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Distribution of *Micromonospora* Isolated from Sugar Cane in Thailand

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Abstract: *Micromonospora* is one of actinomycetes belonging to Gram-positive bacteria. It was found both inside plant tissues and outside tissues as free-living bacteria. The objectives of this study were determined the distribution of *Micromonospora* in sugar cane tissues; root, leave and stem which obtained from 5 provinces in Thailand. The results showed that 147 endophytic actinomycetes were isolated. Most endophytic actinomycetes were found in plant root due to high root exudates in rhizosphere whereas the localization of most *Micromonospora* were found in plant stem. Among these, thirty-four isolates has identified as *Micromonospora* by using morphological character and meso-diaminopimelic acid. They were grouped into 5 different groups based on color of substrate mycelium. In this way, most of them belong to Gr. 1 with 10 members. The representative isolates of each group were analyzed 16S rDNA sequence and construct phylogenetic tree. The phylogenetic tree showed Gr. 1 to Gr. 5 were closed relationship to *Micromonospora coriariae*, *M. brigensis*, *M. humi*, *M. rifamycinica* and *M. saelicesensis*, respectively. This is the first descriptions of the presence of *Micromonospora* inside the sugar cane tissue.

Key words: Sugar cane, endophytic actinomycetes, *Micromonospora*, phylogenetic tree

INTRODUCTION

Thailand is one of the largest sugar cane producers in Southeast Asia region. The sugar cane was used as raw material for sugar and ethanol production. The production has increased drastically every year to approximately more than 100 million tons in 2013 according to demand of consumer. The important factors that effected to plant health is the microorganisms that living in and out-side of the plant (Hirsch and Valdes, 2010). To protect sugar cane from diseases, the control strategies are the use of resistant variety of sugar cane and the application of fungicide. However, the application of chemical control leads to environmental impact. The environmental friendly application was consider for controlling the sugarcane disease.

Actinobacteria are filamentous Gram-positive bacteria. Most members contained the % G+C content higher than 55% (Lo *et al*., 2002). Actinomycetes were accepted source for medical enzymes as uricase, xylanase and bioactive metabolites (Khucharoenphaisan and Sinma, 2011; Khucharoenphaisan *et al*., 2013; Sinma *et al*., 2011). Many Actinomycetes produce bioactive compounds such as antibiotics, including actinomycin and tetracycline (Barrios-Gonzalez *et al*., 2005; Raja *et al*., 2010). Endophytic bacteria were classified into 2 groups of obligate and facultative endophyte base on its life character (Hardoim *et al*., 2008). However, the origin of endophyte came from soil. Endophytic actinomycetes residing in plants are not act as symbiosis but mutually associated as free living microbe (Martinez-Hidalgo *et al*., 2014). These actinomycetes may be an interested source to finding efficient biocontrol agents for plant health. The studies of endophytic actinomycetes were done in various kind of plant such as orchid, mandarin orange, zingiber, cinnamimum leguminous plant, rice etc. (Zin *et al*., 2010). However, endophytic actinomycetes in sugar cane have not been reported. *Micromonospora* is one of endophytic actinomycetes that has long recognized as important producer of secondary metabolite inferior to *Streptomyces*. They can produce various antibiotic (Berdy, 2005) anti-tumor agent, anti-fungal (Ismet *et al*., 2004) vitamin B_{12} and also lignocelluloses degrading enzymes production (El-Shatoury *et al*., 2007). Various species of *Micromonospora* were acknowledged to

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leave inside plant tissue as "Endophyte" such as *M. lupini* (Igarashi *et al*., 2007; Taechowisan *et al*., 2008) *M. aurantiaca* (Valdes *et al*., 2005) and *M. coriariae* (Trujillo *et al*., 2006) and some species have free-life in soils (Mansour, 2003). In this study, distribution of *Micromonospora* on sugar cane was investigated.

MATERIALS AND METHODS

Sugar cane sampling: The healthy sugar canes were collected from fields in 5 provinces of Thailand; Chainat, Petchaboon, Lopburi, Nakhon Pathom and Kanjanaburi. The whole trees were collected for serve as root stem and leaf. The sugar cane samples were kept at 4°C until use.

Isolation and enumeration of endophytic actinomycetes: The sugar canes were separated into root stem and leaf portion and surface sterile by 0.1% Tween 20 for 5 min then rinse with 70% ethanol for 5 min and soak in 1% Na-HCl for 10 min. Final rinse with sterile distilled water for 3 times. The efficiency of surface sterile process was checked by spread 200 μL of final rinse effluent on Humic acid vitamin agar plate and observed the present of microorganisms after incubated at 30°C. Then cut each part of sterile sugar cane to 1×1 cm with sterile blade and glinded in 3 mL of 1/4 Ringer's solution. One hundred micro litters were spread on Humic acid vitamin agar plate which was contained 25 μg mL^{-1} of nalidixic acid and 100 μg mL^{-1} of ketokonazole to prevent the contamination of bacteria and fungi. The pieces of glinding samples were placed on the same media. The culture media were incubated at 30°C for 2 weeks.

Morphological characteristic: All of isolated endophytic actinomycetes were examined their morphological characters on International Streptomyces Project (ISP) medium No. 2 agar under light microscope such as the presence of substrate and aerial mycelium, spore chain morphology, production of soluble pigment. The grouping was performed based on their morphological characters in which was showed different in their colony appearance.

Analysis of diaminopimelic acid (DAP): Representative isolates of endophytic actinomycetes from each group were used. The analysis of DAP was performed using Thin Layer Chromatography (TLC) according to Cuesta *et al*. (2010).

Identification of selected actinomycetes: The 16S rDNA amplification of selected actinomycetes was prepared by PCR using universal primer 9F (5'-GAGTTTGATCCT GGCTCAG-3') and1541R (5'-AAGGAGGTGATCCAGCC-3'). The PCR products were purified and directly sequence using a Big Dye® Terminator V3.1 cycle sequencing kit (Applied Biosystems) and the universal primers9F(5'-GAGTTTGATCCTGGCTCAG-3'), 785F(5'- G GAT TAGATACCCTGGTAGTC-3'), 802R (5'-TACCAGGGTATCTAATCC-3') and 1541R (5'-AAG GAGGTGATCCAG CC-3') (Khucharoenphaisan *et al*., 2012). The nucleotide sequences were compared with other bacteria using the Genetyx version 5.0 program. The phylogenetic tree was constructed by using the neighbor-joining method in MEGA version 4 software. The topology was evaluated by bootstrap analysis based on 1000 resamplings (Felsenstein, 1985).

Statistical analysis: All the data was expressed as Mean±Standard Deviation (SD). The experimental data was analyzed using descriptive statistics followed by Explore. Statistical differences yielding $p<0.05$ were considered significant. The analysis was performed using GNU PSPP Statistical Analysis Software Release 0.6.2.

Place and during time: This study was conducted from September 2012 to January 2014 at Faculty of Science and Technology, Phranakhon Rajabhat University and Department of Soil Sciences Faculty of Agriculture, Kasetsart University Kamphaeng Saen Campus, Nakhon Prathom, Thailand.

RESULTS AND DISCUSSION

Isolation and enumeration of endophytic actinomycetes from sugar cane: One hundred and forty seven of endophytic actinomycetes were isolated from all part of sugar cane which cultivated in 5 provinces of Thailand (Table 1). The dominant distribution was found in root portion followed by stem and leaf, respectively as show in Table 1. The result showed that most endophytic actinomycetes were found in plant root due to high root exudates on rhizophere. The distribution of actinomycetes was similar to other report of endophytic actinomycetes in other plant. Kaur *et al*. (2013) and Zhao *et al*. (2011) reported that the majority of endophytic actinomyces were recover from root followed by stem and leave of rice, cabbage, potato, tomoto, mustard, wheat, radish, turmeric, holybasil and medical plants. However, Shutsrirung *et al*. (2013) reports that the large number of endophytic actinomyces were found from leave more than that root of mandarin orange grown in Northern of Thailand.

Table 1: Distribution of endophytic actinomycetes isolated from sugar cane cultural in different province, Thailand

Collecting site and plant parts	Distribution (CFU g^{-1} wet wt. of sugar cane)	No. of isolate
Chainat		
Root	$2.8\pm0.010\times10^{5}$	18
Stem	$9.1\pm0.025\times10^{3}$	0
Leaf	$4.5\pm0.049\times10^{2}$	4
Nakhonprathom		
Root	$2.7\pm0.013\times10^{4}$	7
Stem	$1.7\pm0.001\times10^{3}$	11
Leaf	0	0
Petchaboon		
Root	$4.2\pm0.031\times10^{3}$	29
Stem	$1.4\pm0.003\times10^{3}$	1
Leaf	$3.5\pm0.027\times10^{2}$	1
Lopburi		
Root	$3.0\pm0.004\times10^{3}$	9
Stem	$2.5\pm0.021\times10^{3}$	0
Leaf	$1.5\pm0.014\times10^{3}$	2
Kanjanaburi		
Root	$1.3\pm0.027\times10^{5}$	31
Stem	$4.2\pm0.005\times10^{4}$	38
Leaf	0	0

Table 2: Grouping and characterization of endophytic actinomycetes of genus *Micromonospora* isolated from sugar cane based on morphological characters and diaminopimelic acid

Gr.	Spore mass colour	Substrate mycelium	Soluble pigment	Isolate	Spore chain morphology	DAP
1	Non	Black-dark orange	Non	1 scs 12, 5 scr 36, 5 scs 15, 1 scs 8, 2 sc l1, 5 scs 4, 1 scs 9, 1 scr 9, 3 scs 3, 2 scs 4	Single spore	Meso
2	Non	Orange-black	Non	1 scs 2, 1 scs 1, 5 scs 8, 5 scs 6, 5 scs 1, 5 scs 27, 3 scs 6, 3 scs7	Single spore	Meso
3	Non	Brown	Non	5 scs 31, 5 scs 30, 5 scs 28, 5 scs 24	Single spore	Meso
4	Non	Orang-brown	Non	5 scs 16, 5 scs 23, 5 scs 22, 5 scs 21, 5 scs 11	Single spore	Meso
5	Non	Orange	Non	5 scs 17, 1 scr 3, 1 scs 2, 5 scs 5, 1 scr 5, 1 scr 4,1 scr 2	Single spore	Meso

scs: Sugar cane stem, scr: Sugar cane root and scl: Sugar cane leave

Morphological and chemotaxonomical characteristic: All isolates were aerobic, Gram-positive, non-acid alcohol-fast actinomycetes that forms extensively branched substrate mycelia. Among these, thirty-four isolates has morphological character under microscopic as single spore. These isolates produced different color of substrate mycelium from orange to black-dark orange and brown with lack of aerial mycelium (Table 2). Moreover, these strains contained meso-diaminopimelic acid of the peptidoglycan in the whole-cell hydrolysate. This result tentatively assigned 34 isolates to genus *Micromonospora* (Williams *et al*., 1989). All of the *Micromonospora* isolates were grouped in to 5-different groups based on color of substrate mycelium (Table 2). In this way, most of them belong to Gr. 1 with 10 members whereas Gr. 2, Gr. 5, Gr. 4 and Gr. 3 were lesser, respectively. The result also showed that most *Micromonospora* were found from stem of sugar cane (Table 2).

Molecular identification and phylogenetic tree: The representative *Micromonospora* isolates of each group were selected to analyze 16S rDNA sequence and construct phylogenetic tree. The phylogenetic tree showed high diversity in the isolates (Fig. 1). The representative isolates were scattered over the phylogenetic tree. By the way, Grs. 1-5 were in closed relationship to *Micromonospora coriariae*, *M. brigensis*, *M. humi*, *M. rifamycinica* and *M. saelicesensis*, respectively. *Micromonospora coriariae* was already reported as endophytic bacteria isolated from coriaria (Trujillo *et al*., 2006).

Micromonospora could be isolated from soils, wheat root, aquatic environments (Coombs and Franco, 2003), plant nodules (Carro *et al*., 2013), casuarina (Valdes *et al*., 2005) and from coriaria (Trujillo *et al*., 2006). Taechowisan *et al*. (2008) also found *Micromonospora* in the root of *Alpinia galangal* but less number of isolate about 1.6% of isolated strains. This is the first descriptions of the presence of *Micromonospora* inside the sugar cane tissue. The results of this study indicated that the presence of *Micromonospora* in sugar cane tissue is common and widespread for plants. However, the function of these *Micromonospora* isolates

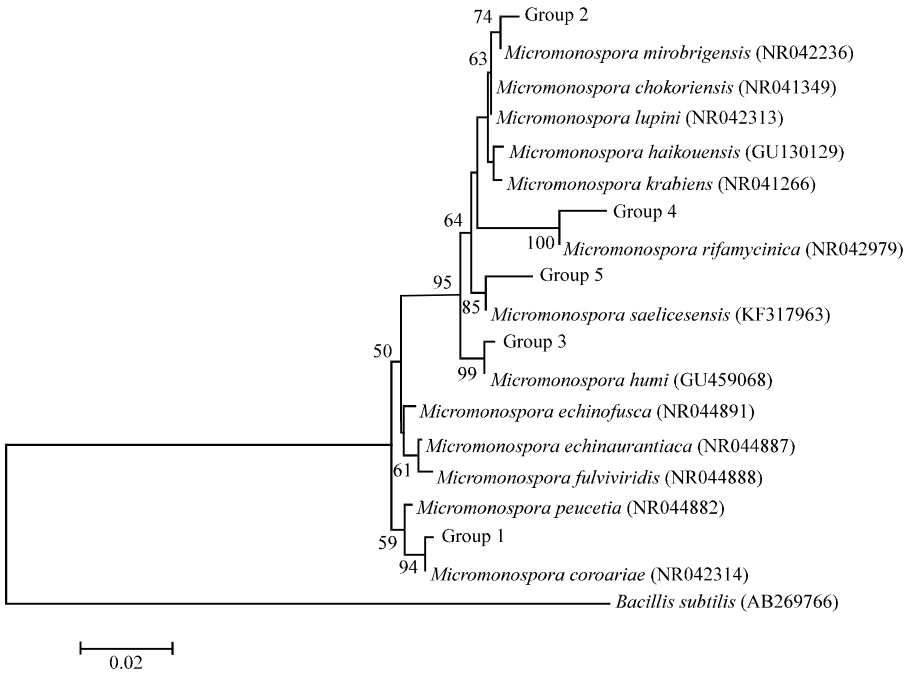

Fig. 1: Phylogenetic tree of nucleotide sequence analysis of 16S rDNA of representative of group 1-5 with related species *Micromonospora* contructed by Neighbor-joining method from MEGA4 program. The tree is rooted by the nuecleotide sequence of *Bacillus subtilis*. Scale bar shown distance values under the tree means 0.02 substitutions per nucleotide position. Bootstrap analyses were performed with 1000 re-samplings and percent values (>50) are shown at the branching points

in the sugar cane was unknown at the moment. The probable they would help the plant fight off the numerous soil pathogens.

CONCLUSION

Thirty-four isolates of *Micromonospora* were isolated from sugar cane tissues; root, leave and stem that obtained from 5 provinces of Thailand. In this way, all of the *Micromonospora* isolates could be grouped in to 5-different groups based on color of substrate mycelium. The phylogenetic tree showed that Gr. 1 was assigned to *Micromonospora coriariae* whereas Gr. 2, Gr. 3, Gr. 4 and Gr. 5 were assigned to *M. brigensis*, *M. humi*, *M. rifamycinica* and *M. saelicesensis*, respectively. The genus *Micromonospora* could be found as free living bacteria and endophytic bacteria. However, *Micromonospora coriariae*, *M. brigensis*, *M. humi*, *M. rifamycinica* and *M. saelicesensis* could find inside plant. They may have specific role for plant as in symbiosis. Further study needs to be determine the bioactive compounds production from those isolates and analyze the mechanism of the bioactive compounds. It might be considered as a candidate source for biological control agent.

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REFERENCES

Barrios-Gonzalez, J., F.J. Fernandez, A. Tomasini and A. Mejia, 2005. Secondary metabolites production by solid-state fermentation. Malaysian J. Microbiol., 1: 1-6.

Berdy, J., 2005. Bioactive microbial metabolites: A personal view. J. Antibiot., 58: 1-26.

Carro, L., P. Pujic, M.E. Trujillo and P. Normand, 2013. Micromonospora is a normal occupant of actinorhizal nodules. J. Biosci., 38: 685-693.

Coombs, J.T. and C.M.M. Franco, 2003. Isolation and identification of actinobacteria from surface-sterilized wheat roots. Applied Environ. Microbiol., 69: 5603-5608.

Cuesta, G., R. Garcia-de-la-Fuente, M. Abad and F. Fornes, 2010. Isolation and identification of actinomycetes from a compost-amended soil with potential as biocontrol agents. J. Environ. Manage., 95: S280-S284.

El-Shatoury, S., H. Abdulla and A. Dewedar, 2007. Factorial design for optimization of rice straw incorporation into soil using *Micromonospora chalcea*. Res. J. Microbiol., 2: 74-80.

Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 39: 783-791.

Hardoim, P.R., L.S. van Overbeek and J.D. Elsas, 2008. Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol., 16: 463-471.

Hirsch, A.M. and M. Valdes, 2010. *Micromonospora*: An important microbe for biomedicine and potentially for biocontrol and biofuels. Soil Biol. Biochem., 42: 536-542.

Igarashi, Y., M.E. Trujillo, E. Martinez-Molina, S. Yanase and S. Miyanaga *et al*., 2007. Antitumor anthraquinones from an endophytic actinomycete *Micromonospora lupini* sp. nov. Bioorg. Med. Chem. Lett., 17: 3702-3705.

Ismet, A., S. Vikineswary, S. Paramaswari, W.H. Wong and A. Ward *et al*., 2004. Production and chemical characterization of antifungal metabolites from *Micromonospora* sp. M39 isolated from mangrove rhizosphere soil. World J. Microbiol. Biotechnol., 20: 523-528.

Kaur, T., D. Sharma, A Kaur and R.K. Manhas, 2013. Antagonistic and plant growth promoting activities of endophytic and soil actinomycetes. Arch. Phytopathol. Plant Prot., 46: 1756-1768.

Khucharoenphaisan, K. and K. Sinma, 2011. Production and partial characterization of uric acid degrading enzyme from new source *Saccharopolyspora* sp. PNR11. Pak. J. Biol. Sci., 14: 226-231.

Khucharoenphaisan, K., K. Sinma and C. Lorrungruang, 2013. Efficiency of actinomycetes against phytopathogenic fungus of chilli anthracnose. J. Applied Sci., 13: 472-478.

Khucharoenphaisan, K., N. Sripairoj and K. Sinma, 2012. Isolation and identification of actinomycetes from termite's gut against human pathogen. Asian J. Anim. Vet. Adv., 7: 68-73.

Lo, C.W., N.S. Lai, H.Y. Cheah, N.K.I. Wong and C.C. Ho, 2002. Actinomycetes isolated from soil samples from the Crocker range Sabah. ASEAN Rev. Biodivers. Environ. Conserv., 9: 1-7.

Mansour, S.R., 2003. The occurrence and distribution of soil actinomycetes in saint catherine area, south Sinai, Egypt. Pak. J. Biol. Sci., 6: 721-728.

Martinez-Hidalgo, P., J. Olivares, A. Delgado, E. Bedmar and E. Martinez-Molina, 2014. Endophytic *Micromonospora* from *Medicago sativa* are apparently not able to fix atmospheric nitrogen. Soil Biol. Biochem., 74: 201-203.

Raja, A., P. Prabakaran and P. Gajalakshmi, 2010. Isolation and screening of antibiotic producing psychrophilic actinomycetes and its nature from rothang hill soil against viridans *Streptococcus* sp. Res. J. Microbiol., 5: 44-49.

Shutsrirung, A., Y. Chromkaew, W. Pathom-Aree, S. Choonluchanon and N. Boonkerd, 2013. Diversity of endophytic actinomycetes in mandarin grown in northern Thailand, their phytohormone production potential and plant growth promoting activity. Soil Sci. Plant Nutr., 59: 322-330.

Sinma, K., K. Khucharoenphaisan, V. Kitpreechavanich and S. Tokuyama, 2011. Purification and characterization of a thermostable xylanase from *Saccharopolyspora pathumthaniensis* S582 isolated from the gut of a termite. Biosci. Biotechnol. Biochem., 75: 1957-1963.

Taechowisan, T., N. Chuaychot, S. Chanaphat, A. Wanbanjob and Y. Shen, 2008. Biological activity of chemical constituents isolated from *Streptomyces* sp. Tc052 and endophyte in *Alpinia galanga*. Int. J. Pharmacol., 4: 95-101.

Trujillo, M.E., R.M. Kroppenstedt, P. Schumann, L. Carro and E. Martinez-Molina, 2006. *Micromonospora coriariae* sp. nov., isolated from root nodules of *Coriaria myrtifolia*. Int. J. Syst. Evol. Microbiol., 56: 2381-2385.

Valdes, M., N.O. Perez, P. Estrada-de Los Santos, J. Caballero-Mellado, J.J. Pena-Cabriales, P. Normand and A.M. Hirsch, 2005. Non-Frankia actinomycetes isolated from surface-sterilized roots of *Casuarina equisetifolia* fix nitrogen. Applied Environ. Microbiol., 71: 460-466.

Williams, S.T., M. Goodfellow and G. Alderson, 1989. Genus *Streptomyces* Waksman and Henrici 1943, 339^{AL}. In: Bergeys Manual of Systematic Bacteriology, Williams, S.T., M.E. Sharpe and J.G. Holt (Eds.). Williams and Wilkins Co., Baltimore, ISBN: 0-683-09061-5, pp: 2453-2492.

Zhao, K., P. Penttinen, T. Guan, J. Xiao and Q. Chen *et al*., 2011. The diversity and anti-microbial activity of endophytic actinomycetes isolated from medicinal plants in Panxi Plateau, China. Curr. Microbiol., 62: 182-190.

Zin, N.M., C.S. Loi, N.M. Sarmin and A.N. Rosli, 2010. Cultivation-dependent characterization of endophytic actinomycetes. Res. J. Microbiol., 5: 717-724.