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Research Article

Molecular Identification of Rhizospheric Fungi Associated with 'Saba' Banana via the Amplification of Internal Transcribed Spacer Sequence of 5.8S Ribosomal DNA

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Abstract

Background and Objective: The banana ranks among the top fruits in the world. Production of bananas is beset with problems of pest and disease infestation. The local Philippine cultivar 'Saba' has been reported to possess resistance to major diseases affecting other banana cultivars. This study assessed fungal species present in the soil rhizosphere of 'Saba' banana. **Materials and Methods:** Fungal isolates obtained and purified from the soil and were characterized morphologically in a previous study. These were identified by amplifying the ITS-5.8S rDNA sequences. Prior to amplification, the isolation of fungal DNA was optimized by the freeze-thaw method where mycelia were collected and stored at -80°C for 48 h. Then, a modified CTAB method was used to extract DNA. The PCR fragments were amplified using the primers Forward: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and Reverse: ITS 4 (5'–TCCTCCGCTTATTGATATGC–3). The cycling conditions were as follows: Initial denaturation at 95°C for 2 min, denaturation at 95°C for 1 min, annealing at 60°C for 1 min, 35 cycles, extension at 72°C for 1.5 min and final extension at 95°C for 5 min. Multiple sequence alignment and phylogenetic analyses were performed. **Results:** Fungal DNA has been successfully isolated. Two out of three fungal species whose morphological characteristics were earlier reported to conform with *Aspergillus* were validated in this study. Isolate 2, *Aspergillus niger* strain was deposited and assigned Genbank Accession No. KX093813. Isolate 3 (Genbank Accession No. KX073814) was identified as another strain of *Aspergillus*. They were shown to belong to a common clade with *Aspergillus* strains that were derived from rhizospheric soil. Strains of *Aspergillus* have been reported to possess various roles among which are as causal organisms of fruit rot, agents for bioremediation, antagonists of *Fusarium* species as well as producers of organic acids, enzymes and nutraceuticals. **Conclusion:** The amplification of ITS-5.8S rDNA sequences is a powerful tool in the identification of fungal species. Knowledge of fungal communities associated with plants are key to managing their health and future coping mechanism against potential pests.

Key words: Internal transcribed spacer, *Aspergillus*, saba banana, cell wall lysis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Banana is one of the most important food crops in the world next to rice, wheat and maize[1]. The Philippines is the 5th largest producer of bananas in the world having produced an average of 6,188,378.71 million mt per year from 1993-2013[2]. According to the Department of Agriculture, the most commonly grown cultivars are 'Saba', 'Lakatan', Latundan', 'Bungulan' and Cavendish[3]. Although cavendish is the major export cultivar grown, it is the least preferred banana. Saba (*Musa acuminata*× *balbisiana*), a cooking banana is most favored for its various uses. Saba may be eaten raw when ripe, cooked into desserts or processed as chips.

Problems in the banana industry include huge postharvest losses due to diseases cause by pathogens, thus, chemicals have been widely used in banana industries to reduce postharvest diseases but application of such is discourages due to economic, environment and health concerns[4]. Susceptibility of many cultivars to a few extremely serious diseases and pests and the continuing spread of "New" diseases into areas not previously infected are the major factors affecting yield and production costs in bananas and plantains worldwide. The major diseases and pests are black sigatoka and black leaf streak caused by *Mycospharella fijiensis* (*Fusarium* wilt caused by various races of *Fusarium oxysporum* f. sp. *cubense*), root and rhizome rot caused by the nematode *Radopholus similis* with association with a borer (*Cosmopolites*), Bunchy-top caused by a virus and Moko disease caused by *Pseudomonas solanacearum*[5]. Saba is susceptible to 'Bugtok' disease (caused by the bacterium *Pseudomonas solanacearum*) which hardens the fruit pulp, making the fruit inedible[6]. However, it is very resistant to diseases that infect other cultivars such as bunchy top.

Researches on banana show that fungicides, along with insecticides and nematicides applied to lessen the impact of soil-borne pests are the main pesticides on dessert banana crops. Application of these synthetic pesticides may eventually cause resistance of the said pathogens and can post a threat in the environment as well as to the health of people[7]. Research efforts are exerted towards finding alternative methods such as biological control. Hence, more knowledge on the fungal components present in soil samples of banana farms are needed for baseline information regarding the activity of fungal pathogens.

This study identified fungal isolates associated with 'Saba' banana by amplifying the Internal Transcribed Spacer (ITS) sequences. In mycological study, ITS is widely used for species identification. The ITS, a non-coding region has numerous copies and high variability within the fungal genome[8]. The ITS region is perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genic regions of rDNA (small sub-unit and large sub-unit), variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS (intergenic spacer) regions. In addition to the standard ITS1+ITS4 primers used by most laboratories, several taxon-specific primers have been described that allow selective amplification of fungal sequences[9]. The nuclear-encoded ribosomal DNA genes (rDNA) of fungi exist as a multiple-copy gene family comprised of highly similar DNA sequences (typically from 8-12 kb each) arranged in a head-to-toe manner[10]. Each repeat unit has coding regions for one major transcript (containing the primary rRNAs for a single ribosome), punctuated by one or more intergenic spacer (IGS) regions. In some groups (mostly basidiomycetes and some ascomycetous yeasts), each repeat also has a separately transcribed coding region for 5S RNA whose position and direction of transcription may vary among groups. Several restriction sites for *Eco*RI and *Bgl*II are conserved in the rDNA of fungi. Nearly all basidiomycetes that have been studied share an *Eco*RI site within the 5.8S RNA gene along with a *Bgl*II site halfway into the LSU RNA sequence. Primers 5.8SR and LR7 include these restriction sites, which makes them also convenient for cloning[10].

MATERIALS AND METHODS

Collection of fungal samples: Samples were collected from the soil of healthy banana banana farms in Davao, Philippines. The protocol for collection of samples described in this study are detailed in our previous work[11]. It reported that the morphological and biochemical characterization of several micro-organisms from Philippine banana cultivars. For fungi, morphological features of colonies recorded in that study were pigmentation, shape and form. Size and shape of conidia were also determined microscopically. Three fungal isolates from 'Saba' that were used here were initially identified as two strains of *Aspergillus* (Isolates 2 and 3) and one strain of *Cylindrocladium* (Isolate 1). These fungal isolates were revived and grown in potato dextrose agar, then their morphological characteristics were revalidated microscopically. Mycelia were harvested for DNA isolation.

Fungal DNA isolation: The DNA was isolated using the protocol of Kumar *et al*.[12] with modifications by Al-Samarrai and Schmid[13]. Two modifications of the CTAB protocol for cell

wall disruption were employed prior to DNA isolation: treatment of mycelia with liquid nitrogen and freeze-thaw method where mycelia collected were stored at -80°C for 48 h and isopropanol precipitation was made overnight. Collected mycelia were freeze-dried and kept at -20°C. Freeze-dried mycelia (50-100 mg) were ground using mortar and pestle. Ground mycelium of each fungal isolate was transferred to a 1.5 mL sterile microcentrifuge tube. Five hundred microliters of cetyltrimethylammonium bromide (CTAB) buffer was added to the ground mycelium followed by 10 µL of β-mercaptoethanol. The mixture was shaken well and incubated at 65°C for 1 h in a water bath. After incubation, the mixture was centrifuged at 15,100xg for 15 min. Supernatant was decanted into a new 1.5 mL Eppendorf tube and was added with equal amount of choloform: isoamylalcohol (24:1). The upper layer was collected into a fresh microcentrifuge tube and was added with an equal volume of chilled isopropanol. The solution was incubated at 20°C to facilitate DNA precipitation. The sample was centrifuged at 15,100xg for 15 min. After centrifugation, supernatant was discarded and the pellet was added with 500 µL 70% ethanol for washing. The mixture was mixed well and centrifuged at 5,700xg for 5 min. Supernatant was discarded and pellet was re-suspended in 70 µL of TE buffer.

PCR amplification of ITS-5.8S ribosomal DNA regions: These regions were amplified from the fungal isolates using the universal primers[14] were used for amplification: Forward: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and Reverse: ITS 4 (5'–TCCTCCGCTTATTGATATGC–3). The following components of the *GoTaq* Green Mastermix kit (Promega, USA) were added into a sterile sterile 1.5 mL microcentrifuge tubes: 5 µL Green *GoTaq* Buffer, 200 µM dNTP, 0.2 µM for each of the 2 primers, 2 µL DNA sample and 1.25 U *GoTaq* polymerase. Optimized $MgCl_2$ concentration was 2 mM while the primer concentration was 0.2 uM. Sufficient amount of molecular grade water to reach 25 µL was added. A thermocycler (Veriti Dx 96-well 16 Thermal Cycler, Applied Biosystems, USA) was used for PCR. For amplification of ITS, the cycling conditions were: Initial denaturation at 95°C for 2 min, denaturation at 95°C for 1 min, annealing at 60°C for 1 min, 35 cycles, extension at 72°C for 1.5 min and final extension at 95°C for 5 min. The PCR products were sent for sequencing to the Philippine Genome Center, University of the Philippines, Quezon. The DNA sequences were analyzed using BLASTn in NCBI. Multiple sequence alignment and phylogenetic analysis was performed using Clustal-Omega (EMBL-EBI). The software employs the Neighbour-Joining Method to construct a phylogenetic tree.

RESULTS

Three fungal isolates as reported in our earlier study[11] when revived were found to conform with the published descriptions. Isolate 1 (morphological identification, *Cylindrocladium*, Fig. 1) had conidiophores which were hyaline, branched and erect. They were mainly penicillate, bearing spore masses at phialides on the corresponding

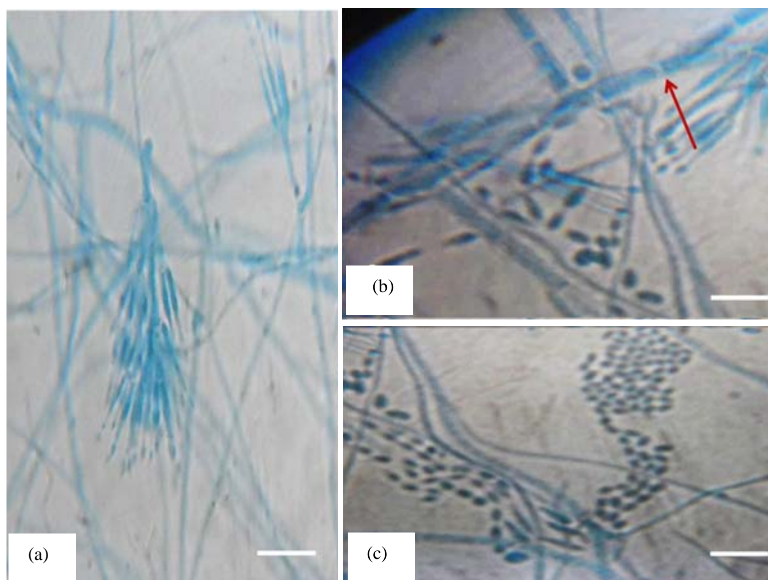

Fig. 1(a-c): Isolate 1, (a) *Cylindrocladium*, (b) Arrangement of branched conidiophore and (c) Septated hypha (red arrow), spread out condiospores. Bar = 10 µm

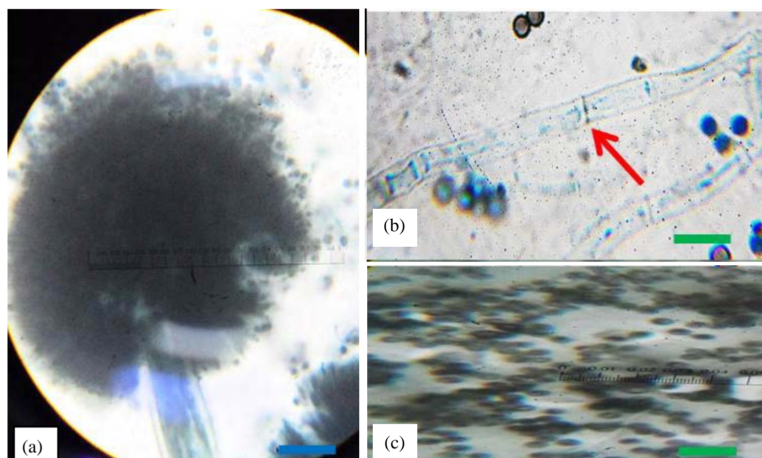

Fig. 2(a-c): Isolate 2, (a) *Aspergillus*, (b) Conidiophore and (c) Septated hypha (red arrow), spread out conidiospores. Blue bar = 20 μm, Green bar = 10 μm

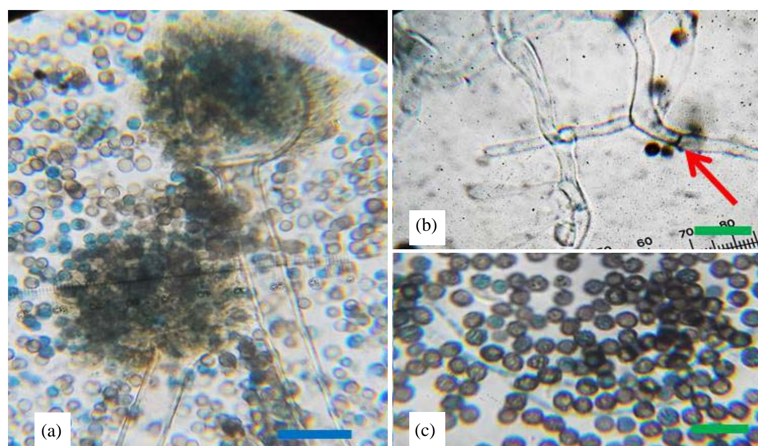

Fig. 3(a-c): Isolate 3, (a) *Aspergillus*, (b) Conidiophore and (c) Septated hypha (red arrow), spread out conidiospores. Blue bar = 20 μm, Green bar = 10 μm

branches with stipes and terminal vesicles. Conidia were hyaline, cylindrical and phialosporous. Conidia (phialospores) were born singly but held together in bundles. Isolates 2 and 3 (morphological identification, *Aspergillus*, Fig. 2, 3) had simple conidiophore and septated hyphae. Conidiophores were erect, hyaline to pale brown. Conidia were oriented in chains which are originating from a globular fruiting body. Spores were green to pale brown in color and are globular in shape.

Fungal DNA was successfully extracted using the CTAB protocol (Fig. 4). The freeze-dry method at -800°C for 48 h as an additional produced optimal DNA yield. Revival of colonies and DNA isolation were not successful for Isolate 1. The ITS fragments which were about 1,000 bp in size (Fig. 5) were successfully amplified in PCR. The BLAST analysis showed that their molecular identities were consistent with previously reported morphological identities, those of ITS sequences of various *Aspergillus* strains. Table 1 shows the summary of results for Isolate 2, which was assigned the Accession No. KX093813 when deposited in Genbank. Isolate 2 shared 99% sequence identity with *A. niger* strains A228, A223, 209 and ZSF16. Table 2 shows the sequence similarity search results for Isolate 3 (Genbank Accession No. KX073814). Isolate 3 also shared 99% identity with *A. niger* strains Z09, HRN004, PFS08 and clone WT-1-5. Figure 6 and 7 shows the deduced gene sequences of the two isolates. Phylogenetic analysis revealed that both Isolates 2 and 3 belong to one clade with a common ancestor with *Aspergillus niger* strain ZSF16 which was also obtained in rhizospheric soil (Fig. 8).

Fig. 4: DNA extracted from saba fungal isolates 2 and 3

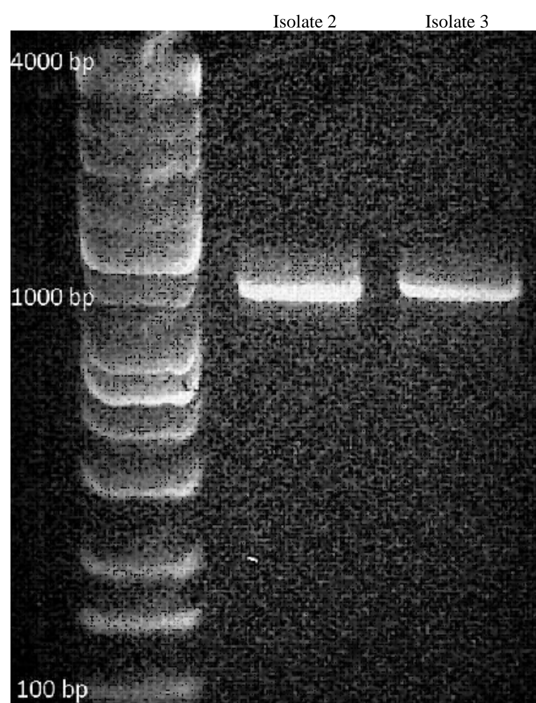

Fig. 5: Putative ITS fragments amplified from Isolates 2 and 3

Table 1: Sequence similarity search results for isolate 2, *Aspergillus* sp. from 'saba' banana (Genbank Accession No. KX093813)

Strain	Description	Maximum score	Total score	Query cover (%)	Identified (%)
Aspergillus niger strain A228	18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence and internal transcribed spacer 2, partial sequence isolated from ancient parchments[15]	917	917	100	99
Aspergillus niger strain A223	18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence and internal transcribed spacer 2, partial sequence. Isolated from ancient parchments[15]	917	917	100	99
Aspergillus niger strain Z09	18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence. Isolated from tobacco-cultivated field	917	917	100	99
Aspergillus niger strain ZSF16		917	917	100	99

DISCUSSION

Fungi contain metabolites that were shown to have antimicrobial properties against human and plant pathogens and therefore, it can be used in modern medicine, agriculture and industry[16]. The higher the diversity of soil-borne microorganisms is the greater the possibility of finding antagonistic microbes that can help in biological control of

5'-
GGTTGGAAAACGTCGGCAGGCGCCGGCCAATCCTACAGAGC
ATGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGC
CGCCGCTGCCTTTCGGGCCCGTCCCCACCGGAGAGGGGGAC
GGCGACCCAACACACAAGCCGGGATTGAGGGCAGCAATGAC
GCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAAT
GTGCGTTCAAAGACTAGATGATTCACTGAATTCTGCAATTCA
CATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAA
CCAAGAGATCCATTGTTGAAAGTTTTAACTGATTGCATTCAA
TCAACTCAGACTGCACGCTTTCAGACAGTGTTCGTGTTGGGG
TCTCCGGCGGGCACGGGCCCGGGGGGCAAAGGCGCCCCCCC
GGCGGCCGACAAGCGGCGGGCCCGCCGAAGCAACAGGGTAT
AATAGACACGGATGGNAGGTTGGGCCCAAAGGACCCGCACT
CGGTAATGATC – 3'

Fig. 6: Isolate 2 identified as *Aspergillus niger* 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence, GenBank Accession no. KX093813

5'-
GGTCAACCTGGAAAAAANGGTTTGGAAAACGTCGGCAGGCG
CCGGCCAATCCTACAGAGCATGTGACAAAGCCCCATACGCTC
GAGGATCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCC
CCCCGGAGAGGGGGACGGCGACCCAACNCACAAGCCGGGCT
TGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAA
TACCANGGGGCGNNCAATGTGCGTTCAAAGACTCGATGATT
CACTGAATTCTGCAATTCACATTAGTTATCGCATTTCGCTGCG
TTCTTCATCGATGCCGGAACCAAGAGATCCATTGTTGAAAGT
TTTAACTGATTGCATTCAATCAACTCAGACTGCACGCTTTCA
GACAGTGTTCGTGTTGGGGTCTCCGGCGGGCACGGGCCCGG
GGGGCAAAGGCGCCCCCCCGGCGGCCGACAAGCGGCGGGCC
CGCCGAAGCAACAGGGTATAATAGACACGGATGGNAGGTTG
GGCCCAAAGGACCCGNACTCGGTAATGATCCTTCCGCAGGTT-3'

Fig. 7: Isolate identified as *Aspergillus niger* 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence, GenBank Accession no. KX073814

Table 2: Sequence similarity search results for Isolate 3, *Aspergillus* sp. from 'saba' banana (Genbank Accession No. KX073814)

Strain	Description	Maximum score	Total score	Query cover (%)	Identified (%)
Aspergillus niger strain Z09	18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence and internal transcribed spacer 2, partial sequence. Isolated from tobacco-cultivated field	968	968	100	99
Aspergillus sp. HRN004	18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence isolated from *Oxytropis* groots	968	968	100	99
Aspergillus niger strain PFS08	18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer isolated from soil by Basu *et al.*[17]	968	968	100	99
Uncultured *Aspergillus* WT-1-5	18S ribosomal RNA gene, clone partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence isolated from feces from vegetarian human	968	968	100	99

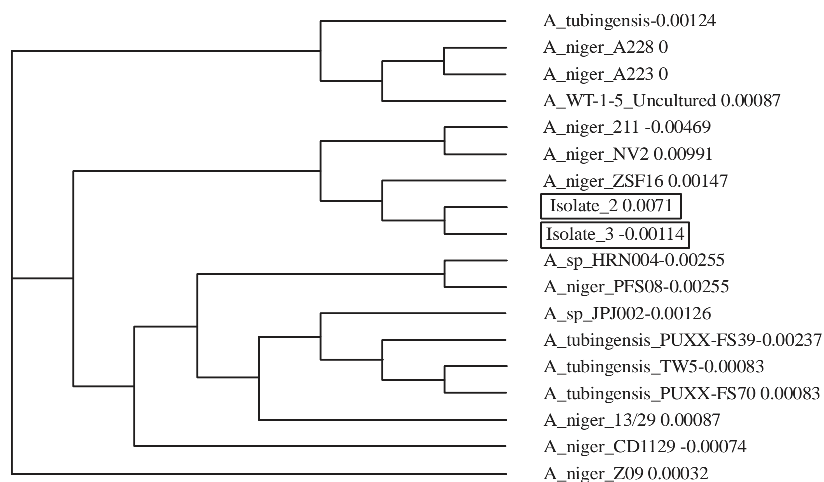

Fig. 8: Phylogenetic relationships of Isolate 2 and Isolate 3 with published *Aspergillus* strains

plant pathogens[18]. Studies on the fungal components of soil along banana plantations are critical in order to attain knowledge on their biological roles and activities and to know if they serve as biological control against plant pathogens. In this study, there was a low turn-out of fungal isolates due to the active application of herbicides containing glyphosates. According to study, glyphosate is a broad-spectrum herbicide that is toxic to a wide array of organisms including lichens[19], nitrogen-fixing bacteria[20-23] and beneficial mycorrhizal fungi[24].

Although, there are numerous published protocols for fungal DNA extraction, this step remains a bottleneck in molecular methods involving fungi. When cell lysis of microbes are not successful, additional steps such as freeze-thawing or application of liquid nitrogen are employed. Liquid nitrogen application requires many intermediate steps to be efficient and sometimes not practical nor available. Fredricks *et al.*[25] reported that liquid nitrogen works well when obtaining large-scale DNA samples from culture. In our study, DNA extraction was only small scale (50 mg mycelia) and so liquid nitrogen addition might have created a harsh condition, thus, producing poor quality DNA. Gonzalez-Mendoza *et al.*[26] also used liquid nitrogen in extracting DNA from filamentous fungi and showed poor results.

Cell disruption is efficient using freezing and thawing. This method allows the formation and successive melting of ice crystals in cell surface. Larger crystals are formed through gradual freezing and thus can disrupt cells more extensively. Compared to other methods, freeze-thaw method is gentle and causes no significant effects on the overall cell integrity[27]. Jin *et al.*[28] froze mycelia of *Aspergillus fumigatus* at 70°C for DNA extraction. They reported that freezing mycelia and bead beating worked well in DNA isolation of more unknown fungi. Freezing and thawing led to successful isolation of DNA in this study.

Identification of the species and strains of both fungal isolates is of high importance in the study of banana and biological control of its pests. *Aspergillus* has been reported to cause fruit rot in various crops[29]. This fungus has also been reported to have played a role in bioremediation due to its ability to degrade organophosphates[30]. Sudarma and Suprapta[16] identified *A. niger* as a potential antagonist of *Fusarium oxysporum* f. sp. *cubense*. *Aspergillus* also produces various organic acid, enzyme, nutraceuticals and other compounds[31]. Hence, antimicrobial assay and isolation and profiling of metabolites unique to both strains reported here are the next steps in determining their future practical applications and utilize their benefits in both agricultural and medical industries.

CONCLUSION

The characterization and molecular identification of micro-organisms associated with a plant is an important step towards the understanding of its health staus as well as its overall ecological balance. This study confirmed two *Aspergillus* strains in the 'Saba' banana rhizosphere. An optimized protocol for fungal DNA isolation which involved a modified CTAB method followed by an additional lysis step (freeze-drying at -800°C for 48 h) was also established.

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REFERENCES

1. Perrier, X., E. De Langhe, M. Donohue, C. Lentfer and L. Vrydaghs *et al*., 2011. Multidisciplinary perspectives on banana (*Musa* spp.) domestication. Proc. Nat. Acad. Sci., 108: 11311-11318.
2. FAO., 2015. FAOSTAT: Download data. Statistics Division, Food and Agriculture Organization of the United Nations, Rome, Italy. http://faostat3.fao.org/download/Q/QC/E.
3. Department of Agriculture, 2010. Banana. Agriculture and Fisheries Information Service, Quezon City, Philippines, pp: 14.
4. Amin, M.N. and M.M. Hossain, 2012. Reduction of postharvest loss and prolong the shelf-life of banana through hot water treatment. J. Chem. Eng., 27: 42-47.
5. Persely, G.J. and E.A. De Langhe, 1986. Banana and plantain breeding strategies. Proceedings of the International Workshop on Banana and Plantain Breeding Strategies, October 13-17, 1986, Cairns, Australia, pp: 13.
6. Dela Cruz, Jr. F.S., L.S. Gueco, O.P. Damasco, V.C. Huelgas and F.M. Dela Cueva *et al*., 2008. Farmers' Handbook on Introduced and Local Banana Cultivars in the Philippines. Bioversity International, Rome, Italy, ISBN: 978-971-91751-8-6, Pages: 67.
7. Risede, J.M., 1994. [Partial characterization of *Cylindrocladium* sp. a root pathogen of banana in Martinique]. Fruits, 49: 167-178, (In French).
8. Michaelsen, A., F. Pinzari, K. Ripka, W. Lubitz and G. Pinar, 2006. Application of molecular techniques for identification of fungal communities colonising paper material. Int. Biodeterior. Biodegrad., 58: 133-141.
9. Gardes, M. and T.D. Bruns, 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol. Ecol., 2: 113-118.
10. Bruns, T.D., R. Vilgalys, S.M. Barns, D. Gonzalez and D.S. Hibbett *et al*., 1992. Evolutionary relationships within the fungi: Analyses of nuclear small subunit rRNA sequences. Mol. Phylogenet. Evolut., 1: 231-241.
11. Novero, A.U., E.G.H. Alcantara, A.A. Blah, J.O.S. Enriquez and J.V. Gapate *et al*., 2015. Diversity of soil microorganisms associated with Philippine banana cultivars free of *Fusarium* wilt. Asian J. Microbiol. Biotechnol. Environ. Sci., 17: 809-817.
12. Kumar, M.S., G. Kaur and A.K. Sandhu, 2014. Genomic DNA isolation from fungi, algae, plant, bacteria and human blood using CTAB. Int. J. Sci. Res., 3: 617-618.
13. Al Samarrai, T.H. and J. Schmid, 2000. A simple method for extraction of fungal genomic DNA. Lett. Applied Microbiol., 30: 53-56.
14. White, T.J., T.D. Bruns, S.B. Lee and J.W. Taylor, 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: PCR Protocols: A Guide to Methods and Applications, Innis, M.A., D.H. Gelfand, J.J. Sninsky and T.J. White (Eds.). Academic Press, San Diego, CA., USA., ISBN-13: 9780123721808, pp: 315-322.
15. De Carvalho, H.P., N. Mesquita, J. Trovao, J.P. da Silva and B. Rosa *et al*., 2016. Diversity of fungal species in ancient parchments collections of the Archive of the University of Coimbra. Int. Biodeterio Biodegrad., 108: 57-66.
16. Yu, H., L. Zhang, L. Li, C. Zheng and L. Guo *et al*., 2010. Recent developments and future prospects of antimicrobial metabolites produced by endophytes. Microbiol. Res., 165: 437-449.
17. Basu, A., S. Mutturi and S.G. Prapulla, 2015. Modeling of enzymatic production of isomaltooligosaccharides: A mechanistic approach. Catal. Sci. Technol., 5: 2945-2958.
18. Sudarma, M.I. and D.N. Suprapta, 2011. Diversity of soil microorganisms in banana habitats with and without *Fusarium* wilt symptom. J. Int. Soc. Southeast Asian Agric. Sci., 17: 147-159.
19. Brown, D.H., C.J. Standell and J.E. Miller, 1995. Effects of agricultural chemicals on lichens. Cryptogamic Bot., 5: 220-223.
20. Carlisle, S.M. and J.T. Trevors, 1986. Effect of the herbicide glyphosate on nitrification, denitrification and acetylene reduction in soil. Water Air Soil Pollut., 29: 189-203.
21. Moorman, T.B., J.M. Becerril, J. Lydon and S.O. Duke, 1992. Production of hydroxybenzoic acids by *Bradyrhizobium japonicum* strains after treatment with glyphosate. J. Agric. Food Chem., 40: 289-293.
22. Martensson, A.M., 1992. Effects of agrochemicals and heavy metals on fast-growing rhizobia and their symbiosis with small-seeded legumes. Soil Biol. Biochem., 24: 435-445.
23. Tu, C.M., 1994. Effects of herbicides and fumigants on microbial activities in soil. Bull. Environ. Contam. Toxicol., 53: 12-17.
24. Sidhu, S.S. and P. Chakravarty, 1990. Effect of selected forestry herbicides on ectomycorrhizal development and seedling growth of lodgepole pine and white spruce under controlled and field environment. Eur. J. For. Pathol., 20: 77-94.
25. Fredricks, D.N., C. Smith and A. Meier, 2005. Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. J. Clin. Microbiol., 43: 5122-5128.
26. Gonzalez-Mendoza, D., R. Argumedo-Delira, A. Morales-Trejo, A. Pulido-Herrera, L. Cervantes-Diaz, O. Grimaldo-Juarez and A. Alarcon, 2010. A rapid method for isolation of total DNA from pathogenic filamentous plant fungi. Genet. Mol. Res., 9: 162-166.

27. Jayakar, S.S. and R.S. Singhal, 2012. Development of an efficient cell disruption method for release of lipoic acid from *Saccharomyces cerevisiae*. Global J. Biotechnol. Biochem., 7: 90-99.
28. Jin, J., Y.K. Lee and B.L. Wickes, 2004. Simple chemical extraction method for DNA isolation from *Aspergillus fumigatus* and other *Aspergillus* species. J. Clin. Microbiol., 42: 4293-4296.
29. Sharma, R., 2012. Pathogenicity of *Aspergillus niger* in plants. Cibtech J. Microbiol., 1: 47-51.
30. Ramadevi, C., M.M. Nath and M.G. Prasad, 2012. Mycodegradation of malathion by a soil fungal isolate, *Aspergillus niger*. Int. J. Basic Applied Chem. Sci., 2: 108-115.
31. Krimitzas, A., I. Pyrri, V.N. Kouvelis, E. Kapsanaki-Gotsi and M.A. Typas, 2013. A phylogenetic analysis of Greek isolates *Aspergillus* species based on morphology and nuclear and mitochondrial gene sequences. BioMed Res. Int. 10.1155/2013/260395.