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Screening of endophytic microorganisms from sweetpotato for the production of antimicrobial compounds

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ABSTRACT

This study was an attempt to isolate endophytic microorganism with antimicrobial properties from sweetpotato that are grown in the Philippines. Endophytic microorganisms were isolated from surface-sterilized stem cuttings of selected Philippine sweetpotato varieties such as BSP-SP-17, BSP-SP-22, and NSIC-SP-25. The isolates were purified and tested for antimicrobial activities using spot and streak inoculation methods against *Lasiodiplodia theobromae* (sweetpotato rot-causing mold), *Colletotrichum gloeosporioides* (yam anthracnose-causing mold), *Diplodia natalensis* (watermelon and citrus stem end rot-causing mold), and *Bacillus subtilis* (potato soft rot-causing bacteria). Twenty-one isolates (12 bacteria and 9 fungi) exhibited antimicrobial activities against one or more indicator microorganisms tested. The bacteria were identified through 16S rDNA sequencing analyses as *Bacillus megaterium*, *Bacillus pumilus*, *Microbacterium arborescens*, *Micrococcus luteus* and *Bacillus subtilis*. The fungi were also identified through their partial 26S rDNA and ITS region sequencing analyses as *Phomopsis* sp., *Aspergillus* sp., *Penicillium* sp. and two belong to *Mycosphaerellaceae* and *Polyporaceae* family respectively. The supernatant of these isolates were further tested against the above four indicator microorganisms and four other types of indicators such as *B. subtilis* AHU 2035, *A. flavus* var. *asper* AHU7051, *Alternaria* sp. S-1 and *C. albicans* CAI4. All identified isolates possessed wide spectrum of activity against the indicator microorganisms. *B. subtilis*, *B. megaterium*, *Penicillum* sp. and one isolate belonging to *Mycosphaerellaceae* family were able to inhibit 6 or more of the indicator microorganisms tested.

Keywords: sweetpotato, endophytes, zone of inhibition, bacteria, fungi, screening

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INTRODUCTION

The beneficial role of endophytic microorganisms which are found in a wide range of plants and plant tissues has been extensively studied by various researchers. The isolation of endophytes is mostly done in temperate regions but recently, endophytic mycobiota of topical ecosystems have been investigated. It is believed that with hot and moist climate, a very rich flora of endophytic fungi can be found (Carrol, 1990).

Endophytes are usually fungi or bacteria, which grow symbiotically inside the plant tissues. Virtually, wide spectrum of interactions is involved between endophytes and plant hosts. However, the asymptomatic or beneficial effects of endophytes in host plants are mostly emphasized in various researches. Their growth does not lead to any pathogenic symptoms in plants. These microorganisms are found to increase the plant vigor, drought tolerance, resistance to predation and diseases and ability to withstand other stresses (Latch, 1994). Consequently, the growth and proliferation of endophytic microorganisms in agricultural plants lead to an increase in crop yield. Moreover, endophytes are increasingly recognized as a group of microorganisms that could provide new secondary metabolites that are useful in biotechnology and agriculture (Bills and Polyshook, 1992).

Alkaloid compounds, which act as natural insecticides within the plants, are secreted by endophytes (Emerald Seed and Supply, 2001). Antibiotic compounds are also produced by endophytes from ericaceous plants (Fisher *et al.*, 1984). Secondary metabolites with broad antifungal activity are likewise produced by endophytic strain of *Cryptosporiopsis* (Noble *et al.*, 1991). An anti-cancer drug called taxol was also reported as an important compound by produced by endophytic fungi such as *Taxomyces andreanae*, *Pestalotia*, *Pestalotiopsis*, *Fusarium*, *Alternaria*, *Pithomyces* and *Monochaetia* (Strobel *et al.*, 1996).

The Philippine Root Crop Research and Training Center (PhilRootcrops) at the Visayas State University, Baybay, Leyte has developed high-yielding and appropriate varieties of sweetpotato for specific uses. At present, the problem of non-resistance of these hybrid varieties to pests and diseases has not been fully addressed. These developed varieties generally possess special characteristics that are suitable for processing of food products but are found

to be susceptible to pests and diseases. Thus, supply of raw materials becomes limited and production of processed products is also hampered. While PhilRootcrops is equally engaged in developing varieties that are highly resistant to pests and diseases, the control of these pests and diseases is also one of the priority goals for the Center. In this study therefore, the endophytic microorganisms from selected varieties of sweetpotato were collected, identified and screened for production of antimicrobial compounds against some selected indicator microorganisms, most of which are disease-causing microorganisms in rootcrops.

MATERIALS AND METHODS

Collection and preparation of samples for microbial analysis

Branches of sweetpotato plants were collected and used in this study. The plant samples were washed in running water for 10 minutes and branches were cut in 1 cm length ad surface-sterilized by dipping in 75% ethanol for 1 minute then dipped in 5.3% sodium hypochlorite solution for 5 minutes and finally dipped in 75% ethanol for 0.5 minutes. The treated samples were dried and cut into halves in sterilized slides under the clean bench and mounted on nutrient agar (NA) and potato dextrose agar (PDA) plates. Incubation was done from 3 days to 3 weeks at 27°C or until abundant growth of bacteria and fungi appeared. Bacteria and fungi that grew from underneath the samples that were incubated unto the agar were transferred in NA and PDA slants respectively and incubated at 27°C. All isolates were purified using serial dilution method and distinct single colonies were pick out from the agar plate and transferred into the agar slant. The purity of the isolates grown in agar was confirmed through microscopic examination.

Testing of anti-microbial activities of the isolates

All isolates were tested for their antimicrobial activities using spot and streak inoculation methods against the indicator microorganisms such as *Lasiodiplodia theobromae* (SP rot-casuing mold), *Colletotrichum*

gloeosporioides (yam anthracnose-causing mold), *Diplodia natalensis* (watermelon and citrus stem end rot-causing mold), and *Bacillus subtilis* (potato soft rot-causing bacteria). In spot inoculation method, the test and indicator microorganisms were inoculated side-by-side 3 cm apart in the plate agar. Positive inhibition is demonstrated by negation of growth of indicator microorganisms. In streak plate method, the test organism was streaked perpendicularly across the indicator microorganisms and inhibition of growth of indicator microorganisms at the intersection showed positive activity. Other indicator microorganisms such as *Bacillus subtilis* AHU 1035, *Candida albicans* CA14, *Aspergillus flavus* var. *asper* AHU 7051 and *Alternaria* sp. S-1 were used in screening further the isolates the exhibited positive activities against *L. theobromae*, *C. gloeosporioides*, *D. natalensis*, and *B. subtilis* using the supernatant of test microorganisms. The bacteria were grown in Nutrient Broth for 1-2 days while fungi were grown in Potato Dextrose Broth with Yeast Extract and Malt Yeast Extract Broth for 2-3 days. The bacteria and fungi were grown in their respective media and the supernatant were assayed for biological activity against the above indicator microorganisms.

Identification of the isolates

The isolates with positive activities against one or more test microorganisms were characterized taxonomically through 16S rDNA (bacteria) and partial 26S rDNA and ITS (internal transcribed space) region (fungi) sequencing analyses. For the sequence analysis, bacterial and fungi DNA were extracted using ISOPLANT (Nippon gene). The genes of 16S rRNA, partial 26S RNA and ITS region including 5.8S rRNA were amplified using primer pairs of 27F (5'-AGAGTTTGATCCTGGCTCAG-3')-1522R (5'-GGCTACCTTGTTACGACT-3'), NLI (5'GCATATCAATAAGCGGAGGAAAAG-3')-NL4(5'-GGTCCGTGTTTCAAGACGG-3') and ITS4-ITS5-3') respectively in the following conditions (2.5 min at 95°C, 30 cycles of 30 s at 94°C, 1 min at 50°C and 1 min at 72°C and one final step of 5 min at 72°C for bacteria, 3 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 53°C and 2 min at 72°C and one final step of 10 min at 72°C for fungi). The amplified products were re-amplified using the following primers; (27F, 357F (5'-CTACGGGAGGCAGCAG-3'), 520R (5'-ACCGCGGGGTGCTGGC-3'), 920F (5'-AAACTCAAAGGAA

Table 1. Types and number of microorganisms isolated from some selected varieties of sweetpotato

Sweetpotato Varieties	Number of Isolates		Total Number of Isolates With Biological Activities*		Total Number of isolates With Biological Activities
	Bacteria	Fungi	Bacteria	Fungi	
BSP-SP-17	24	11	5	2	7
BSP-SP-22	18	8	3	3	6
NSIC-SP-25	25	10	4	4	8
Total	67	29	12	9	21

*Total number of isolates with biological activities against one or more indicator microorganisms such as *B. subtilis*, *Colletotrichum gloespoiroides*, *Lasiodiplodia theobromae* and *Diplodia natalensis* in spot and streak inoculation.

TTGACGG-3'), 1080R (5'-CCCAACATCTCACGAC-3') AND 1522R for the 16S rDNA and NL1, NL4, ITS4, ITS5 for the partial 26S rDNA or ITS region and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) containing fluorescent dye. An extension reaction was carried out by PCR following the procedure recommended by the manufacturer. The fragments were sequenced in both directions using an ABI PRISM 3100 DNA Sequencing System according to the manufacturer's instructions. To identify the isolates, the sequences obtained were complied and compared with those in the GenBank databases through the National Center for Biotechnology Information (NCBI) using the basic linear alignment of sequences tool (BLAST; Altschul *et al.*, 1990).

RESULTS AND DISCUSSION

Endophytic microorganisms were isolated from sweetpotato plant. Table 1 presents the type and number of isolates collected from some selected varieties of sweetpotato. A total of 96 isolates from branches of sweetpotato (var. BSP-SP-17, BSP-SP-22, and NSIC-25) were cultured and purified. Bacteria were found to be in greater number compared with fungi. Twenty-one isolates were observed to possess antimicrobial activities against one or

Table 2. Biological activities of isolates against the indicator microorganisms using spot and streak inoculation

Isolates(Code)	Spot/Streak Inoculation			
	A	**B**	**C**	**D**
Bacteria				
17114 (01)	-	-	+	-
17132(02)	+	-	-	-
1721(03)	-	+	+	-
17134(04)	+	-	-	-
1722(05)	-	+	+	-
223211(06)	-	+	-	-
223212(07)	-	+	-	-
2211(08)	-	-	+	-
2532(09)	-	+	+	+
25133(10)	+	-	-	-
25131(11)	+	-	-	-
2531(12)	+/-	+	+	+
Fungi				
1723(13)	+	+	+	-
172212(14)	-	-	+	-
2212(15)	+	-	-	-
22312(16)	+	-	-	-
22334(17)	+	-	-	-
25132(18)	+	-	-	-
2513(19)	-	-	+	-
25122(20)	-	-	+	-
25322(21)	+	-	-	-

Note: With inhibitory activity (+); Without inhibitory activity (-); Slightly clear zone of inhibition (+/-)*B. subtilis* (**A**); *Colletotrichum Gloespoiroides* (**B**); *Lasiodiplodia theobromae* (**C**); *Diplodia natalensis* (**D**).

Table 3. Identification of the isolates with their corresponding biological activities

Isolate No.	Identification (Family/Genus/ Species)	Biological Activity							
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Bacteria									
(01)	*Bacillus megaterium*	-	-	+/--	+/--	+/-	+	+/-	+/-
(03, 05, 08)	*Bacillus pumilus*	-	-	+/--	+/--	-	-	+	-
02, 04, 10, 11)	*Microbacterium arborescens*	-	-	+/--	+/--	-	-	+	-
(06, 07)	*Micrococcus luteus*	+	-	-	+/--	-	+	+	-
(09, 12)	*Bacillus subtilis*	+/-	+	+/--	+/-	+/-	-	+	+/-
Fungi				-					
(13)	*Phomopsis sp.*	-	+/-	+/--	+/--	-	-	-	-
(14)	*Aspergillus sp.*	-	-	+/-	+/--	-	-	+	+/-
(15,16,17,18,21)	*Penicillium spp.*	+	+/-	+/--	+/-	-	+*	+	+/-
(19)	*Mycosphaerellaceae*	+/--	-	+/--	+/--	+	+	+	-
(20)	*Polyporaceae*	-	-		+/--	-	-	-	-

Note: Extremely clear inhibitory activity (++); Very clear zone of inhibition (+); Slightly clear zone of inhibition (+/-); Unclear zone of inhibition (+/--); No Clear zone of inhibition (-); *B. subtilis* (**A**); *C. gloeosporioides* (**B**); *L. theobromae* (**C**); *Diplodia natalensis* (**D**); *B. subtilis* AHU 2035 (**E**); *Aspergillus flavus* var. *asper* AHU 7051 (**F**);*Alternaria* sp. S-1 (**G**); *C. albicans* CAI4 (**H**); Positive only in Isolate No. 17(*).

more indicator microorganisms tested using spot and streak inoculation. There were 12 bacteria and 9 fungi isolates that exhibited inhibitory growth zones in agar seeded with one or more indicator microorganisms. Majority of the isolates were able to inhibit the growth of only one or two indicator microorganisms except isolates 09, 12 and 13 which inhibited 3 or almost all indicator tested. These isolates were able to inhibit *C. gloeosporioides* and *L. theobromae*.

The bioactive components produced by these isolates were further screened by testing their supernatant against the indicator microorganisms used in spot and streak inoculation and four other types of indicators such as *B. subtilis* AHU 2035, *A. flavus* var. *asper* AHU 7051, *Alternaria* sp. S-1 and *C. albicans* CAI4. Table 3 summarizes the biological activities of the 21 isolates, which produce bioactive compounds and their corresponding family or genus/species identification. Taxonomic identification of the isolates through DNA sequencing analysis showed that of the 21 isolates, only 5 were identified up to species level as exhibited by their 100% homology with those in the GenBank databases through NCBI using BLAST. These isolates were *Bacillus megaterium* (01), *Bacillus pumilus* (03, 05, 08), *Bacillus subtilis* (09, 12). The other isolates which were characterized up to genus level only were *Phomopsis* sp. (13), *Aspergillus* sp. (14), *Penicillium* spp. (15, 16, 17, 18, 21), *Mycosphaerellaceae* (19) and *Polyporaceae* (20). Isolates 09 and 12 consistently exhibited wide range of activity spectra. Notably, *B. megaterium*, *B. subtilis*, *Penicillium* spp. and one isolate from the *Mycosphaerellaceae* family exhibited wide range of activity spectra. All of these isolates were able to inhibit at varying degrees, *L. theobromae*, *D. natalensis* and *Alternaria* sp. S-1. *Penicillium* sp. and *Mycosphaerella* can also inhibit the growth of *B. subtilis* and *A. flavus* var. *asper* AHU 7051 while *Penicillium* and *B. subtilis* can also inhibit *C. gloeosporioides* and *C. albicans* to a lesser degree. Interestingly, the identified *B. subtilis* was observed to produce a compound that could inhibit the growth of the same species, *B. subtilis* AHU 1035. Other fungal isolates belonged to *Aspergillus*, *Phomopsis* sp. and *Polyporaceae* family and other bacterial isolates were identified as *B. pumilus*, *Microbacterium arborescens* and *Micrococcus luteus*. These isolates also exhibited inhibitory activity against *D. natalensis*. Generally, all the identified isolates possessed inhibitory activities against two or more indicator microorganisms tested.

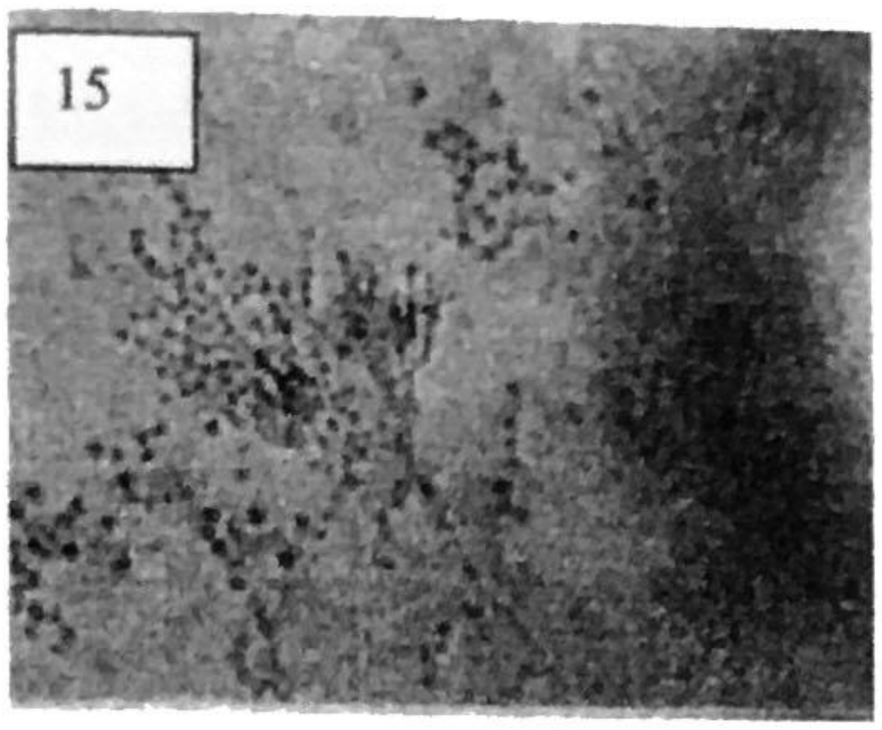
The isolation of culturable endophytic microorganisms with antimicrobial activities from sweetpotato plant is suggestive of various types of microbial

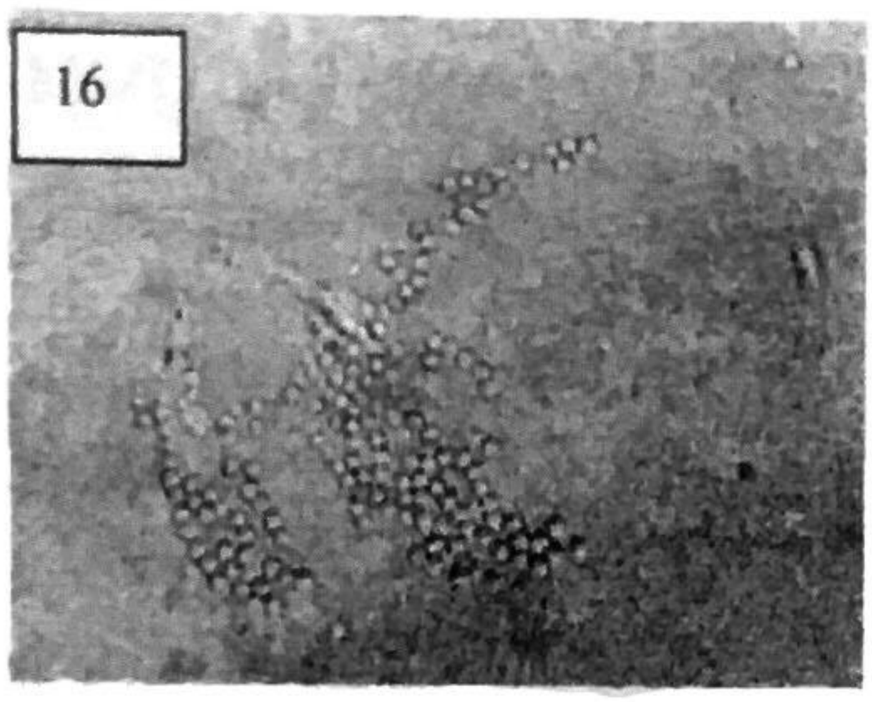
interactions and production of various types of substances inside the plant. Three species of *Bacillus*, namely, *Bacillus megaterium*, *Bacillus pumilus*, and *Bacillus subtilis*, were characterized and identified. *B. subtilis*, and occasionally *B. megaterium*, *B. cereus*, *B. pumilus*, and *B. polymixia* have the characteristics of high thermal tolerance, rapid growth in liquid culture, ready formation of resistant spores, and are considered to be safe biocontrol agents and have high potential to be used as biocontrol agents (Shoda, 2000).

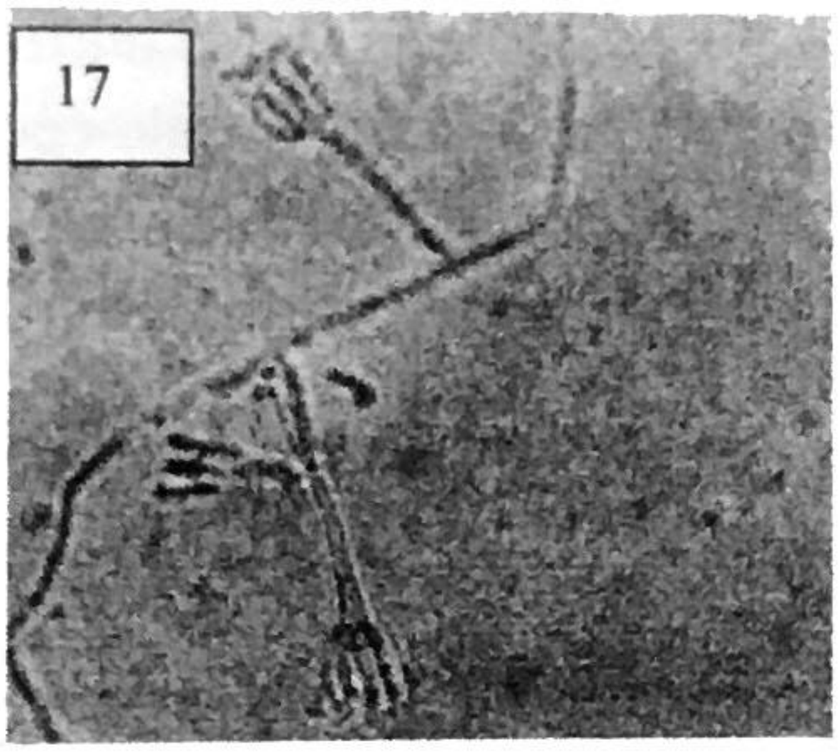
The use of the gram-positive *Bacillus* species as a biocontrol agent has been relatively rare and has been studied less intensively than that of the gram-negative bacteria. *B. subtilis* which acts on the competitive exclusion principle by occupying the identical ecological niche within the plant has been used as biological control agent against *Fusarium moniliforme* in maize Bacon *et al.*, 2001). Due to its high thermal tolerance, *Bacillus subtilis* was able to grow and proliferate in sweetpotato plant that grows in tropical countries like the Philippines.

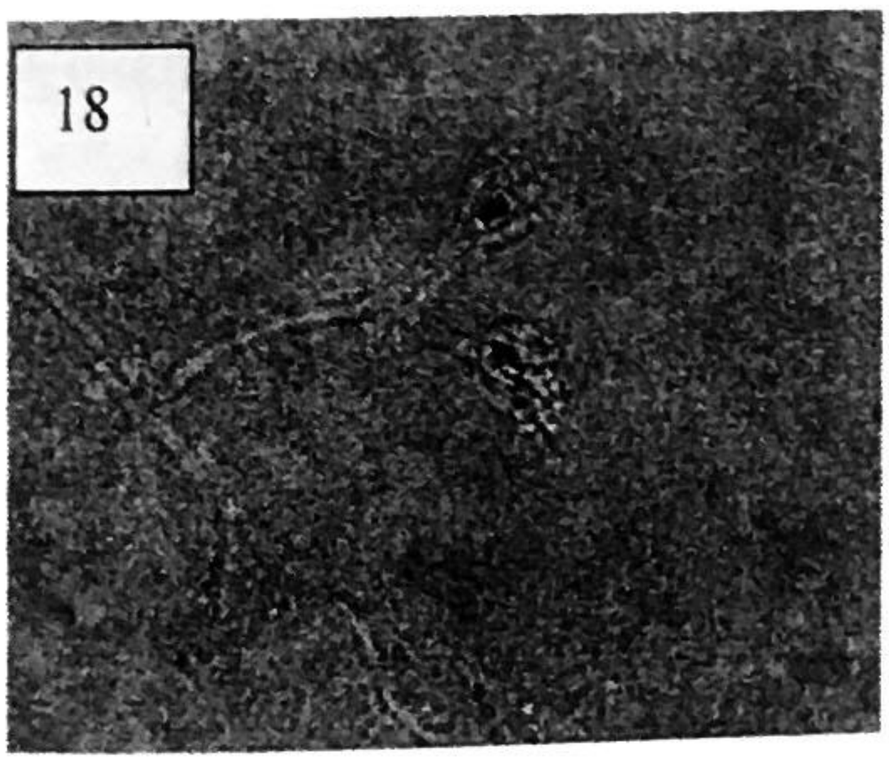
Fungal isolates belonging to *Phomopsis*, *Aspergillus*, *Penicillium* spp. and *Mycosphaerellaceae* and *Polyporaceae* family were also found in sweetpotato plant. *Penicillium* spp. (Figure 1) and isolate identified to be a member of *Mycosphaerellaceae* family exhibited wide spectrum of activities against the indictor microorganisms tested. *Phomopsis* sp., an endophyte from leaves of *Aspidosperma tomentosum* and twigs of *Spondias spombin* has also been reported to exhibit activity against bacteria such as *E. coli* and *P. aeruginosa*; yeast such as *C. albicans* and *S. cerevisiae* and fungi such as *Aspergillus niger* and *Fusarium oxysporum* (Corrado *et al.*, 2004). *Phomopsis* sp. originating from twigs of *Salix graclostyla* var. *melanostachys* produced a novel cytochalasan called Phomopsichalasin, which showed antimicrobial activities against *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella gallinarum*, and *Candida albicans* (Tan and Zou, 2001). In Tuscany, species of *Aspergillus*, a fungal endophyte from grapevine, *Vitis viniferia*, has been tested to be an antagonist of *Plasmopara viticola*, a pathogen of grapevines that cause lesions on leaf tissues and "oily spot" symptoms (Musetti *et al.*, 2003).

Generally, all the identified isolates possessed inhibitory activities against two or more indicator microorganisms tested. Interestingly, majority of the isolates were able to inhibit the growth of pathogenic microorganisms that are associated with rootcrop diseases. It is no doubt therefore, that such endophytes









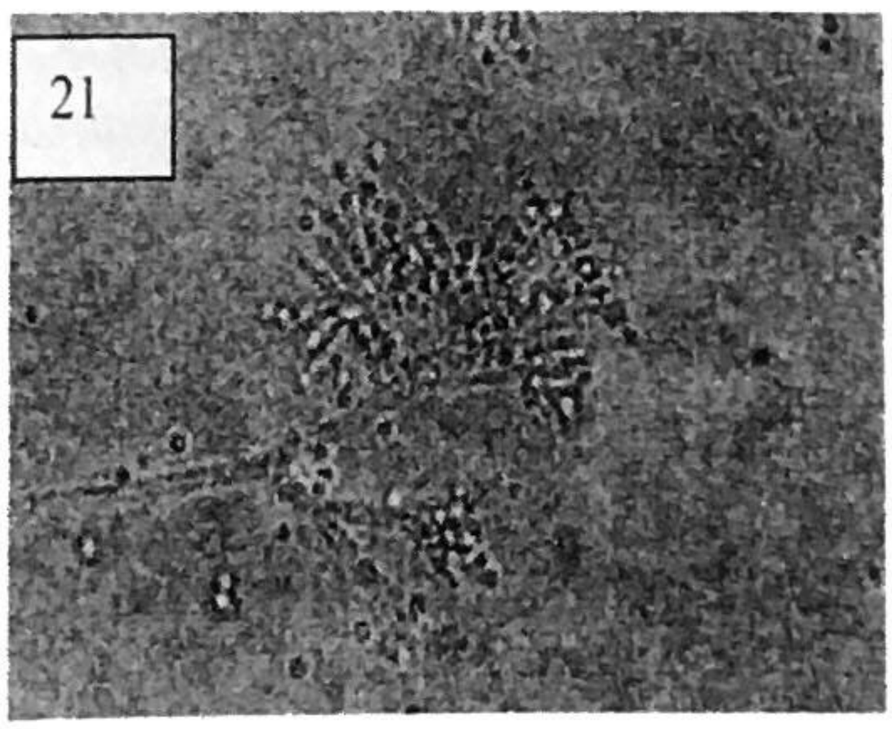


Figure 1. *Penicillium* species with wide spectrum of activities

can contribute a clear solution in eradicating some important diseases in rootcrops.

CONCLUSION AND RECOMMENDATION

Endophytic microorganisms which were tested to possess inhibitory activities against some selected disease-causing microorganisms in plants especially in rootcops were identified as *Bacillus megaterium*, *Bacillus pumilus*, *Microbacterium arborescens*, *Micrococcus luteus*, *Bacillus subtilis*, *Phomopsis* sp. *Aspergillus* sp. *Penicillium* sp., *Mycosphaerellaceae* and *Polyporaceae* family. It is believed that the supernatants of these endophytes contain antimicrobial compounds that are responsible for inhibiting the indicator microorganisms. It is therefore recommended to purify and identify specific compounds that cause growth inhibition of disease-causing microorganisms for their practical applications in rootcrops and possibly in other important agriculture products in the tropical countries like the Philippines.

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