The 45th
Congress on
Science and Technology of Thailand
การประชุมวิชาการวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย ครั้งที่ 45 (วทท45)

PROCEEDINGS BOOK

"Seedling Innovation
For Sustainable Development"
"ต้นกล้านวัตกรรมสู่การพัฒนาอย่างยั่งยืน"

7 – 9 October 2019
Mae Fah Luang University, Chiang Rai
THAILAND

Content

A_023_PF: DETECTION OF *atzB* GENE IN TROPICAL *Trichoderma harzianum* ISOLATED FROM ATRAZINE CONTAMINATED SOIL IN CENTRAL REGION OF THAILAND

Gun Anantasomboon[1]*, Intira Tampayak[2]
[1]Anatomy Unit,
[2]Microbiology Unit, Department of Medical Science, Rangsit University, Patumthani, Thailand
*e-mail: ananta_rsu@yahoo.com

Abstract: *Trichoderma* spp. is tropical fungus currently used as biological control agent due to their ability to antagonize other plant pathogenic fungi, as well as to degrade some agrochemicals. Among herbicides, atrazine, is intensively used in sugarcane, corn and sorghum fields. Due to the toxicity and persistence of atrazine in the environment, bioremediation using microorganisms have been studied to remove atrazine from contaminated soil and water. This study aimed to investigate the tropical *Trichoderma* spp. that may has unique capability in atrazine degradation or toleration. Fifty isolates of fungal strain from atrazine contaminated corn and sugar cane fields from Kampahaengphet, Nakornpathom and Ratchaburi provinces were cultured in modified medium agar containing 50 mg/L of atrazine for 15 days, then the *atzB* gene investigations of eight survival isolates were determined by using PCR analysis. All of eight survival isolates were finally identified for specific fungal stain by PCR and DNA sequencing analyses, respectively. The results showed that six fungus isolates were positive *atzB*-PCR analysis and classified as *Trichoderma harzianum*. Therefore, the selected strain of tropical *T. harzianum* from central region of Thailand may benefit for the agriculture and the global environment to reduce or to degrade atrazine in contaminated soil.

Introduction: Atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine) is a selective herbicide widely used in agricultural fields to control the emergence of broadleaf and grassy weeds in corn, sugar cane, sorghum, cotton, residential lawns, golf courses and other crops, which is moderately mobile and highly persistent in soil[1], surface water, groundwater and agricultural products. The persistence of this herbicide in environment is a serious problem for economy loss and health[2], as well as the limitation of biocontrol of plant diseases by some competitive bacteria and fungi. Effects of atrazine for health are risks for cancer formation, malformation of embryo and fetus, respiratory and digestive signs. In 2004, atrazine is a prohibited herbicide for the European Union[3]. In Thailand, atrazine has been imported and used for many decades. Annual cost of atrazine imports in the year 2013 is high as a top-ten of the world countries[4]. The reduction of atrazine is found to be influenced by the metabolic action of habituated microorganisms[5]. The usage of selective microorganisms for atrazine degradation is probably in a practical. Alabouvette[6] reported that some bacteria and fungi in temperate zone such as *Aspergillus*, *Rhizopus*, *Fusarium*, *Pseudomonas*, *Agrobacterium*, *Clavibacter*, *Arthrobacter* and *Escherichia coli*[5] have a capacity in atrazine destruction and reduction. Cai and co-workers[7] reported that the *Arthrobacter* sp. isolated from waste water unit in atrazine producing plant in China has a specific property in atrazine destruction and used atrazine as a nitrogen source for its growth. In the year 2010, mechanism for atrazine degradation in bacteria was firstly reported that it begins from the Hydrolytic dechlorination by using atrazine chlorohydrolase (AtzA), controlled by *atzA* (*trzA*) gene, followed by the Hydrolytic deamination processes by using Hydroxyl-atrazineethylamino-hydrolase (AtzB) and N-isopropyl-ammelide isopropyl-amino-hydrolase (AtzC), controlled by *atzB* (*trzB*) and *atzC* (*trzC*), respectively. After that, atrazine was transformed as cyanuric acid and finally changed to be non-toxic substances, CO_2 and NH_3[8]. *Trichoderma viridae* is known as a competitive fungus for control of plant diseases in temperate zone. It also found to degrade the atrazine rapidly[9]. However, there is lacking data of some tropical fungi, such as *Trichoderma* spp. that may contain an atrazine degradation property. This research aimed to

examine the *atzB* gene of atrazine-tolerant fungi that were isolated from contaminated agricultural fields in central region of Thailand. Selection of tropical *Trichoderma* spp. with atrazine degrade element is beneficial for plant disease control and environmental protection.

Methodology:

Fungal isolation from atrazine contaminated fields: Atrazine contaminated soils were collected from 6 corn and sugarcane fields in Kampahaengphet, Nakornpathom and Ratchaburi provinces. One kilogram of surface soil from each source was kept, filtered and preserved at 4 °C until used. Fifty mg of soil was hydrated with 25 ml sterile water. Then, the fungal culture was performed on Martins'agar plate with Dilution Plate Technique (0.5, $0.5x10^{-1}$, $0.5x10^{-2}$ ml) at 28 °C, for 5 days.

In vitro selection of atrazine tolerant fungus: Selection of each fungal isolates on the center of Czapek-dox agar (D-glucose 1% w/v), with atrazine (50 mg/L) at 28 °C, for 15 days. Identify the surviving fungus under microscope by its morphology and color of hypha[10].

analysis of atzB gene in atrazine tolerant fungus: Pick up hyphae of each isolate of atrazine tolerant fungus from the previous step into 1.5 ml microcentrifuge tube, add liquid nitrogen, lysis buffer and then extract for DNA[11]. DNA samples were preserved at -70 °C. Then, the PCR analysis with a specific primer set[12] (Table 1.) was applied to detect *atzB* gene fragment of atrazine tolerant fungus.

Table 1.Demonstrating the specific primers[12] for *atzB* gene examination of *Trichoderma* spp.

atzB-F primer: 5' TCACCGGGGATGTCGCGGGG 3'
atzB-R primer: 5' CTCTCCCGCATGGCATCGGG 3'

Identification of fungal stain: The atrazine tolerant isolates were sent to investigate for fungal stain at the First BASE laboratories Co. Ltd., Selangor, Malaysia.

Results and Discussion:

Fungal isolation from atrazine contaminated fields: Fungal isolation from 6 corn and sugarcane fields in Kampahaengphet, Nakornpathom and Ratchaburi provinces that were contaminated with atrazine revealed 50 isolates on Martins'agar plate at 28 °C, at 5 days (Figure1).

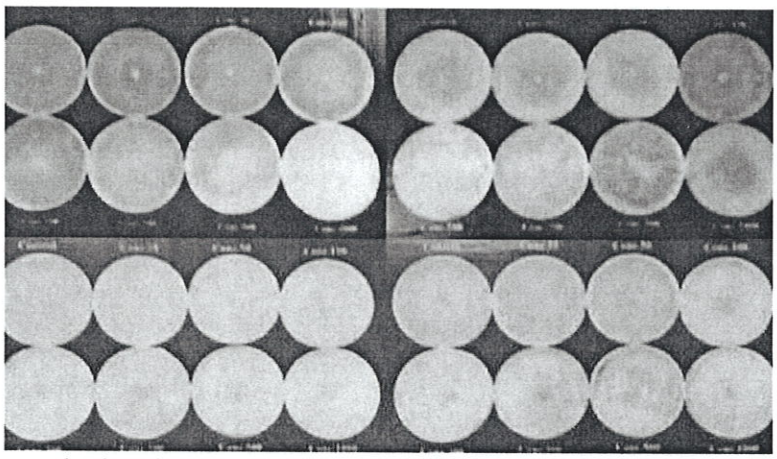

Figure 1. Photograph showing samples of fungal isolated from atrazine contaminated corn and sugarcane fields

In vitro selection of atrazine tolerant fungus: Selection of atrazine tolerant fungus was performed under an Enrichment Culture technique. Fungal isolates from the previous method were separately cultured on Czapek-dox agar with atrazine (50 mg/L), at 28°C. After 15 days of culture period, eight surviving fungal isolates were obtained. The tolerant isolates were fully growth on culture plates. Under microscopic observation, hyphae of all isolates were similar to *Trichoderma* spp.

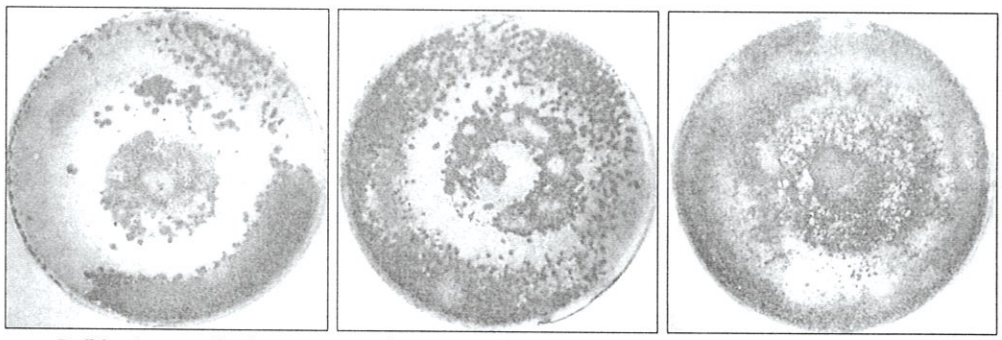

Figure 2. Photograph demonstrating sample of fungal isolates which are survive and tolerant from high concentration of atrazine (50 mg/L) on Czapek-dox agar for 15 days.

PCR analysis of atzB gene in atrazine tolerant fungus: Detection of *atzB* fragment of 8 isolates by using PCR and Agarose Gel Electrophoresis exhibited PCR product at 560 bp with isolates 1-6 and showed negative result with isolates 7-8 (Figure3). Result revealed the detection of *atzB* gene in 6 fungal isolates (lane 1-6).

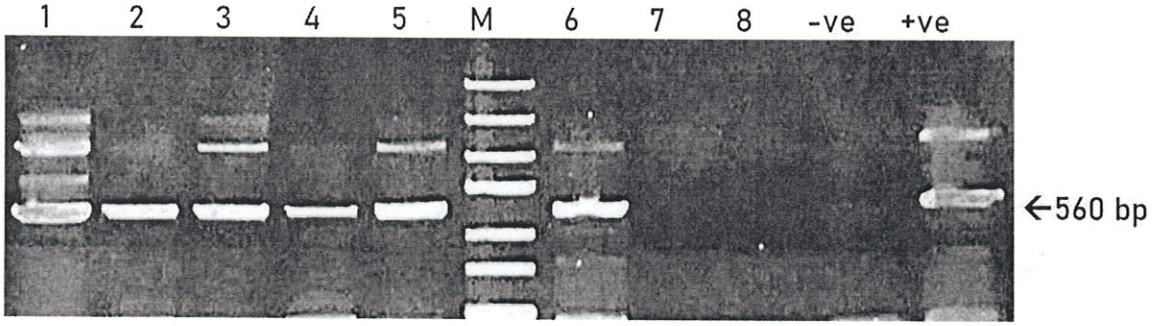

Figure 3. Gel-electrophoresis showing a result of *atzB* gene-PCR analysis of 8 isolates of fungus that are survive and tolerant to a high concentration of atrazine (50 mg/L) on Czapek-dox cultural plate. Positive *atzB*-PCR band (560 bp) was found with 6 isolates (lane 1-6), M = DNA ladder.

4. Identification fungal stain: Result from 18s rRNA fragment analysis revealed that the fungal isolates 1-6 (contains *atzB* gene) were *T. harzianum*, whereas isolates 7-8 were *T. asperellum.*

Conclusion: Since result on the positive PCR detection of *atzB* fragment with 6 isolates of tropical fungus that were survive and tolerant from a high concentration of atrazine (50 mg/L) in Czapek-dox medium with 15 days culture period, all 6 isolates (isolates 1-6) identity to *Trichoderma* spp. by gross morphology and color of hypha. The result correlated to a formation of clear zone during the day 1-5. It was different from the result of 2 isolates that were negative of *atzB*-PCR (isolates 7&8) and did not formation of clear zone. Another result

of fungal stain investigation revealed that all 6 isolates are *T. harzianum*. It is possible that the selected stain of *T. hazianum* from our study has an atrazine degrading property as be found in some bacteria. Normal process for atrazine degradation is involved by many enzymes including the Hydroxyl-atrazineethylamino-hydrolase (AtzB)[8,11]. This study demonstrates a method for selection of tropical *T. harzianum* which is tolerant to high concentration of atrazine *in vitro*. The selected isolates may be further used as a biocontrol of plant disease in atrazine contaminated fields[12,13], as well as for atrazine elimination form soil[14].

References:

1. Cohen SZ, Greege SM, Carsel RF, Enfield CG. Treatment and Disposal of Pesticide Wastes. 1984;p.297-325.[doi:10.1021/bk-1984-0259.ch018]
2. Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, Vonk A. Proceedings of the National Acad of Sci. 2002;99(8):5476-5480.
3. Sass JB, Colangelo A. Int J Occup Environ Health. 2006;12(3):260-267.
4. Tawatsin A, Thavara U, Siriyasatien P. Med Res Archiv. 2015;3:1-10.
5. Solomon RDJ, Kumar A, Santhi VS. J Zhejiang University SCIENCE B. 2013;14(12):1162-1172.
6. Alabouvette C, Olivain C, Steinberg C. European J of Plant Pathol. 2006;114(3):329-341.
7. Cai B, Han Y, Liu B, Ren Y, Jiang S. Letters in Appl Microbiol. 2003;36(5):272-276.
8. Sene L, Converti A, Secchi GAR, Simão RDCG. Brazilian Archives of Biol and Technol. 2010;53(2):487-496.
9. Muthuselvam M, Arunkumar S. J Pure and Appl Microbiol. 2009;3(2):661-666.
10. Howell CR. Plant Disease. 2003;87(1):4-10.
11. Chomczynski P, Sacchi N. Analy Biochem. 1987;162(1):156-159.
12. De Souza ML, Wackett LP, Sadowsky MJ. Appl Environ Microbiol. 1998;64(6):2323-2326.
13. Arbeli Z, Fuentes C. FEMS Microbiol Ecol. 2010;73(3):611-623.
14. Pelcastre MI, Ibarra JRV, Navarrete AM, Rosas JC, Ramirez CAG, Sandoval OAA. Tropical Subtropical Agroecosystems. 2013;16(2):265-276.

Acknowledgements: This study was supported by a research grant for permanent staffs (No.7/54) from the Research Institute, Rangsit University, Patumthani, Thailand. We would like to appreciate for this funding.