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The potential of fungi collected from earthworm gut and vermicompost producing auxin under tryptophan and non-tryptophan culture

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Abstract The earthworm gut consists of many microorganisms which are important to soil improvement. Fungi colonize in the gut of earthworm can produce some secondary metabolites. The result showed thirty-one fungal isolates could produce IAA under PDBt added 0.4% L-tryptophan (53.54 µg/mL) and without (24.05 µg/mL). Three highest IAA producing fungi were similar to *Fusarium solani (*Mpe7*)*, *Aspergillus terreus (*YC2*)* and *Trichoderma* sp. (RL2), respectively. The biological test revealed that the supernatant of isolates Mpe7, YC2 and RL2 can enhance the root number of *Vigna radiata* more than the non-trated control on day 7. The test demonstrated that the increasing of IAA concentration resulted to decrease the root length. It is concluded that the fungi isolated from earthworm guts and developed products can produce IAA with 0.4% L-tryptophan more than without L-tryptophan and enhanced the root growth of *V. radiata.*

Keywords: Earthworm, Auxin, Secondary metabolite, Vermicomposting liquid, Vermicompost

Introduction

Earthworm gut consists of many microbial species (Gate, 1939). More than 343 species were identified and reviewed by Tancho (2013). The microorganisms were spread by earthworm associated processing into the soil via excreting vermicast (Szlavecz *et al*., 2018; Chang *et al*., 2017). They can improve soil fertility and stimulate plant growth and activities (Schmidt *et al*., 2019).

Perionyx sp. 1 is one of the terrestrial earthworms which has also been cultured and studied at Natural Agriculture Research and Development Center, Maejo University, Chiang Mai, Thailand. For decades, this strain has been spreading throughout Thailand and nearby countries. Previously, a researcher from our station studied them due to their high efficiency to

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convert the wastes decomposition (Vermicompost) through gut associated processing microorganism (Tancho, 2013). Interestingly, it did not only make vermicompost, but also produced the brown liquid (vermicomposting liquid) by microbial degradation activities through the alimentary tract processing. Consequently, it is a premium earthworm liquid production for plant growth and soil improvement.

Formerly, some studies from our research indicated that some bacteria from the product can produce a high amount of indole-3-acetic acid (Arraktham *et al*., 2016). However, the test of potential fungi which collected from earthworm gut has less information.

The objective was to apply *in vitro* for measuring total IAA from fungal strains collected from earthworm guts and some products (vermicompost and vermicomposting liquid) with 0.4% L-tryptophan to test for their bioactivity in early growth of mung bean seed germination.

Materials and methods

Fungal isolation and fungal identification

Three gut of earthworms *(Metaphire peguana, M. Posthruma* and *Perionyx* sp.1) and vermicompost were collected. They were diluted (10^{-3}, 10^{-4}, 10^{-5}) and spread on potato dextrose agar (PDA) (pH 5.6-6.0) with 30% streptomycin and yeast malt agar (YMA) (pH 4.0) with rose bengal and 30 % cholemphenicol. After five days, the different fungal colonies were isolated until getting pure cultures, and maintained in potato dextrose agar (PDA). The fungal isolates were transferred in 15% glycerol and frozen in -20 ^{o}C refrigerator. After fungal cuture in potato dextrose broth (PDBt), the fungal colony was cut at peripheral colony and transferred to 1.5 ml tube for DNA extraction kit (BIO-HELIX) after 3-day incubation. All cell wall was destroyed using Lyticase enzyme (10,000 Unit/µl) at 60 ^{o}C by dry heat box. Then, mycelium was moved into the tube of DNA extraction. DNA was eluted through TE buffer as 100 µl and stored at -20 ^{o}C. Each DNA sample was measured for the quality and quantity via nanodrop. For identification, the PCR reaction using ITS1 (5 pmol/µl) and ITS4 (5 pmol/µl) primer were prepared. In PCR reaction, added 5 µl of genomic DNA + 25 µl one PCR + 1 µl forward primer + 1 µl reverse primer + 23 µl deionized water were done. All samples were set for PCR steps starting by 96 ^{o}C for 1 min for pre-denaturation, 35 cycles; denaturation (96 ^{o}C, 1 min), annealing (55 ^{o}C, 2 min), extension (96 ^{o}C, 1 min). The PCR product was checked in 2.0% agarose gel electrophoresis, and afterward cleaned up the PCR product before DNA sequencing at Macrogen company, Korea. Finally, aligning the nucleotides and blast sequence with NCBI database was confirmed the fungal species.

IAA measurement and biological test

Auxin production by fungi, both with and without 0.4 % L-tryptophan, was determined colorimetrically in terms of IAA equivalents (Sarwar and Frankenberger Jr., 1992). 14 day-old fungal cultures were grown in darkness at 35 degree C in PDBt. The supernatant was then kept by filtration via sterile straining cloth in to a sterile tube. Three milliliters of supernatants were mixed with 2 mL Salkowski's reagent (12 gL^{-1} $FeCl_3$ in 429 mL L^{-1} H_2SO_4). Mixtures were incubated at room temperature for 30 min for color development, and absorbance was messured at 530 nm using a spectrophotometry. Auxin concentration produced by fungal isolates was determined using a standard curve for IAA prepared from serial dilutions of 0.1-1,000 µg/ml. In the preliminary test, preparing all standard IAA solution as 1 µg/ml, 10 µg/ml, 100 µg/ml, 1,000 µg/ml and mung green seed for germination test. Seeds were sown directly in plastic box containing five seeds in three replicas. Pure water was sprayed on all seeds eventually. After one day seedling, 1 cm-long roots were cut from their tip and treated with fungal supernatant for 7 days. All root number and length were measured before analysing the variance (ANOVA).

Results

The result showed 73 fungal isolates which could produce IAA when treated with 0.4% L-tryptophan and without. Only 31 fungal isolates (YC2, YC4, YC5, RY3, YY2, RD4, RD5, YD10, RK3, RK4, RK7, RK8, YL2, RL2, RC1, RC2, RC5, RC6, RC9, VL3, Pe4, Pe7, MG4, MG5, MG6, MG8, Mpe3, Mpe6, Mpe7 MP4, MP5) produced IAA when cultured in PDBt added 0.4% L-tryptophan (average 53.54 µg/mL) more than two times (average 24.05 µg/mL), all of which, Mpe7, YC2 and RL2, were the three highest produced IAA as 170.41 µg/mL, 182.00 µg/mL and 151.33 µg/mL, respectively (Table **1)**. The *ITS1* region was used to identify three fungal isolates, and the NCBI database showed that isolate Mpe7 is similar to *Fusarium solani* (KX064991.), YC2 is similar to *Aspergillus terreus (*KM924436.1*)* and RL2 is similar to *Trichoderma* sp. (MK871174.1), respectively (Table 2). In mung bean germination, the pure IAA treated with mung bean seeds showed statistical difference from supernatant of Mpe7, YC2, RL2 both the root number and root length. On pure IAA, it revealed the increses of root number followd by concentration opposite to the root length. The supernatant of Mpe7, YC2, RL2 showed the fluctuation (Figure 1, Table 3).

Table 1. The comparison of the amount of IAA concentration from seventy-three fungal isolates cultured in PDBt with 0.4% L-tryptophan added and without

fungal isolate	without 0.4% L-tryptophan (µg/ml) ± SD	added 0.4% L-tryptophan (µg/ml) ± SD	fungal isolate	without 0.4% L-tryptophan (µg/ml) ± SD	added 0.4% L-tryptophan (µg/ml) ± SD
YC1	23.79±0.024^{d}	25.84±0.002^{f}	RC8	31.89±0.006^{b}	16.71±0.013^{h}
YC2	32.00±0.001^{b}	182.00±0.010^{a}	RC9	16.51±0.013^{c}	87.64±0.121^{c}
YC3	28.30±0.010^{c}	22.25±0.006^{g}	VL1	24.36±0.004^{d}	35.02±0.483^{e}
YC4	20.66±0.001^{d}	132.20±0.023^{b}	VL2	31.33±0.003^{b}	10.87±0.005^{i}
YC5	22.25±0.001^{d}	94.66±0.005^{bc}	VL3	31.18±0.006^{b}	150.92±0.42^{ab}
YC6	24.51±0.012^{d}	19.54±0.003^{h}	VL4	37.33±0.012^{a}	31.48±0.014^{f}
YC7	23.64±0.002^{d}	19.28±0.004^{h}	Pe1	24.05±0.025^{d}	12.56±0.003^{i}
YC8	22.25±0.006^{d}	19.54±0.001^{h}	Pe2	30.97±0.004^{b}	52.36±0.011^{de}
YC9	14.77±0.005^{c}	17.38±0.003^{h}	Pe3	20.46±0.006^{d}	33.54±0.09^{e}
YC10	22.87±0.008^{d}	26.25±0.005^{f}	Pe4	21.43±0.005^{d}	138.46±0.139^{b}
YC11	21.48±0.020^{d}	4.51±0.005^{k}	Pe5	31.79±0.005^{b}	19.53±0.026^{h}
RY1	21.64±0.017^{d}	13.79±0.008^{i}	Pe6	32.15±0.009^{b}	1.84±0.003^{k}
RY2	17.02±0.007^{e}	7.74±0.013^{j}	Pe7	31.89±0.006^{b}	139.69±0.278^{b}
RY3	25.48±0.003^{c}	107.43±0.05^{bc}	MG1	35.43±0.006^{a}	6.97±0.017^{j}
YY2	17.79±0.006^{e}	93.84±0.006^{bc}	MG2	3.18±0.003^{h}	8.05±0.001^{j}
RD4	24.25±0.013^{d}	124.87±0.042^{b}	MG3	22.25±0.014^{d}	36.35±0.006^{e}
RD5	7.54±0.010^{g}	86.15±0.004^{c}	MG4	30.61±0.004^{b}	82.00±0.722^{c}
YD10	23.84±0.005^{d}	100.10±0.18^{bc}	MG5	21.02±0.002^{d}	42.66±0.003^{e}
RK2	22.82±0.010^{d}	32.56±0.002^{e}	MG6	23.54±0.005^{d}	63.43±0.006^{d}
RK3	21.54±0.005^{d}	49.54±0.008^{de}	MG7	22.46±0.008^{d}	19.23±0.001^{h}
RK4	26.87±0.003^{c}	136.46±0.002^{b}	MG8	8.77±0.001^{g}	140.15±0.281^{b}
RK7	26.51±0.009^{c}	56.92±0.003^{de}	Mpe1	24.46±0.014^{d}	21.22±0.036^{g}
RK8	23.95±0.009^{d}	99.59±0.003^{bc}	Mpe2	27.07±0.025^{c}	30.56±0.037^{f}
YK4	28.97±0.007^{c}	14.82±0.005^{i}	Mpe3	20.61±0.007^{d}	96.25±0.142^{bc}
YL1	22.66±0.005^{d}	16.61±0.001^{h}	Mpe4	22.51±0.003^{d}	18.46±0.008^{h}
YL2	18.36±0.007^{e}	36.25±0.001^{e}	Mpe5	23.13±0.004^{d}	30.35±0.005^{f}
YL3	29.48±0.012^{c}	30.15±0.003^{f}	Mpe6	26.82±0.002^{c}	70.66±0.041^{d}
RL1	18.87±0.004^{e}	23.02±0.003^{g}	Mpe7	23.79±0.005^{d}	170.41±0.217^{a}
RL2	17.13±0.007^{e}	151.33±0.004^{ab}	Mpe8	26.05±0.008^{c}	9.43±0.003^{j}
RL3	23.74±0.003^{d}	25.23±0.026^{f}	Mpe9	28.72±0.003^{c}	28.92±0.003^{f}
RC1	30.97±0.006^{b}	78.66±0.001^{cd}	MP1	21.48±0.012^{d}	20.56±0.005^{g}
RC2	27.84±0.003^{c}	72.05±0.006^{d}	MP2	21.95±0.007^{d}	24.86±0.004^{g}
RC3	21.23±0.006^{d}	4.46±0.004^{k}	MP3	27.59±0.015^{c}	20.15±0.006^{g}
RC4	21.84±0.013^{d}	7.12±0.116^{j}	MP4	20.92±0.006^{d}	43.02±0.009^{e}
RC5	24.30±0.011^{d}	73.74±0.003^{d}	MP5	24.10±0.010^{d}	125.74±0.012^{b}
RC6	30.77±0.003^{b}	91.43±0.002^{c}	MP6	19.89±0.100^{e}	18.35±0.007^{h}
RC7	31.95±0.003b	14.25±0.002^{i}	average	24.05	53.54

SPSS statistic test at $P < 0.05$ (Duncan)

Table 2. Three fungal isolates identification

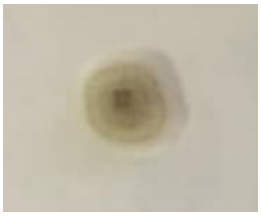
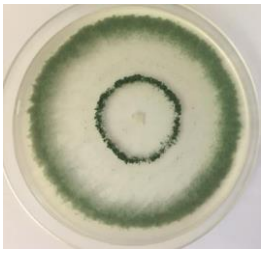
Isolate	ITS1 identification	Accession	Identity
Mpe7	*Fusarium solani*	KX064991.	99.00%
			
YC2	*Aspergillus terreus*	KM924436.1	89.00%
			
RL2	*Trichoderma* sp.	MK871174.1	93.31%
			

Table 3. Preliminary test of green bean germination with IAA fungal supernants

Treatment	IAA concentration (μg/ml)	root number ± SD	root length ± SD (mm)
A	0.00	0.67 ± 0.57^{f}	2.26 ± 0.75^{e}
B	1,000.00	31.67 ± 4.16^{a}	15.56 ± 0.7^{d}
C	100.00	9.33 ± 1.15^{c}	21.03 ± 0.90^{b}
D	10.00	7.33 ± 2.51^{cd}	21.13 ± 0.24^{b}
E	1.00	3.67 ± 1.15^{d}	30.03 ± 0.57^{a}
Mpe7 (F)	170.41	25.00 ± 1.00^{b}	20.63 ± 0.74^{cd}
YC2 (G)	182.00	4.33 ± 0.57^{e}	0.93 ± 0.20^{f}
RL2 (H)	151.33	3.67 ± 1.52^{d}	14.23 ± 1.0^{d}

SPSS statistic test at $P < 0.05$ (Duncan)

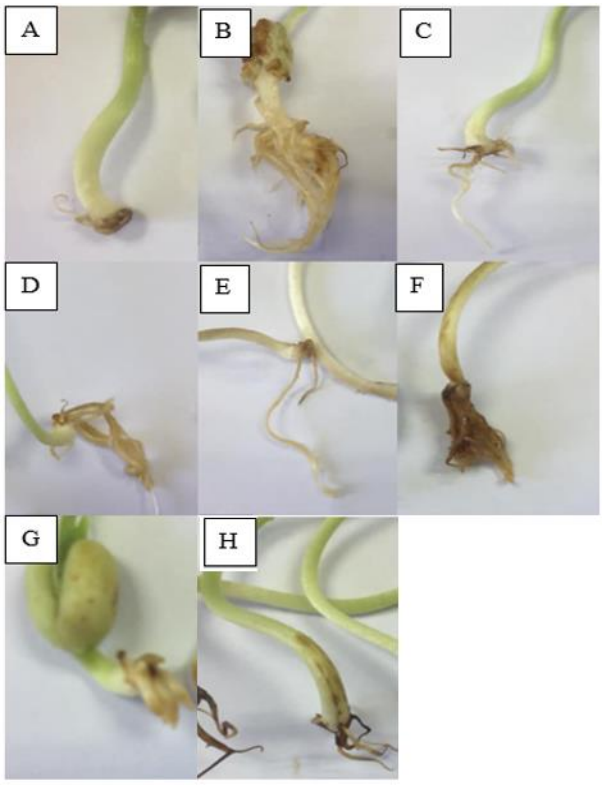


Figure 1. The test fungal supernatants and pure IAA on green bean germination. A = Control (sterile water), B – E = 100% IAA (1,000 µg/ml, 100 µg/ml, 10 µg/ml and 1 µg/ml respectively), F – I = Fungal supernatant (F = Mpe7, G = YC2, H = RL2)

Discussion

This study demonstrated that the seventy-three fungal isolates could produce indole-acetic acid (IAA). 31 out of 73 fungal isolates could produce IAA added 0.4% L-tryptophan more than twice without (YC2, YC4, YC5, RY3, YY2, RD4, RD5, YD10, RK3, RK4, RK7, RK8, YL2, RL2, RC1, RC2, RC5, RC6, RC9, VL3, Pe4, Pe7, MG4, MG5, MG6, MG8, Mpe3, Mpe6, Mpe7 MP4, MP5) (Table 1). This study is similar to Bilkey *et al*. (2010); Patil *et al*. (2011); Zhao and Zhong (2013) and Naveed *et al*. (2014) describing that some fungus used L-tryptophan to produce indole-3-pyruvic acid in the step of L-tryptophan pathway (Tsavkelova *et al*., 2007). the present study, it revealed that three fungus out of all was the highest IAA producing fungi which are Mpe7, YC2, RL2 as 170.41 µg/ml, 182.00 µg/ml and 151.33 µg/ml, respectively. The NCBI data base (https://www.ncbi.nlm.nih.gov/nucleotide/) indicated that Mpe7 isolate as *Fusarium solani (*KX064991*.)*, YC2 was similar to *Aspergillus terreus* (KM924436.1) and RL2 closed to *Trichoderma* sp. *(*MK871174.1*)* ,

respectively (Table 2). Our result is similar to Yadav *et al.* (2011) which explained that *Aspergillus niger*, *Trichoderma harzianum* and *Penicillium citrinum* was the best IAA secretion as 85, 65 and 52 µg/ml, respectively. Moreover, Uniyai *et al*. (2016) argued that *Aspergillus niger*, *A. flavus* and *Penicillium cotrinum* cultured in PDBt could produce a few IAA as 82, 67 and 61 µg/ml, respectively. In addition, Bilkey *et al*. (2010) found that fungi in genus *Aspergillus* is the best fungi producing IAA cultured in Czapek-Dox broth added 0.1% L-tryptophan. In the germination test, it indicated that pure IAA can stimulate the increase of the root number ($P < 0.05$) from 1 µg/ml (3.67), 10 µg/ml (7.33), 100 µg/ml (9.33) and 1,000 µg/ml (31.67), respectively. In term of length, it found that the high concentration effect to shorter root than higher (Figure 1, Table 2). The result is related to Padmavathi *et al*. (2015) which demonstrated that1 mg/ml of IAA could stimulate the length as 1.00 cm less than 5 mg/ml as 0.40 cm. However, the IAA from supernatant from the selected fungi showed unexpected data. It may occur from the supernatant consisting of many substrances.

It can be concluded that fungi from the earthworm gut, Vermicompost and Vermicomposting, liquid has more efficiency to produce IAA, especially to the fungi in genus *Fusarium*, *Aspergillus* and *Trichoderma* which is the highest ability to produce IAA under 0.4% L-tryptophan. Hence, this is important data for improving our product under the Research and Development Natural Agriculture, Maejo University.

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