primers showing the similarities of the isolates. All the isolates produced DNAbanding profiles which had some monomorphic and polymorphic bands.URP2F clustered together Py 25and 30 with a 63% similarity level as the highest level of similarity for the primer. Py 19 was 18% similar to Py 15, Py 21, Py 25, Py 30 and Py64. A 17% similarity level was shown in the **Fig 5.0** between Py 19 and Py 14. Py 20 was 15% similar to Py 19 and the primer had a 36, 7% polymorphism level showing its effectiveness in distinguishing between the pythiumisolates.

URP 2R

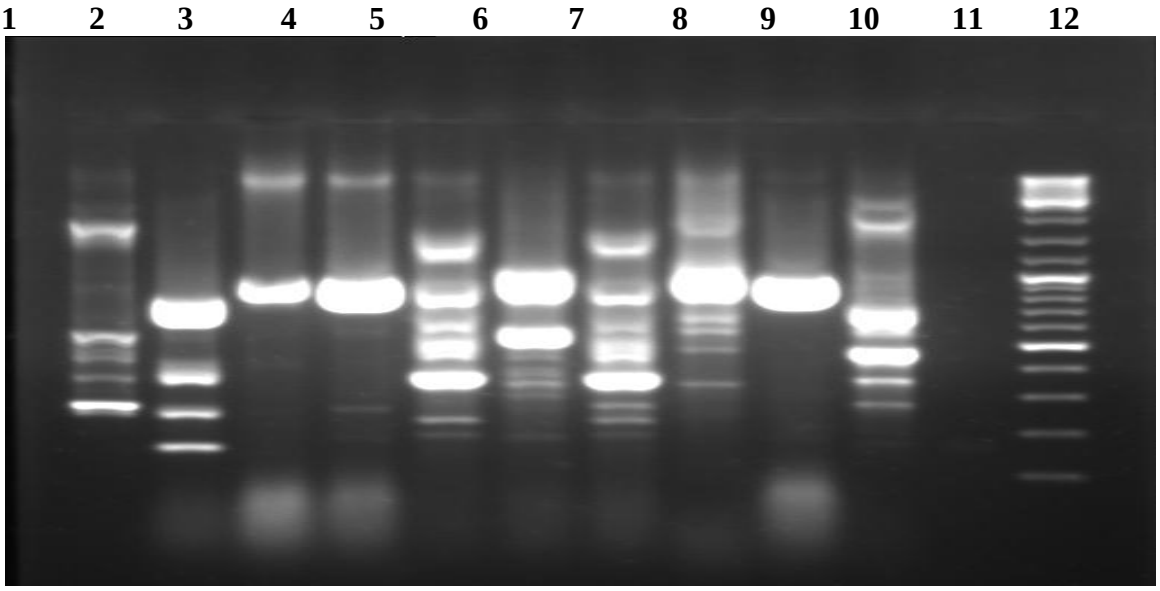

Fig 2.0 *Amplification products of the 9 Pythium isolates using primer URP 2R. Clear URP 2R bands range from 200-10 000 bp in size. Lane 1-9: Py 14, Py 15, Py 20, Py 21, Py 25, Py 29, Py 30, Py 64, Py 19 (+ve control), Lane 10: RhizoctoniaSolani (-ve control), Lane 11: Water (-ve control), Lane 12: DNA Ladder Mix*

URP 9F

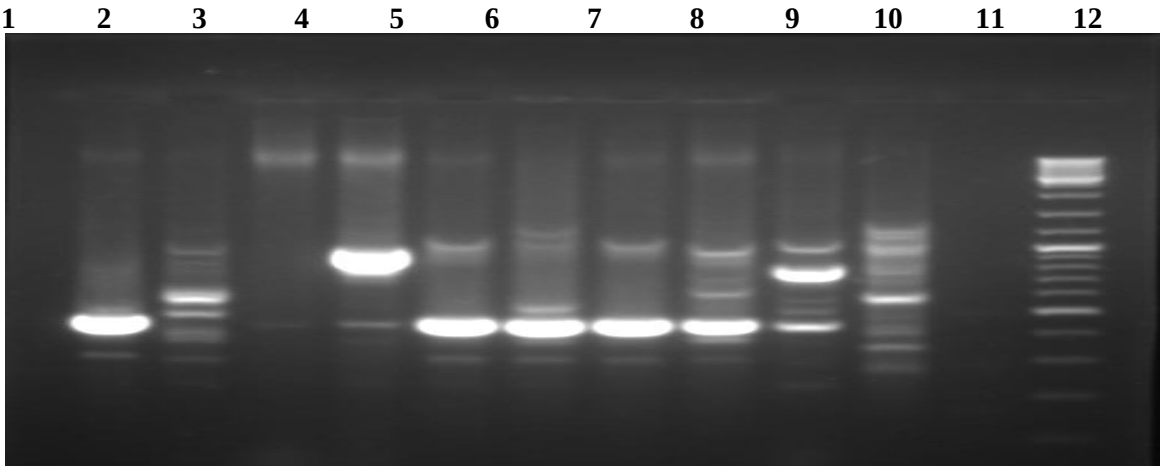

Fig 3.0*Amplification products of the 9 Pythium isolates using primer URP 2F. Clear URP 2F bands range from 200-10 000 bp in size. Lane 1-9: Py 14, Py 15, Py 20, Py 21, Py 25, Py 29, Py 30, Py 64, Py 19 (+ve control), Lane 10: RhizoctoniaSolani (-ve control), Lane 11: Water (-ve control), Lane 12: DNA Ladder Mix*

URP 13R

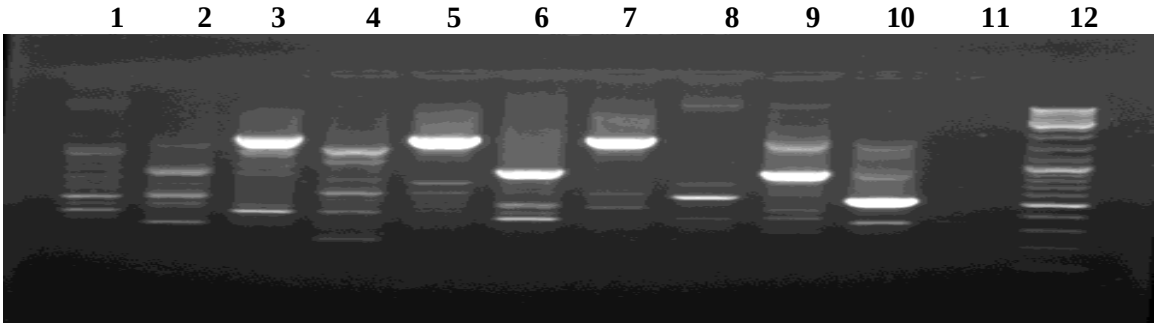

Fig 4.0*Amplification products of the 9 Pythium isolates using primer URP 2F. Clear URP 2F bands range from 200-10 000 bp in size. Lane 1-9: Py 14, Py 15, Py 20, Py 21, Py 25, Py 29, Py 30, Py 64, Py 19 (+ve control), Lane 10: RhizoctoniaSolani (-ve control), Lane 11: Water (-ve control), Lane 12: DNA Ladder Mix*

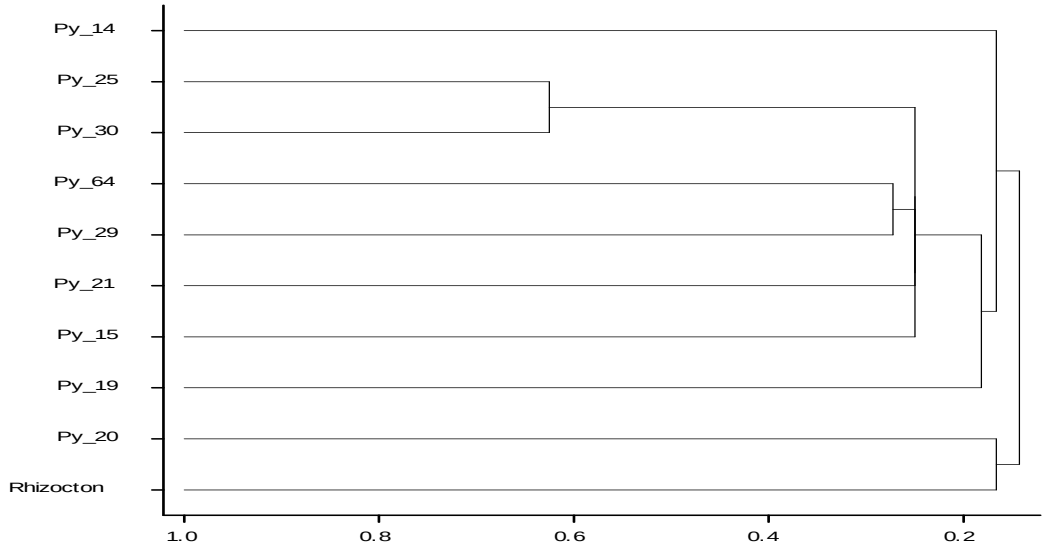

Fig 5.0 *Adendrogram constructed for results obtained using URP 2F primer*

The dendrogram above shows the similarities of *Pythium* isolates amplified by URP 2F. As seen on Fig 4.5 Py 25 and 30 are 63% similar and these two isolates are 25% similar to Py 15, 21, 29 and 64. Py 29 and 64 are shown to be 28% similar to each other. Py 19 is 18% similar to Py 15, 21, 25, 29, 30, and 64 whilst, Py 14 is 17% similar to these isolates including Py 19. Py 20 is 15% similar to all the other isolates.

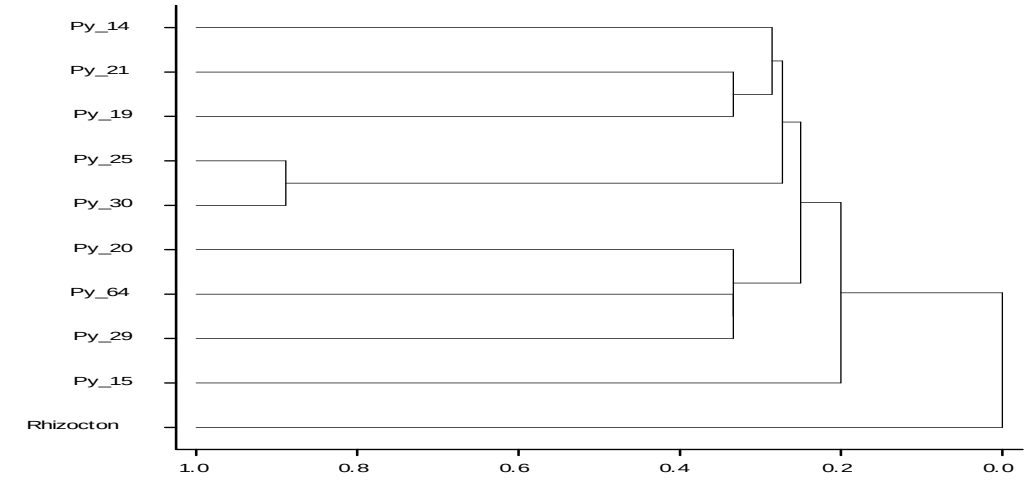

Fig 6.0 *A dendrogram constructed from the URP 2R gel scoring data after PCR*

The dendrogram shows the relationship between the *Pythium* isolates. The primer was able to distinguish between the species as seen in the dendrogram. Py 25 and 30 were 90% similar to each other and these two isolates are 28% similar to Py 14, 19 and 21. Py 20, 29 and 64 are 34% similar to each other and they are 25% similar to Py 14, 19, 21, 25 and 30. Py 19 and 21 are 34% similar to each other and are 29% similar to Py 14. Py 15 is 21% similar to all the *Pythium* isolates

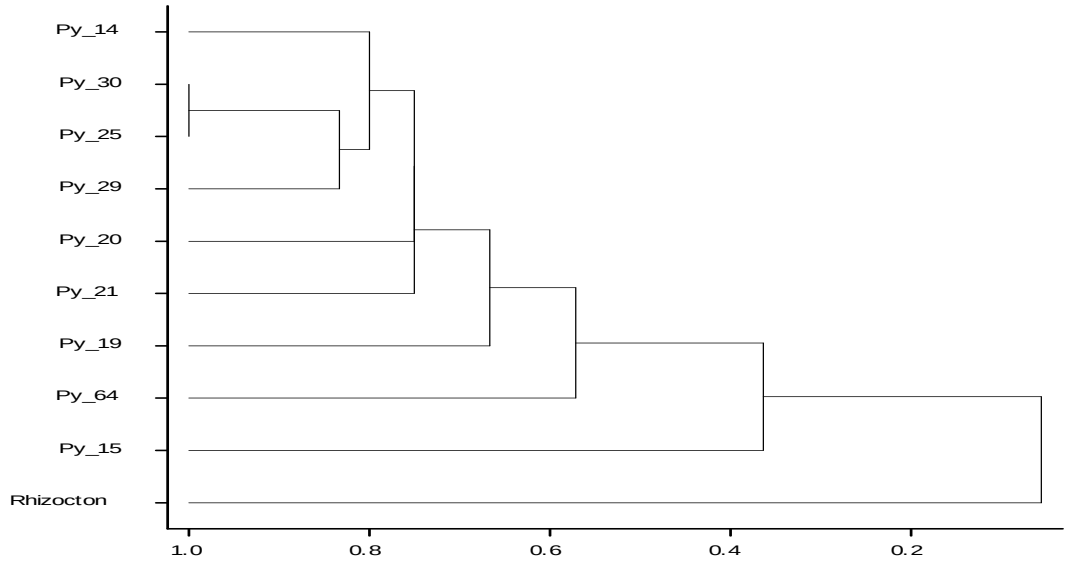

Fig 7.0 *A dendrogram constructed for results obtained using URP 9F primer*

As seen on the dendrogram, Py 25 and 30 are 100% similar to each other and they are 84 % similar to Py 29. Py 14 is 80% similar to Py 25, 29 and 30. The dendrogram shows that Py 20 and 21 are 75% similar to each other and are also 75% similar to Py 14, 25, 29 and 30. Py 19 is 67% similar to Py 14, 20,21,25,29 and 30. Py 64 is 58% similar to all the *Pythium*isolates except Py 15, whilst Py 15 is 37% similar to all the *Pythium* isolates.

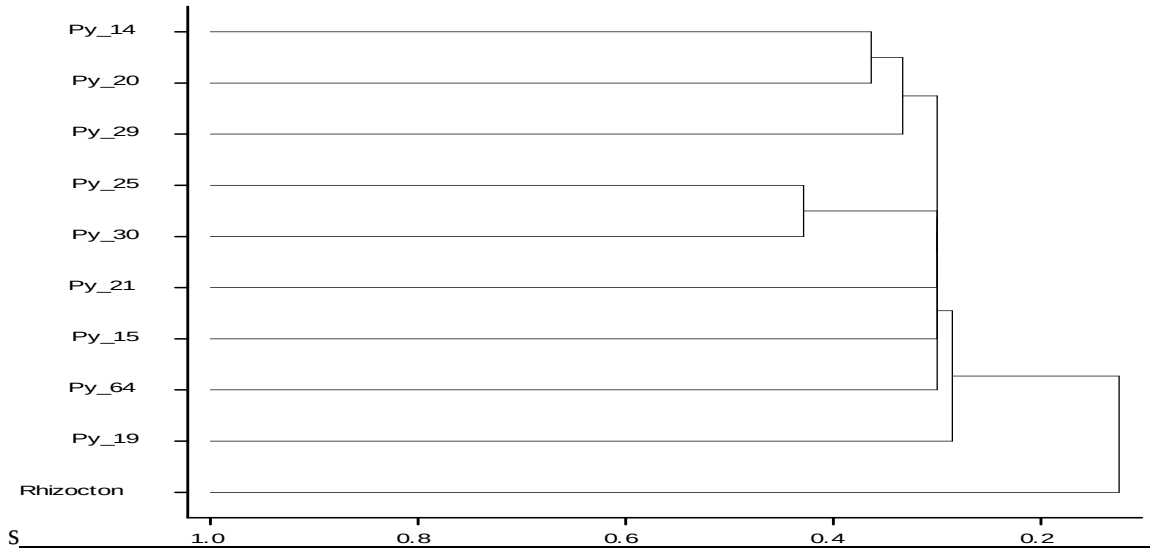

Fig 8.0 *Adendrogram constructed from the URP 13R gel scoring data after PCR*

The dendrogram shows the clustering patterns, with Py 25 and 30 being 43% similar and also being the highest level of similarity for the primer. Py 14 and 20 are 37% similar and they are 33%similar to Py 29. All the isolates except Py 19(which is 29% similar to all other *Pythium* isolates) are 30% similar as shown in Fig 4.0.

URP 2R clustered Py 25 and Py 30 together with a 90% similarity coefficient and these two isolates together with Py 14 and Py 21 were 28% similar to Py 19. Py 20, Py 29 and Py 64 were

25% similar to Py 19 whilst Py 14 was 29% similar to Py 19. Py 15 was shown to be 21% similar to Py 19. The primer had a 26,9% polymorphism.

URP 9F, as shown if Fig 7.0, showed that Py 25 and Py 30 were identical whilst they were 67% similar to Py 19 together with Py 14, Py 20 and Py 21. Py 64 was 58% similar to Py 19 whilst, Py 15 was 37% similar to Py 19. The primer had a 27, 1% polymorphism. Fig 8.0 showed the relationship between the Pythium isolates when amplified with URP 13R. As seen in the dendogram the highest similarity level was recorded for Py 25 and Py 30 which was at 43%. All the isolates were 29% similar to Py 19 and the primer had a 27,1% polymorphism also showing its ability to distinguish between the isolates. The URP – PCR technique proved useful in detecting and distinguishing between the Pythium species. The technique can be employed in detecting or monitoring the fungus from plant material, soil or water samples. The URP- PCR technique its usefulness as it was able to show high levels of similarity between the Pythium isolates and also show the differences between the species (which is useful in differentiating between the species). The technique can be used for detecting the Pythium species in soil and water prior to planting of seed beds using the float tray system.

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