Journal of Applied Sciences

ISSN 1812-5654

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Applied Sciences 13 (3): 472-478, 2013
ISSN 1812-5654 / DOI: 10.3923/jas.2013.472.478

Efficiency of Actinomycetes Against Phytopathogenic Fungus of Chilli Anthracnose

[1]K. Khucharoenphaisan, [2]K. Sinma and [3]C. Lorrungruang
[1]Faculty of Science and Technology, Phranakhon Rajabhat University, Bangkok 10220, Thailand
[2]Department of Soil Science, Faculty of Agriculture, Kasetsart University, Kamphaeng Saen Campus, Nakhonpathom 73140, Thailand
[3]Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand

Abstract: Phytopathogenic fungus as *Colletotricum gloeosporioides* is a cause of disease on chilli and wide varieties of agricultural crops resulting in yield loss. The aim of this study was to screened actinomycetes according to its ability to produce various secondary metabolites with inhibition activity against chilli anthracnose. Firstly, actinomycetes from previously study were tested for antagonistic activity toward the fungus by the dual culture technique. Finally, extracellular antifungal metabolites produced by selected isolates were evaluated for antifungal potential toward the fungus with agar core technique. Eighty three strains of actinomycetes were screened for their antifungal as well as phytopathogenic activity. Among these, 26 isolates were shown the inhibition activities against *Colletotricum gloeosporioides* chi in which was isolated from infected chilli. The culture supernatants obtained from 21 actinomycetes strains were affective against the fungus. More interestingly, 7 isolates produced affective thermostable compound that having activity after treated with temperature of 121°C for 20 min. In total, the isolate R58 was most promising on the basis of its interesting antimicrobial activity and it could reduce anthracnose disease of chilli comparing to the absence of biocontrol agent. Based on morphological character, its 16S rDNA sequence and phylogenetic tree analysis, isolate R58 belong to the *Streptomyces malaysiensis*. These findings have increased the scope of agriculturally important actinomycetes.

Key words: Phytopathogenic fungus, actinomycetes, antifungal, *Colletotricum* sp., *Streptomyces* sp.

INTRODUCTION

Chilli belongs to the family *Solanaceae*, is one of important vegetable in Thailand (Rapatsa and Terapongtanakorn, 2010; Rajamanickam *et al*., 2012). *Collectotrichum* sp. such as *C. capsici* and *C. gloeosporioides* was caused of chilli anthracnose due to reduce crop production in Thailand and developed countries (Than *et al*., 2008; Suwan *et al*., 2012; Rahman *et al*., 2003). Actinobacteria are filamentous gram-positive bacteria. Most members contained the % G+C content higher than 55% (Lo *et al*., 2002). They were exceptional source for medical enzymes as uricase and bioactive metabolites production (Khucharoenphaisan and Sinma, 2011). Many actinomycetes produce bioactive compounds such as antibiotics, including actinomycin and tetracycline (Barrios-Gonzalez *et al*., 2005), especially genus *Streptomyces*. This genus has been recognized for many years as a microbial reagents which is important for bioactive compounds production with wide range of activity for antibacterial and antifungal (Usha *et al*., 2011; Reddy *et al*., 2011; Atta and Ahmad, 2009; Khucharoenphaisan *et al*., 2012). Nearly ten-thousand of antibiotics producing by actinomycetes have been identified so far (http://banglapedia.search.com.bd/HT/A_0261.htm). Eighty percentage of an antibiotic derived from *Streptomyces* and less the other as the genus *Micromonospora* (Arifuzzaman *et al*., 2010). Suwan *et al*. (2012) reported that *Steptomyces* species has a potential to reduced chilli anthracnose in pot experiment. *Streptomyces* that obtained from different sources may affect on the performance of inhibition of plant pathogenic fungi, especially symbiosis *Streptomyces* with other organisms. This symbiosis *Streptomyces* may be an interested source to finding efficient *Sterptomyces*.

The aim of this study was screening of 83 actinomycetes which isolated from termite guts against phytopathogenic fungus *C. gloeosporioides* causing chilli anthracnose. The culture broth of those actinomycetes was treated with and without heat treatment to investigate the antifungal activity. Moreover,

Corresponding Author: K. Khucharoenphisan, Faculty of Science and Technology, Pranakhon Rajabhat University, Bangkok 12200, Thailand Tel: +66-02-544-8000 Fax: +66-02-521-0421

the pot experiments were done for the efficiency of selected actinomycetes strain against chilli anthracnose.

MATERIALS AND METHODS

Place and during time: This study was conducted from September 2011 to November 2012 at Faculty of Science and Technology, Phranakhon Rajabhat University, Thailand.

Isolation of phytopathogenic fungus: *C. gloeosporioides* causing anthracnose was isolated from infected chilli. The isolated fungus was cultured on Potato Dextrose Agar (PDA) at room temperature. It was identified as *C. gloeosporioides* chi using morphological character under light microscopic. The colony showed white to pale grey mycelium with abundant mycelia containing bright orange conidial masses produced in concentric rings on the colonies. Conidial shape is cylindrical (length 16 μm and width 5 μm) (Photita *et al*., 2005).

Screening of antifungal activity of actinomycetes: Two hundred and forty eight strains of actinomycetes were cultured on International Streptomyces Project (ISP) medium No. 2 agar (Shirling and Gottlieb, 1966). The cultures of actinomycetes were single streak on one side of PDA (potato dextrose agar) at distance of 1.0 cm and then incubated at room temperature for 7 days. The active mycelium of *C. gloeosporioides* chi was cut and transferred to the middle of cultured actinimycetes plate. The inhibition of mycelium growth was measured after 7 day cultivation. The inhibition was evaluated by radius of fungal colony compared to control culture. Three replications were done.

Test of culture filtrates: The effective actinomycetes were inoculated in ISP medium No.2 broth and then incubated at room temperature for 7 days with shaking condition at 150 rpm. The cultured supernatants were filtrated using sterile cotton as supernatant. The supernatants with and without heat treatment at 121°C for 20 min were tested against phytopathogenic fungus of chilli anthracnose activity using agar well method. The active mycelium of *C. gloeosporioides* chi was cut and transferred to the middle of cultured actinomycetes plate. The fungal plate was incubated at room temperature for 5 days. Thirty microliter of supernatants with and without heat treatment was put to each well and then inhibition of mycelium growth was measured. Three replications were done.

Identification of selected actinomycetes: The selected actinomycetes were tentative identified into genera based on morphological characteristics using crosshatch streak technique (Shirling and Gottlieb, 1966; Khucharoenphaisan *et al*., 2011). The morphological characteristics were observed as colony shape, color, sporulation, soluble pigment on ISP medium No.2 and humic acid vitamin agar after 14-day cultivation at 30±2°C (Cuesta *et al*., 2010).

The 16S rDNA amplification of actinomycetes was prepared by PCR using universal primer 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541R (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 primers 9F (5'-GAGTTTGATCCTGGCTCAG-3'), 785F (5'- GGATTAGATACCCTGGTAGTC-3'), 802R (5'-TACCAGGGTATCTAATCC-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3') (Khucharoenphaisan *et al*., 2012). The nucleotide sequences were compared with other bacteria using the Genetyx version 5.0. 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).

Production of inoculums: The inoculum was prepared by placing 100 g of rice grain into plastic bag and autoclaving at 121°C for 20 min. Then inoculated with *S. malaysiensis* R58 or *C. gloeosporioides* chi and incubated at 30°C for 1 week. The sterile rice grain without inoculation was used as control using in the pot experiment.

Green house inoculation: The colonized rice grain was dispersed in the soil before planting chilli by mixing 5% of inoculums. In total, there were four treatment combinations: (T1) with *C. gloeosporioides* chi and without *S. malaysiensis* R58 and (T2) with containing of *S. malaysiensis* R58 and *C. gloeosporioides* chi (T3) with *S. malaysiensis* R58 and without *C. gloeosporioides* chi; (T4) control without *C. gloeosporioides* chi and *S. malaysiensis* R58. The efficiency of actinomyces was monitored by growth and productivity of the chilli that showed against anthracnose disease. Twelve chilli pots per treatment were investigated.

Data analysis: The student t-test was used to determine significance fungal inhibition by supernatant and living cell of actinomycetes.

RESULTS AND DISCUSSION

Antifungal activity of actinomycetes: The antifungal activities against *C. gloeosporioides* chi were determined on PDA with dual culture technique. The percentage of inhibition was evaluated from colony

Table 1: Anti *Collectotrichum gloeosporioides* chi activities of actinomycetes

Inhibition (%)	No. of actinomycetes (isolate)
0-20	4 (4.8%)
21-40	11 (13.2%)
41-60	28 (33.7%)
61-100	40 (48.2%)

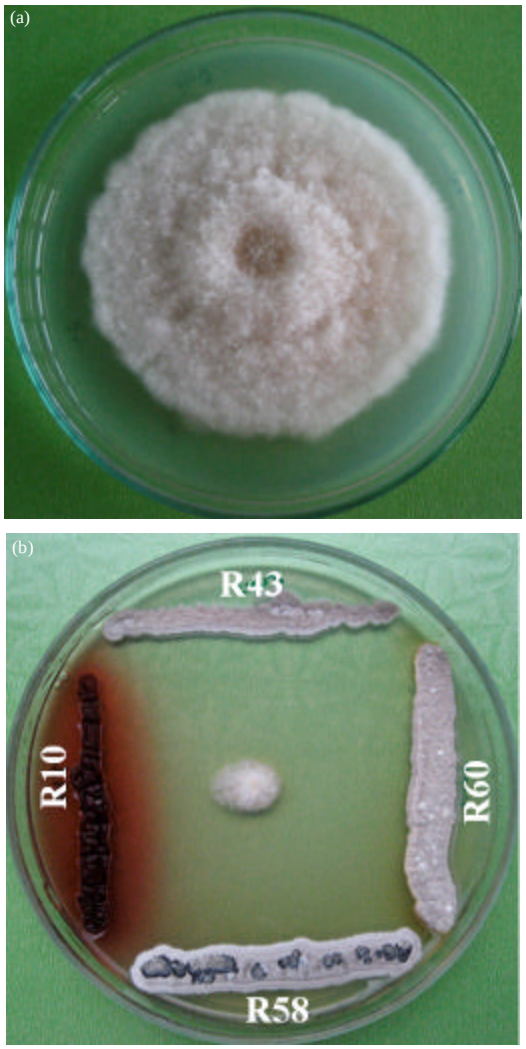

Fig. 1(a-b): Colony of *C. gloeosporioides* chi isolated from infected chilli as a (a) Control and (b) Antifungal activity of actinomycetes stains after incubated on PDA at room temperature for 7 days

diameter of *C. gloeosporioides* chi growth with and without tested actinomycetes. The fully growth of *C. gloeosporioides* chi without tested actinomycetes (control) referred to 0% of inhibition while no growth referred to 100% inhibition. The results revealed that most actinomycetes from termites have wide range abilities to inhibit *C. gloeosporioides* chi growth as shown in Table 1 and Fig. 1. Among 83 isolates of actinomycetes from termites, 79 isolates (95.2%) showed the percentage of inhibition in a range of 21-100%. Table 1 showed the percentage of *C. gloeosporioides* chi inhibition by the tested actinomycetes. There are 40 isolates (48.2%) that shown the high efficience to significantly inhibit the growth of *C. gloeosporioides* chi more than 60%. Furthermore, 28 (33.7%) and 11 isolates (13.2%) were found to have abilities to inhibit *C. gloeosporioides* chi in a range of 41-60% and 21-40%, respectively. Only 4 isolates (4.8%) has less effective on inhibition of the fungal pathogen at low level of 0-20% (Table 1). This suggests that termite may be a suitable source for isolated efficient actinomyces. Moreover, the result indicated that antimicrobial activities obtained from actinomycetes were effective against anthracnose disease in chilli. One of possible reasons was chitinase production from actinobacteria and then degraded cell wall of the fungus. Another one, the actinomycetes produced secondary metabolites such as bioactive compound to inhibit the phytopathogenic fungal growth. The finding agreed with the report of Suwan *et al*. (2012) that *Streptomyces* sp. isolated from soil that collected from Suthep-Pui National Park and chilli plant have effective activity against *C. gloeosporioides*. Moreover, Intra *et al*. (2011) reported that most actinomycetes showing activity against *C. gloeosporioides* and *C. capsici* were *Streptomyces* sp. (87%) especially *S. cavurensis* whereas *Saccharopolyspora*, *Norcardia* and *Nocadiopsis* were lesser than those.

Effect of actinomycetes supernatant: Among 40 isolates that display highly against pathogenic fungal from previous step were cultured and tested activity of supernatant. The result showed that 21 supernatant that obtained from actinomycetes cultures were significantly inhibit the *C. gloeosporioides* chi colony. This positive result might due to presence of extracellular secondary metabolites and antifungal agent in which was produced by actinomycetes. Only 7 isolates producing supernatant with heat treatment at 121°C for 20 min showed the fungal colony inhibition (Fig. 2). The result indicated that supernatant has more effective inhibition of the fungus than supernatant with heat treatment. This due to supernatant was contained living cell of actinomycetes and it produced secondary metabolites against the *C. gloeosporioides* chi cell. Among these, isolates R58 gave the highest inhibition of *C. gloeosporioides* chi in both supernatant with and without heat treatment. The results are agreement with the

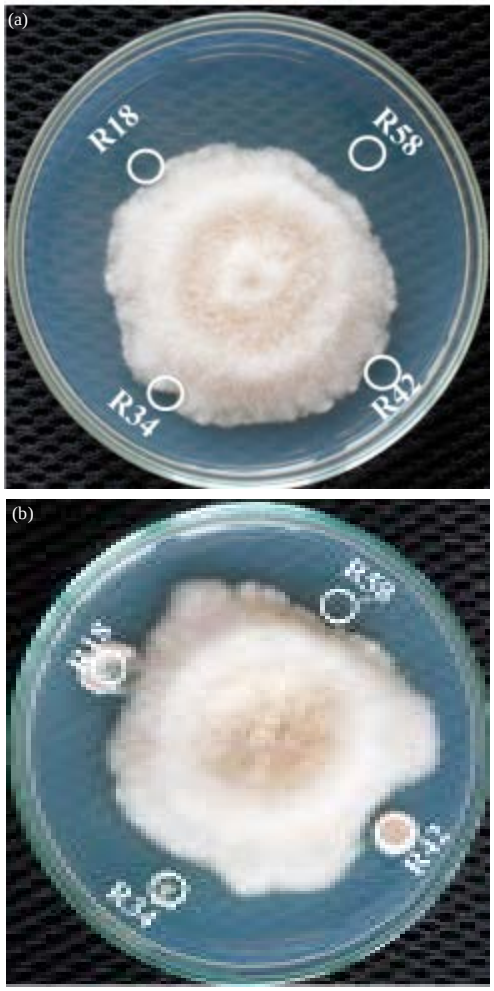

Fig. 2(a-b): Antifungal activity of actinomycetes culture supernatant (a) with and (b) without heat treatment at 121°C for 20 min against *C. gloeosporioides* chi

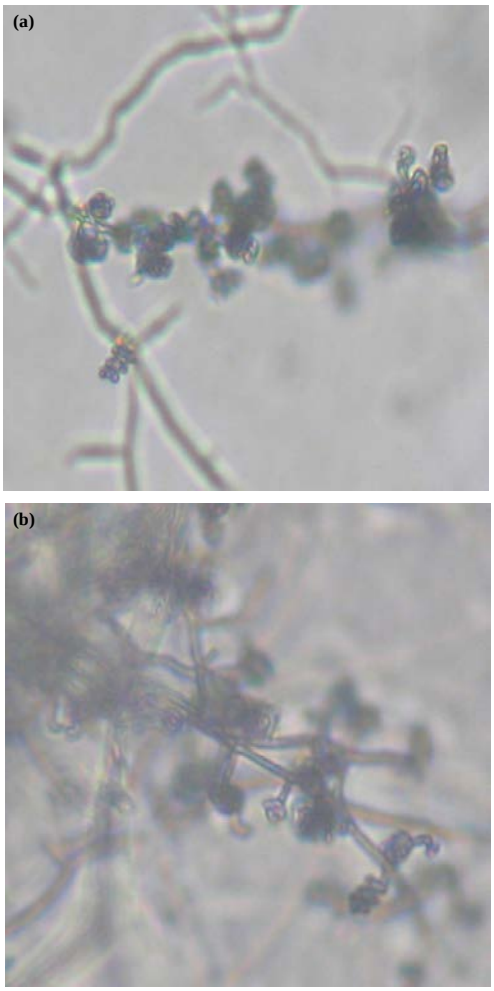

Fig. 3(a-b): Light microscopic micrograph of strain R58 showing substrate mycelium, aerial mycelium and spore chain forming coil on (a) ISP medium No.2 and (b) Humic acid vitamin agar

earlier reports where it was found that cultural filtrates contained active actinomycetes cell showed better inhibition than sterile filtrate (Suwan *et al*., 2012; Bibb, 2005; Prapagdee *et al*., 2008).

Morphological identification: The isolates R58 was inoculated on ISP medium No. 2 and humic acid vitamin agar. It has morphological character under microscopic and agar slant as shown in Fig. 3. The result showed that isolate R58 was aerobic, Gram-positive, non-acid alcohol-fast actinomycetes that forms extensively branched substrate mycelia. This strain produced brown aerial mycelium with longitudinal spirales-type spore chain and light black soluble pigment. The aerial spore color varied from white to gray. This result assigned strain R58 to genus *Streptomyces* Williams *et al*. (1989) and implied to *Streptomyces malaysiensis* (Al-Tai *et al*., 1999).

Molecular identification: The 16S rDNA sequence was generated for strain R58 (1416 nucleotides). Comparison of this nucleotide sequence with members of actinomycetes clearly showed that this strain belong to the genus *Streptomyces* (Fig. 4). The close relationship to *Streptomyces malaysiensis* is supported by both of treeing algorithms and a high bootstrap value (Fig. 4).

Application of strain R58 on chilli anthracnose: The culture of *S. malaysiensis* R58 and *C. gloeosporioides* chi was applied on the 3 month growth chilli pot, except

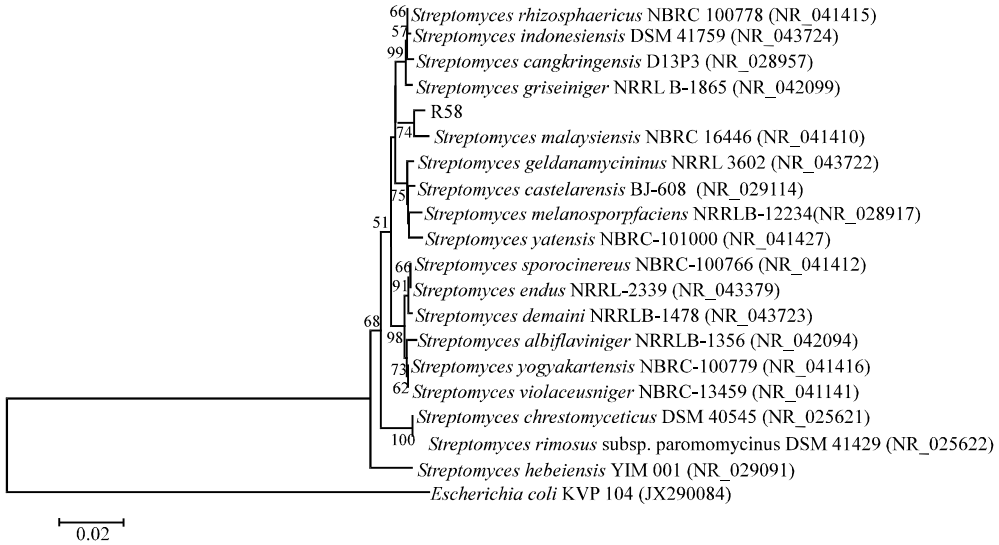

Fig. 4: Phylogenetic tree of nucleotide sequence analysis of 16S rDNA of strain R58 with related species *Streptomyces* constructed by Neighbor-joining method from MEGA4 program, the tree is rooted by the nucleotide sequence of *Escherichia coli* KVP104, scale bar shown distance values under the tree 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, the strain R58 indicated by bold letter

Fig. 5(a-b): Anthracnose disease on (a) Chilli plant and (b) Infected fruit when use *S. malaysiensis* as biological agent. chilli pots were treated with different treatments containing (T1) with *C. gloeosporioides* chi and without *S. malaysiensis* R58 and (T2) with *S. malaysiensis* R58 and *C. gloeosporioides* chi; (T3) with *S. malaysiensis* R58 and without *C. gloeosporioides* chi; (T4) control without *C. gloeosporioides* chi and *S. malaysiensis* R58

negative control as shown in Table 1. One week later, the effects of *C. gloeosporioides* chi and *S. malaysiensis* R58 on chilli pot were checked. The result showed that treatment 1 (T1) which inoculated *C. gloeosporioides* chi in the chilli pot revealed high level of symptoms of the chilli anthracnose on the chilli plants and fruit (Fig. 5a, b). Addition of *S. malaysiensis* R58 on treatment 1, the anthracnose of chilli plants and fruit could be significantly reduce as shown on treatment 2 (T2) in Fig. 5a and b. Moreover, addition of *S. malaysiensis* R58 on chilli pot has not negative effect on the chilli plants as shown in treatment 3. Treatment 3 (T3) and treatment 4 (T4), *C. gloeosporioides* chi were not added to chilli pot so the chilli plants and fruit did not show symptoms of the anthracnose disease. This indicated that *S. malaysiensis* R58 can be use to control anthracnose disease as the biological agent. Antibiosis is considered to provide in biological disease control because microorganism

can produce antibiotic rapidly and contact to the pathogenic fungi (Taechowisan *et al*., 2009). Prapagdee *et al*. (2008) used *Streptomyces hygroscopicus* for biocontrol of anthracnose disease in orchid to inhibit *C. gloeosporioides*. *Streptomyces* sp. SRM1 also applied for biological control for anthracnose disease of banana (Taechowisan *et al*., 2009). Several studies demonstrated many secondary compound produced by *Streptomyces* strain against plant fungal pathogens (Fguira *et al*., 2005). There are many reports that *Streptomyces* can produce chitinases and glucanase enzyme to lysis pathogenic fungal hyphae (Taechowisan *et al*., 2003).

CONCLUSION

The strain R58 has high efficiency against phytopathogenic fungus *C. gloeosporioides* chi. This strain produced thermostable bioactive compound on the culture broth against *C. gloeosporioides* chi and its culture can be use as biological agent to reduce anthracnose disease of chilli in the pot. Based on morphological character and its 16S rDNA sequence, the actinomycetes strain R58 is belonging to *Streptomyces malaysiensis*. Further study needs to be undertaken to analyze the mechanism for the antimicrobial activity of this bioactive compounds. It might be considered as a new candidate source for biological control agent.

ACKNOWLEDGMENTS

We thank Dr. Paiboon Viriyavathana, Faculty of Science and Technology for general supports. This research was supported by Institute of Research and Development Phranakhon Rajabhat University, Thailand.

REFERENCES

Al-Tai, A., B. Kim, S.B. Kim, G.P. Manfio and M. Goodfellow, 1999. *Streptomyces malaysiensis* sp. nov., a new streptomycete species with rugose, ornamented spores. Int. J. Syst. Bacteriol., 4: 1395-1402.

Arifuzzaman, M., M.R. Khatun and H. Rahman, 2010. Isolation and screening of actinomycetes from sundarbans soil for antibacterial activity. Afr. J. Biotechnol., 9: 4615-4619.

Atta, H.M. and M.S. Ahmad, 2009. Antimycin-A antibiotic biosynthesis produced by *Streptomyces* sp. AZ-AR-262: Taxonomy, fermentation, purification and biological activities. Aust. J. Basic Applied Sci., 3: 126-135.

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

Bibb, M.J., 2005. Regulation of secondary metabolism in streptomycetes. Curr. Opin. Microbiol., 8: 208-215.

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.

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

Fguira, L.F., S. Fotso, R.B. Ameur-Mehdi, L. Mellouli and H. Laatsch, 2005. Purification and structure elucidation of antifungal and antibacterial activities of newly isolated *Streptomyces* sp. strain US80. Res. Microbiol., 156: 341-347.

Intra, B., I. Mungsuntisuk, T. Nihira, Y. Igarashi and W. Panbangred, 2011. Identification of actinomycetes from plant rhizospheric soils with inhibitory activity against *Collectotrichum* spp. the causative agent of antracnose disease. BMC Res. Notes, Vol. 4. 10.1186/1756-0500-4-98

Khucharoenphaisan, K., U. Puangpetch, K. Puttaraksa and K. Sinma, 2011. Grouping of actinomycetes isolated from termites using biochemical character. J. Biol. Sci., 11: 314-319.

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., 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. Lai, N.K.I. Wong and C.C. Ho, 2002. Actinomyetes isolated from soil samples from the cocker range sabah. ASEAN Rev. Biodiversity Environ. Conserve, 9: 1-7.

Photita, W., P.W.J. Taylor, R. Ford, K.D. Hyde and S. Lumyong, 2005. Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. Fungal Diversity, 10: 117-133.

Prapagdee, B., C. Kuekulvong and S. Mongkolsuk, 2008. Antifungal potential of extracellular metabolites produced by *Streptomyces hygroscopicus* against phytopathogenic fungi. Int. J. Biol. Sci., 4: 330-337.

Rahman, M.A., T.H. Ansari, M.B. Meah and T. Yoshida, 2003. Prevalence and pathogenicity of guava anthracnose with special emphasis on varietal reaction. Pak. J. Biol. Sci., 6: 234-241.

Rajamanickam, S., K. Sethuraman and A. Sadasakthi, 2012. Exploitation of phytochemicals from plants extracts and its effect on growth of *Colletotrichum capsici* (Syd.) butler and bisby causing anthracnose of chilli (*Capsicum annuum* L.). Plant Pathol. J., 11: 87-92.

Rapatsa, J. and S. Terapongtanakorn, 2010. Some physiological measurements on growth, pod yields and polyamines in leaves of chili plants (*Capsicum annuum* cv. Hua Reua) in relation to applied organic manures and chemical fertilisers. Pak. J. Biol. Sci., 13: 263-270.

Reddy, N.G., D.P.N. Ramakrishna and S.V.R. Gopal, 2011. A morphological, physiological and biochemical studies of marine *Streptomyces rochei* (MTCC 10109) showing antagonistic activity against selective human pathogenic microorganisms. Asian J. Biol. Sci., 4: 1-14.

Shirling, E.B. and D. Gottlieb, 1966. Methods for characterization of *Streptomyces* species. Int. J. Syst. Evol. Microbiol., 16: 313-340.

Suwan, N., W. Boonying and S. Nalumpang, 2012. Antifungal activity of soil actinomycetes to control chilli anthracnose caused by *Collectotrichum gloeosporioides*. J. Agric. Technol., 8: 725-737.

Taechowisan, T., J.F. Peberdy and S. Lumyong, 2003. Isolation of endophytic actinomycetes from selected plants and their antifungal activity. World J. Microbiol. Biotechnol., 19: 381-385.

Taechowisan, T., N. Chuaychot, S. Chanaphat, A. Wanbanjob and P. Tantiwachwutikul, 2009. Antagonistic Effects of *Streptomyces* sp. SRM1 on *Colletotrichum musae*. Biotechnology, 8: 86-92.

Than, P.P., H. Prihastuti, S. Phoulivong, P.W.J. Taylor and K.D. Hyde, 2008. Chilli anthracnose disease caused by *Colletotrichum* species. J. Zhejiang Univ. Sci., 9: 764-778.

Usha R.J., N.H. Shenpagam and D.K. Devi, 2011. Antagonistic activity of actinomycetes isolates against human pathogen. J. Microbiol. Biotech. Res., 1: 74-79.

Williams, S.T., M. Goodfellow and G. Alderson, 1989. Genus *Streptomyces* Waksman and Henrici 1943, 339AL. 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.