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Identification and Controlling *Pythium* sp. Infecting Tomato Seedlings Cultivated in Jordan Valley using Garlic Extract

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ABSTRACT

Garlic extract is well known for its antibacterial and antifungal activity and is used to treat several plant pathogens. *Pythium* sp. was isolated from infected tomato seedlings grown in Jordan Valley (Jordan) and the species was identified as *Pythium ultimum* using morphological and molecular methods. The fungicidal activity of garlic extract with different concentrations in controlling the growth of the isolated *Pythium* sp. was determined *in vitro*. The control activity was highly dependent on Garlic extract concentration. For instance, undiluted garlic extract showed the highest control activity with no growth as compared to the biotic control without the extract whereas diluted garlic extracts 10 and 5% reduced the fungal growth to 15.5 and 41%, respectively. The results of this study show that garlic extract could successfully control *Pythium ultimum* on tomato seedlings and is considered as an environmentally friendly product.

Key words: Natural fungicide, tomato, *Pythium ultimum*, pathogen characterization, garlic, myclial growth, Jordan vally, soil-bourn

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) ranks as the leading fresh and processed vegetable crop in Jordan and many other countries in the world. World production which exceeded 133 million metric tons in 2007, occupied approximately 4.7 million hectares (FAO, 2007). It is grown practically in every country of the world in outdoor fields, greenhouses and net-houses. However, tomato is a host for several pathogens including fungi, fungal like organisms, bacteria, viruses and nematodes. The environmental conditions that are favored to tomato (especially in greenhouse which always has high humidity and moderate to high temperature) enhances the conditions for the growth of specific soilborne pathogens including the two fungal genera *Pythium* and *Phytophthora* (Kerkeni *et al*., 2007; Akaza *et al*., 2009).

Pythium causes seed rot, damping-off and root rot diseases which are the most destructive and economically important agricultural problems worldwide in nursery and greenhouse crops (Agrios, 2005). In spite of the importance of this pathogen, there is a scarcity of studies on the prevalence and the effect of *Pythium* on tomato in Jordan without ignoring the few reports published by the National Center for Agricultural Research and Extension (NCARE) investigating the effect of different pathogens on tomato production (Ministry of Agriculture, 2003).

The genus *Pythium* was established by Pringsheim in 1858 with the description of *Pythium monospermum* Pringsh as the type species (Martin, 1992). Up to now, more than 200 species have been described worldwide (Mathew *et al*., 2003; Abdelghani *et al*., 2004) but only 120 species have been given valid names and the rest were placed in five groups (F, T, G, P and HS) (Levesque and de Cock, 2004; Tambong *et al*., 2006).

Members of the genus *Pythium* are now classified as fungal-like organisms in the phylum *Oomycotoa* in the kingdom *Chromista* and not in the kingdom Fungi (Deacon, 2006). The fungal-like organisms have aseptate mycelium and both molecular and biochemical studies suggest that they are closer to algae than true fungi (Abdelghani *et al*., 2004). However, these organisms infect plants in similar ways to true fungi (Agrios, 2005).

The main and traditional methods for identifying *Pythium* species are based on morphological and physiological studies (Taechowisan *et al*., 2008).

More recently, molecular techniques have been developed to identify *Pythium* spp. and also to understand the evolutionary relationships between organisms (Levesque and de Cock, 2004; Amein, 2006). In eukaryotes, these methods involve amplifying a fragment from the Internal Transcribed Spacer (ITS) region of rDNA by Polymerase Chain Reaction (PCR). Sequence analysis of the ITS region of rDNA has also been used to identify and study the relationships among isolates of *Pythium* species worldwide (Paul, 2000; Bailey *et al*., 2002; Mathew *et al*., 2003; Schurko *et al*., 2003; Levesque and de Cock, 2004; Paul, 2004; Ghadin *et al*., 2008).

Fungicides are the most important method to control diseases caused by *Pythium* spp. and other *Oomycets*. However, many fungicides have negative impacts to beneficial organisms in soil. In addition, the problem of pathogen resistance could emerge against these fungicides under certain circumstances. Moreover, the residual of many fungicides (most of them recalcitrant and not readily biodegradable) could damage the ecosystem and negatively affect human life if they reach our diet or water (Ministry of Agriculture, 2003).

Different approaches may be applied to prevent, reduce or control plant diseases. Many agronomic and horticultural practices were applied using resistant varieties and physical methods to control plant diseases. Biological control is an ideal method for controlling pathogens with no harm to beneficial organisms and to the environment (Sharma *et al*., 2011). Several natural products were also used to control plant pathogens as an alternative of fungicides (Nithyameenakshi *et al*., 2006; Saravanan *et al*., 2010). Garlic extract is a natural product used to treat several plant pathogens (Curtis *et al*., 2004; Reddy *et al*., 2007; Slusarenko *et al*., 2008). The active antimicrobial component here is allicin (Cavallito and Bailey, 1944; Portz *et al*., 2008). Allicin is a chemical compound found in garlic known as 2-propene-1-sulfinothioc acid S-2-propenyl ester; thio-2-propene-1-sulfinic acid S-allyl ester. Obagwu and Korsten (2003) found that using garlic extract was an effective method to control green and blue molds of citrus caused by *Penicillium digitatum* and *P. italicum* and they found that garlic extract can inhibit growth and development of mycelia for both pathogens significantly.

In this study, *Pythium* sp. infecting tomato plants grown in the jordan valley (Jordan) was isolated and identified at the morphological, physical and molecular levels. Moreover, the effect of garlic extract on the growth of the isolated *Pythium* sp. was tested using different concentrations *in vitro*.

MATERIALS AND METHODS

Isolate recovery: *Pythium* species were isolated from roots of diseased tomato seedlings grown in Jordan Valley from four farms. Samples were placed separately in labeled plastic bags and kept at 4°C. Root systems were washed under tap water and root pieces were plated on Potato Carrot Agar (PCA) media. Cultures were incubated in the dark at 25±1°C for 2 days according to (Van der Plaats-Niterink, 1981).

Morphological identification: Morphological studies were carried out microscopically (Olympus CX41RF, Olympus Optical, Philippines) for preliminary identification. The studied microscopic characteristics include sexual structures, appressoria and hyphal swellings. Slides were then prepared from these cultures and stained with lacto-phenol cotton blue according to Parija and Prabhakar (1995) and examined under the microscope. Measurements of hyphal diameters, oogonia, oospores and oospore wall thicknesses were estimated by eyepiece and stage micrometers. Thirty to fifty measurements were made for these structures. Other features studied were: number, shape and arrangement of antheridia; size and shape of oogonial projections (spines) and shape and abundance of appressoria forming at the points of contact with the Petri dish. The examined morphological characteristics were compared to the documented one in Van der Plaats-Niterink (1981).

Sporangia and zoospores were induced from 2- to 4-day-old cultures growing on PCA. Rectangular pieces of agar culture (20 mm square) were subcultured into sterile plastic Petri dishes and flooded with 20 mL sterile distilled water. The dishes were incubated at 4°C for 1-3 h, then at room temperature (25±1°C); the water was changed hourly for the first three hours. Sporangial development was observed using light microscopy at each water change. If no zoospores were observed after the first 24 h, cultures were left for 5 days at room temperature (25±1°C) and re-examined periodically. If no zoospore production was observed in this period they were treated as such in the taxonomic keys used.

Molecular identification: DNA was extracted from the mycelium of the isolated *Pythium* sp. and the Internal Transcibed Spacer (IST1) region of the ribosomal nuclear DNA (rnDNA) was amplified using PCR according to Paul (2000). Sequencing of ITS1 was carried out at a commercial facility (Macrogen Inc., Seoul, South Korea) using the standard method. Sequence analysis was carried out using BLAST search (http://blast.ncbi.nlm.nih.gov) while the phylogram of the isolate and its relatives was created using ClustalW (http://www.ebi.ac.uk).

Colony growth rates at different physicochemical conditions: *Pythium* isolate was inoculated centrally on a Petri dish (90 mm diameter) containing 20 mL PCA with a 5 mm diameter plug taken from the edge of actively growing 3-day-old cultures on PCA incubated at 25±1°C in the dark. Effect of temperature on growth was tested by incubating the isolate at different temperatures including 5, 10, 15, 20, 25, 30, 35 and 40°C. Five replicates were used at each temperature. The colony diameters of the cultures were recorded at 24 and 48 h in two directions

perpendicular to each other. The colony growth in millimetres over a 24 h period was calculated using the mean colony diameters at 24 and 48 h.

Garlic extraction and *Pythium* treatment: The effect of garlic extract against mycelial growth of *Pythium ultimum* was examined in September 2009. Garlic juice was extracted using the method mentioned by Portz *et al.* (2008). Three concentrations (5, 10 and 100%) of garlic extract were applied and sterile distilled water was used as control. Five plates were used for each treatment. Inoculation was carried on by dispersing 1 mL of the garlic extract from each treatment on the surface of each plate. Then, plates were inoculated with 5 mm disc that was cut from the margin of an actively growing *Pythium ultimum* culture (5 days old) on PCA media incubated in the dark at 25±1°C. Treated plates were then incubated at 25±1°C on the dark. Mycelial growth was assessed daily (visual observation) and the final assessment was recorded after 5 days of incubation using colony counter on Petri dish, by which the total area of the mycelial growth on each plate was measured.

Data analysis: All treatments were arranged in Completely Randomized Design (CRD) with 5 replicates for each treatment. Average growth area of each treatment was assessed using colony counter on Petri dish. General Linear Model (GLM) ANOVA was used to find differences ($p \leq 0.05$) between treatment means (SPSS VER 10).

RESULTS

Isolation and identification of *Pythium* sp.: Several fungal isolates were obtained from tomato roots. All isolates in this study were morphologically similar and identified as *Pythium ultimum* using morphology description and DNA sequencing. Colonies isolated on potato-carrot agar with a radiate pattern. Hyphae were up to 11 μm wide (Fig. 1). Sporangia mostly not formed and zoospores very rarely produced through short discharge tubes at 5°C. Hyphal swellings globose, intercalary, sometimes terminal, 20-25 (-29) μm diam. Oogonia terminal, sometimes intercalary, globose, smooth-walled, (14-) 20-24 (-25) (av. 21.5) μm diam; antheridia either 1 (-3) per oogonium, sac-like, mostly monoclinous originating from immediately below the oogonium, sometimes hypogynous or 2-3 and then either monoclinous or diclinous and frequently straight (Fig. 1). Oospores single, a plerotic, globose, (12-) 17-20 (-21) (av. 18) μm diam, wall often 2 μm or more thick (Fig. 1). Cardinal temperatures: minimum 5°C, optimum 25-30°C, maximum 35°C. Daily growth rate on potato-carrot agar at 25°C was 30 mm.

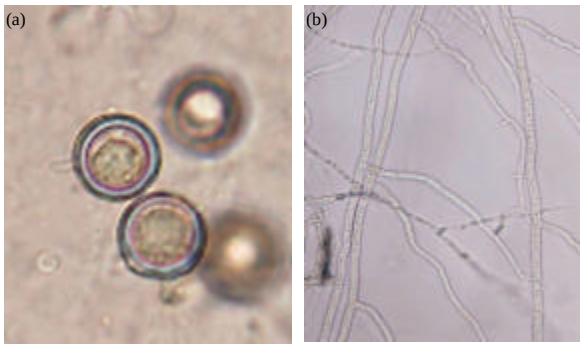

Fig. 1(a-b): Structures of *Pythium ultimum* isolate (a) oospores with antheridia and (b) hyphae

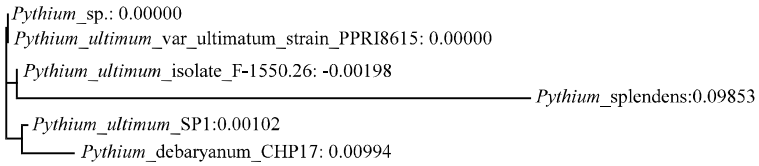

Fig. 2: Phylogram of *Pythium* sp. isolated from tomato roots based on ITS1 sequence. Sequences were retrireved from NCBI database whereas the phylogram was established by ClustalW, EMBL-EBI. Distances are shown on the phylogram

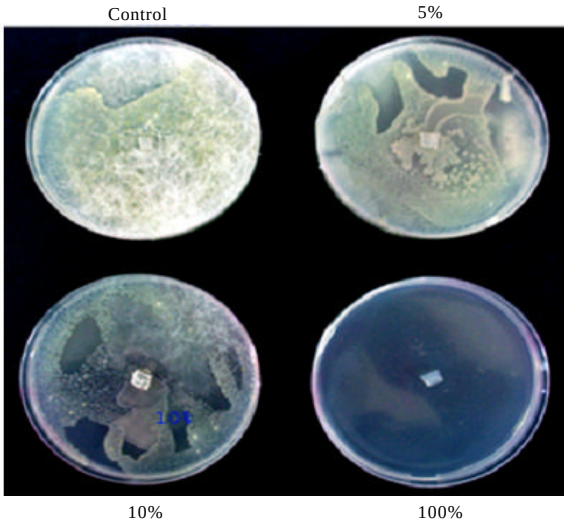

Fig. 3: Effect of different garlic-extracts (5, 10 and 100%) on mycelial growth of *Pythium* sp.

Table 1: The closest relatives of *Pythium* sp. isolated from tomato roots based on BLAST search of ITS1 sequences

Organism	Identity (%)
Pythium ultimum var. ultimum strain PPRI8615	99
Pythium ultimum isolate F-1550.26	99
Pythium ultimum SP1	98
Pythium debaryanum CHP17	97
Pythium splendens	89

Molecular identification based on ITS region of rDNA: ITS1 sequence (781 bp) of one representative isolate was found to be a species of *Pythium* with 99% identity with *Pythium ultimum*. The closest relatives of our isolate, *Pythium* sp., are shown in Table 1 and the phylogram in Fig. 2.

Effect of garlic extract on myclial growth of *Pythium* sp.: Garlic extract was found to be effective to control *Pythium ultimum* isolated from tomato seedlings roots under *in vitro* conditions (Table 2, Fig. 3). One hundred percent Garlic extract showed the highest growth inhibition activity. At this level, the fungus has no growth compared with the control (56.72 cm^2) (Table 2). Moreover,

Table 2: Mean growth area (cm^2) and the percentage of growth inhibition area of *Pythium ultimum* due to treatment with different garlic extract concentrations

Garlic extract conc.	Mean growth (cm^2)	Inhibition percentage (%)
Full concentration 100%	0.00[a]	100.0
10%	8.84[b]	84.4
5%	23.25[c]	59.0
Control	56.72[d]	-

Means followed by the same letter within a column are not significantly different from each other at $p \leq 0.05$. n = 5 for each isolate

diluted garlic extract (10%) reduced the fungal growth to 15.5% (8.84 cm^2) compared with the control. Furthermore, treatment with 5% garlic extract showed growth area of 41% (23.25 cm^2) compared with the control.

DISCUSSION

Pythium ultimum isolated from tomato seedlings grown in Jordan Vally (Jordan) in this study was identified as using morphological and physical characteristics according to the key of Van der Plaats-Niterink (1981), in addition to molecular methods based on ITS1 sequence analysis. The susceptibility of this plant pathogen to garlic extract was established in this study.

Identification of plant pathogens is very important in helping to find effective disease control or management methods. Incorrect identification could lead to control strategies and control methods that are ineffective. Fungi, in general and Oomycetes in particular, have traditionally been identified using morphological characteristics but this is difficult since many species produce overlapping characteristics which are often hard to differentiate (Agrios, 2005). Many morphological features are similar among different groups of species and intraspecific morphological variation is frequently observed in different field isolates (Van Os, 2003).

More recently, molecular techniques based on several DNA methods have been developed to identify species and understand the relationships between them (Levesque and de Cock, 2004; Drenth *et al*., 2006), with the aim of re-examining identifications and relationships determined by morphological methods. The sequences of the Internal Transcibed Spacer (ITS1) region of the ribosomal DNA (rDNA) for *Pythium* isolate were obtained for identification to species level. This isolate was found to be 99% identical to *Pythium ultimum* in the Gene-Bank database (Table 1, Fig. 2). This finding confirms the identification by morphological and physical characterization.

Garlic extract in the present study was found to be effective in reducing growth of *Pythium ultimum in vitro*. Portz *et al*. (2008) found that garlic extract inhibit the germination of sporangia and cysts and subsequent germ tube growth by *Phytophthora infestans* both *in vitro* and *in vivo* on the leaf surface of cucumber. Similarly, garlic juice was found effective in reducing the production of conidiophores and oospores on *Hyaloperonospora parasitica* the casual agent of downy mildew on *Arabidopsis* (Curtis *et al*., 2004). Furthermore, garlic extract was found to have an antibacterial activity against different pathogenic bacteria (Saravanan *et al*., 2010).

In the present study, garlic extract as low as 5% showed a great potential for reducing mycelial growth area to 23.25 cm^2 compared with the control (Table 2). These results demonstrate that garlic extract even with low concentrations could be used to control root rot disease on tomato caused by *Pythium ultimum*. Moreover, other garlic extracts concentrations used in the present study show reduction to complete inhibition of growth of the mycelia. These results agreed with the results from Portz *et al*. (2008) who found disease severity of *Phytophthora infestans* on tomato seedlings was reduced when they sprayed leaves with garlic juice containing allicin over the range tested

(55-110 μg mL^{-1}) with an effectiveness ranging from approximately 45-100%. Other studies found that different concentrations of garlic extract were cased decrease the incidence of seed-borne fungi such as *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium moniliforme*, *Penicillium* spp. and *Rhizopus stolonifer* (Amin *et al*., 2009).

Therefore, garlic extract as low as 10% (v/v) inhibited mycelial growth of all of the fungal pathogens tested in this study. Garlic extract with concentration 100% showed the highest control activity, were the fungus has no growth compared with the control 56.72 cm^2 (Table 2). Moreover, garlic extract with concentration 10% reduced the fungal growth to 8.84 cm^2 compared with the control (Table 2). Furthermore, treatment with concentration 5% of garlic extract showed growth area of 23.25 cm^2 compared with the control (Table 2).

CONCLUSION

The present study shows that garlic extract can control soil-bourn pathogen *Pythium ultimum* under the lab conditions. This result will help in producing a natural pesticide that reduces the losses in the yield and have better crops. Moreover, this natural pesticide will help in reducing the environmental damage and human health caused by chemical pesticides. Further study is needed to assess the effect of this pesticide under field conditions.

REFERENCES

Abdelghani, E.Y., K. Bala and B. Paul, 2004. Characterisation of *Pythium paroecandrum* and its antagonism towards *Botrytis cinerea*, the causative agent of grey mould disease of grape. FEMS Microbiol. Lett., 230: 177-183.

Agrios, G.N., 2005. Plant Pathology. 5th Edn., Academic Press, San Diego, USA.

Akaza, M.J., J.A.K.N. Goran, S.P.A.N. Guetta, B.I. Kebe, G.M. Tahi and A. Sangare, 2009. Resistance to *Phytophthora palmivora* (Butler) butler assessed on leaf discs of cacao (*Theobroma cacao* L.) hybrid trees. Asian J. Plant Pathol., 3: 106-118.

Amein, T., 2006. Soil-borne pathogens infecting sugar beet in Southern Sweden. Plant Pathol. J., 5: 356-361.

Amin, A.B.M., M.M. Rashid and M.B. Meah, 2009. Efficacy of garlic tablet to control seed-borne fungal pathogens of cucumber. J. Agric. Rural Dev., 7: 135-138.

Bailey, A.M., D.J. Mitchell, K.L. Manjunath, G. Nolasco and C.L. Niblett, 2002. Identification to the species level of the plant pathogens *Phytophthora* and *Pythium* by using unique sequences of the ITS1 region of ribosomal DNA as capture probes for PCR ELISA. FEMS Microbiol. Lett., 207: 153-158.

Cavallito, J. and J. Bailey, 1944. Allicin, the antibacterial principle of *Allium sativum* L. Isolation, physical properties and antibacterial action. J. Am. Chem. Soc., 66: 1950-1951.

Curtis, H., U. Noll, J. Stormann and A.J. Slusarenko, 2004. Broad-spectrum activity of the volatile phytoanticipin allicin in extracts of garlic (*Allium sativum* L.) against plant pathogenic bacteria, fungi and Oomycetes. Physiol. Mol. Plant Pathol., 65: 79-89.

Deacon, J.W., 2006. Fungal Biology. Blackwell, Malden, MA.

Drenth, A., G. Wagels, B. Smith, B. Sendall, C. O'Dwyer, G. Irvine and J.A.G. Irwin, 2006. Development of a DNA-based method for detection and identification of *Phytophthora* species. Aust. Plant Pathol., 35: 147-159.

FAO, 2007. A global spatial database of subnational agricultural land-use statistics. http://www.fao.org/corp/statistics.

Ghadin, N., N.M. Zin, V. Sabaratnam, N. Badya, D.F. Basri, H.H. Lian and N.M. Sidik, 2008. Isolation and characterization of a novel endophytic streptomyces SUK 06 with antimicrobial activity from malaysian plant. Asian J. Plant Sci., 7: 189-194.

Kerkeni, A., M. Daami-Remadi, N. Tarchoun and M.B. Khedher, 2007. *In vitro* and *in vivo* suppression of *Pythium ultimum* the causal agent of the cucumber damping-off by some compost fungi. Asian J. Agric. Res., 1: 50-58.

Levesque, C.A. and W.A.M. de Cock, 2004. Molecular phylogeny and taxonomy of the genus *Pythium*. Mycological Res., 108: 1363-1383.

Martin, F.N., 1992. Methods for Research on Soil Borne Phytopathogenic Fungi, In: Singleton, L.L., J.D. Mihail and C.M. Rush (Eds.). The American Phytopathological Society, St. Paul, Minnesota, USA., pp: 39-49.

Mathew, R., K.K. Singh and B. Paul, 2003. *Pythium campanulatum* sp. nov., isolated from the rhizosphere of maize, its taxonomy, ITS region of rDNA and comparison with related species. FEMS Microbiol. Lett., 226: 9-14.

Ministry of Agriculture, 2003. The National Strategy for Agricultural Development, 2002-2010. Amman, Jordan.

Nithyameenakshi, S., P.R. Jeyaramraja and S. Manian, 2006. Investigations on phytotoxicity of two new fungicides, azoxystrobin and difenoconazole. Am. J. Plant Physiol., 1: 89-98.

Obagwu, J. and L. Korsten, 2003. Control of citrus green and blue molds with garlic extracts. Eur. J. Plant Pathol., 109: 221-225.

Parija, W. and P.K. Prabhakar, 1995. Evaluation of lacto-phenol cotton blue for wet mount preparation of feces. J. Clin. Microbiol., 33: 1019-1021.

Paul, B., 2000. ITS1 region of the rDNA of *Pythium megacarpum* sp. nov., its taxonomy and its comparison with related species. FEMS Microbiol. Lett., 186: 229-233.

Paul, B., 2004. A new species of *Pythium* isolated from burgundian vineyards and its antagonism towards *Botrytis cinerea*, the causative agent of the grey mould disease. FEMS Microbiol. Lett., 234: 269-274.

Portz, D., E. Koch and A. Slusarenko, 2008. Effects of garlic (*Allium sativum*) juice containing allicin on *Phytophthora infestans* and downy mildew of cucumber caused by *Pseudoperonospora cubensis*. Eur. J. Plant Pathol., 122: 197-206.

Reddy, K.R.N., C.S. Reddy and K. Muralidharan, 2007. Exploration of ochratoxin a contamination and its management in rice. Am. J. Plant Physiol., 2: 206-213.

Saravanan, P., V. Ramya, H. Sridhar, V. Balamurugan and S. Umamaheswari, 2010. Antibacterial activity of *Allium sativum* L. on pathogenic bacterial strains. Global Veterinaria, 4: 519-522.

Schurko, A.M., L. Mendoza, C.A. Levesque, N.L. Desaulniers, A.W.A.M. De Cock and G.R. Klassen, 2003. A molecular phylogeny of *Pythium insidiosum*. Mycol. Res., 107: 537-544.

Sharma, N., K.P. Sharma, R.K. Gaur and V.K. Gupta, 2011. Role of chitinase in plant defense. Asian J. Biochem., 6: 29-37.

Slusarenko, A.J., A. Patel and D. Portz, 2008. Control of plant diseases by natural products: Allicin from garlic as a case study. Eur. J. Plant Pathol., 121: 313-322.

Taechowisan, T., A. Wanbanjob, P. Tuntiwachwuttikul, Y. Shen and S. Lumyong, 2008. Synergistic activities of 4-arylcoumarins against phytopathogenic fungi. Res. J. Microbiol., 3: 237-245.

Tambong, J.T., A.W.A.M. de Cock, N.A. Tinker and C.A. Levesque 2006. Oligonucleotide array for identification and detection of *Pythium* species. Applied Environ. Microbiol., 72: 2691-2706.

Van Os, G., 2003. Ecology and Control of Pythium Root Rot in Flower Bulb Culture. Uitgeverij Eigen Beheer, Amsterdam, pp: 157.

Van der Plaats-Niterink, A.J., 1981. Monograph of the Genus *Pythium*. Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, pp: 242.