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Original Article

Effect of *Streptomyces* sp. St1 on growth of and potential to stimulate anthracene removal by sunn hemp (*Crotalaria juncea*) grown in anthracene-contaminated soil

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Abstract

This study was performed to investigate the ability of *Streptomyces* sp. St1 that has indole-3-acetic acid (IAA) production and phosphate solubilizing ability on the growth and remediation efficiency of sunn hemp cultivated in 202.9 mg/kg of anthracene-contaminated soils. *Streptomyces* sp. St1 immobilized in rice straw and coconut husk could increase sun hemp growth. On day 36 of the experiment, shoot length, shoot fresh weight, shoot dry weight, root fresh weight and total chlorophyll content in sunn hemp leaves were around 41.2-44.4 cm, 2.81-3.45 g, 0.44-0.53 g, 0.46-0.55 g and 47.4-79.2 mg/ml, respectively, in soil amended with *Streptomyces* sp. St1 immobilized in rice straw and coconut husk. Sunn hemp cultivated in anthracene-spiked soil without *Streptomyces* sp. St1 inoculation died at 36 d after germination. Additional immobilized cells+spores of *Streptomyces* sp. St1 in rice straw and coconut husk in soil planted with sunn hemp maintained the cell density to a better extent than the free cells+spores. However, both free and immobilized cells+spores of *Streptomyces* sp. St1 did not improve anthracene removal from the soil with or without sunn hemp planting.

Keywords: anthracene, *Crotalaria juncea*, phytoremediation, plant growth promoting bacteria

1. Introduction

Anthracene is a polycyclic aromatic hydrocarbon (PAH), which is mainly dispersed in the environment from industrial use as a diluent for wood preservatives and manufacture of dyes and pigments. Even though anthracene is not carcinogenic or mutagenic to humans, it is one of the priority pollutants listed by the US EPA (Abdel-Shafy & Mansour, 2016). Anthracene toxicity is a skin sensitizer, eye irritation, causes nausea, vomiting and confusion (Igwe & Ukaogo, 2015). The level of anthracene contamination in the environment was variable. For example, the mean concentration of anthracene in the top soil of petroleum pollution sites on the Loess Plateau, located in the north-central part of China, was 272.76 µg/kg (Wang, Ma, Li, & Zhang, 2018) and the anthracene concentration in the soils collected around the coal spoil piles in Yangquan coal mine, located in the Shanxi Province, China, was 7,710 µg/kg (Li, Zhang, Gao, Qi, & Wang, 2019). However, the available data about anthracene and other PAHs contamination in the environment of Thailand remains deficient, despite various sources of PAHs contamination having been observed (Abdel-Shafy & Mansour, 2016).

Rhizodegradation is a technique of using a plant to stimulate the bioremediation of organic pollutants by soil microorganisms (Hussian *et al.*, 2018). Successful rhizodegradation is dependent on the desirable growth of the plant root system at the contaminated site, as the growth of the root will provide a habitat and metabolic activity for the soil

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microorganisms (Hussian *et al.*, 2018). However, various plants were sensitive to the petroleum hydrocarbon component, including PAHs (Somtrakoon & Chouychai, 2013). Exposure to PAHs can cause stress in the plant (Song *et al.*, 2011). Under stressful conditions, plant growth is usually inhibited and decreased plant biomass is also detected (Gerhardt, Huang, Glick, & Greenberg, 2009). Other stress conditions for the plants at contaminated sites could be low soil fertility (Gerhardt, Gerwing, & Greenberg, 2017) and inadequate microbial populations to promote plant growth or microbial degradation (Hussian *et al.*, 2018). One way to improve the plant ability to defeat environmental stress and grow in the contaminated site is by using plant growth promoting bacteria (Gerhardt *et al.*, 2009). Possible mechanisms of plant growth promoting bacteria to encourage organic pollutant phytoremediation include synthesis of plant growth regulators and specific compounds (enzymes, biosurfactants, siderophores, organic acids and antibiotics), which are accountable for phytopathogens elimination, improved nutrient uptake and tolerance to abiotic stresses (Hou *et al.*, 2015; Yadav *et al.*, 2018). The synergistic effects of plant growth promoting bacteria lead to organic pollutant phytoremediation in contaminated sites (Hong, Ryu, Kim, & Cho, 2011).

Most studies usually inoculate plant growth promoting bacteria in the form of free cells (Hong *et al.*, 2011; Hou *et al.*, 2015). However, the inoculation of a free cell bacterial suspension into soil without a suitable carrier usually rapidly decreases the bacterial population in the soil environment. This may be due to the unprotected bacterial inoculum not being able to compete with indigenous bacteria, so they cannot endure the predation by other soil microorganisms (Bashan, de Bashan, Prabhu, & Hernandez, 2014). Thus the aim of this study was to investigate the ability of free cells+spores and immobilized cells+spores of *Streptomyces* sp. St1 (97% similarity to *Streptomyces niveoruber* St1 based on 16S rDNA gene sequences) in rice straw or coconut husk to stimulate the growth of sunn hemp grown in anthracene-contaminated soil. *Streptomyces* sp. St1 is anthracene tolerant and has plant growth promoting activity with the ability to solubilize phosphate and produce IAA (approximately 15 mg/l when cultured on a half formula of potato dextrose broth + 0.1 mg/l of tryptophane for 20 d) (Somtrakoon, Sangdee, & Chouychai, 2019). The materials used for immobilizing cells+spores of *Streptomyces* sp. St1 were rice straw and coconut husk because these materials are cheap and naturally abundant. Both rice straw and coconut husk have been reported to be high quality carriers of immobilized microbial cells for hydrocarbon degrading cells (Zhang, Shang, Zheng, & Zhong, 2016) and phenol degrading cells (Shazryenna *et al.*, 2014), respectively. The advantage of immobilizing cells in a natural carrier is to maintain the microbial cell density in the environment and maintain the long-term activity of the bacterial cells (Zhang *et al.*, 2016). Sunn hemp was used as a model plant in this study because it is a cover crop in tropical countries, and it produces plant biomass and nitrogen within a short period of growth (Balkcom, Massey, Mosjidis, Price, & Enloe, 2011). Sunn hemp has been used in the phytoremediation of zinc and cadmium (Stanbrough, Chuaboonmee, Palombo, Malherbe, & Bhave, 2013). Sunn hemp is interesting for anthracene removal in this study for several reasons. Firstly, sunn hemp has never been used in PAHs phytoremediation. Moreover, sunn hemp belongs to the Fabaceae, and root exudates of plants in this family have been previously reported to stimulate PAH degradation by increasing the PAH availability and have been shown to stimulate PAH removal by rhizodegradation processes rather than via phytoaccumulation (Hall, Soole, & Bentham, 2011).

2. Materials and Methods

2.1 Preparation of anthracene-contaminated soils

Soil without a previous history of PAHs contamination was collected from Nakhonsawan Rajabhat University Yanmatsri, Payuhakiri District, Nakhon Sawan Province, Thailand, and air dried at room temperature (30-35 ºC) for 4-5 days until a constant dry weight of the soil was achieved. The physical and chemical characteristics of the soil were analyzed at the Central Laboratory (Thailand) Co., Ltd. This soil contained 39.72 mg/kg available potassium, 7.66 mg/kg available phosphorus, 0.2% total nitrogen, 1.82% soil organic matter and pH 6.79. The texture of the soil was sandy loam (64.9% sand, 29.97% silt and 5.08% clay).

The soil was subject to autoclaving at 121 ºC for 15 min before being used to prepare the anthracene-contaminated soil or uncontaminated soil. To prepare anthracene-contaminated soil, the soil was mixed with anthracene (Sigma Aldrich, Germany) using the method described in Somtrakoon, Chouychai, and Lee (2018). Firstly, 200 mg of anthracene was dissolved in 50 ml of dichloromethane. The anthracene solution was poured into 1 kg dry weight of soil and mixed thoroughly to give anthracene-contaminated soil. Then, this soil was left under a fume hood until the dichlromethane was completely evaporated. Each pot of anthracene-contaminated soil was prepared separately, and samples of these soils were randomly collected from the pot to determine the initial concentration of anthracene in the soil. The initial anthracene concentration in the soil was 202.09 mg/kg. The soil used in the experimental control pot was prepared according to the same method but only dichloromethane was added to the soil.

2.2 Preparation of sunn hemp seedlings

Seeds of sunn hemp were obtained as commercial seeds from Rai Kaset Ta Yai, Nakhon Rachasima Province, Thailand. Surface sterilization of the sunn hemp seeds were performed before planting (sunn hemp seeds were soaked in 0.6% sodium hypochlorite for 5 min and rinsed with sterilized distilled water for 1 min, three times). Then, five surface sterilized sunn hemp seeds were planting in each experimental pot (pot containing anthracene-contaminated soils or uncontaminated soil). After seed germination, only one healthy seedling with comparable size was allowed to grow in each experimental pot (one seedling in one experimental pot). The other seedlings in each experimental pot were pulled out and removed.

2.3 Preparation of free cells+spores and immobilized cells+spores of *Streptomyces* sp. St1

Streptomyces sp. St1 was isolated from soil in

Kosumpisai District, Mahasarakham Province, Thailand. The colony morphology, mycelium and spore chain of *Streptomyces* sp. St1 are showed in Figure 1. The *Streptomyces* sp. St1 culture was maintained in a half formula of PDA and incubated at 37 ºC for 48 hrs, and then transferred to a culture plate to incubate at room temperature for 12 days. To prepare free cells+spores of *Streptomyces* sp. St1, sterilized 0.85% NaCl was added into the culture plate of 14 d old *Streptomyces* sp. St1, and the cells+spores of *Streptomyces* sp. St1 were scrapped from the colony surface with a sterilized loop. Then, the cells+spores suspension of *Streptomyces* sp. St1 was pipetted from the culture plate to sterilized containers and this cells+spores suspension was used as the inoculum for the cells+free spores. To prepare the rice straw- and coconut husk-immobilized cells+spores of *Streptomyces* sp. St1, rice straw or coconut husk was cut into small pieces (5x5 cm) and sterilized by autoclaving at 121 ºC for 15 min. Small pieces of rice straw or coconut husk were cooled to room temperature and soaked in the cells+spores suspension of *Streptomyces* sp. St1 for 3 h to give immobilized cells+spores of *Streptomyces* sp. St1.

2.4 Experimental design

The experiment was performed under a completely randomized design with five treatments for the study using *Streptomyces* sp. St1 to stimulate the growth of sunn hemp (uncontaminated soil and anthracene-contaminated soil without bacterial inoculum, anthracene-contaminated soil with bacterial inoculum as free cells+spores, immobilized cells+spores in rice straw or coconut husk), with each treatment performed as seven replicates. Eight treatments to test the ability of *Streptomyces* sp. St1 to stimulate the removal of anthracene from soil (unplanted soil without bacterial inoculum, unplanted soil with bacterial inoculum as free cells+spores, immobilized cells+spores in rice straw or coconut husk, planted soil without bacterial inoculum, planted soil with bacterial inoculum as free cells+spores, immobilized cells+spores in rice straw or coconut husk), with each treatment performed as seven replicates. The first day of the experiment was determined to be the first day of sunn hemp germination.

Suun hemp seedlings were grown in experimental pots containing 1 kg of anthracene-contaminated soil for 36 days. Free cells+spores were poured onto the experimental pot on the 1st and 24th days after seed sowing (final concentration of viable cells after plating on half formula of PDA was 10^6 cfu/g of soil). In addition, 7 g of rice straw-immobilized cells+spores and coconut husk-immobilized cells+spores were placed on the surface of each experimental pot on the 1st day after seed sowing (final concentration of used cells after plating on half formula of PDA was 10^6 cfu/g of rice straw or coconut husk). Re-inoculation of immobilized cells+spores was performed on the 24th day after seed sowing, when free cells+spores were slowly poured onto the rice straw or coconut husk that had already been overlay on the 1st day in the experimental pot. Autoclaved water was poured into the experimental pot every day to maintain the soil moisture content. Plant and soil samples were collected on day 12 and 36 of the experiment for analysis of the plant growth parameters (shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, root dry weight, number of nodules per plant, flowering, chlorophyll content in leaves) and anthracene remaining in the soil. The number of total bacteria which similar to *Streptomyces* sp. cells in the soil of each treatment was also counted on half formula of PDA by the spread plate technique.

Figure 1. Colony morphology (A) and spore chain (B) of *Streptomyces* sp. St1 when cultured on arginine glycerol mineral agar for one week

2.5 Analysis of anthracene remaining in soil

The extraction of the anthracene from soil and the condition of the anthracene analysis was done by the method described in Somtrakoon *et al.* (2018). Briefly, the 1 g soil sample was mixed with anhydrous sodium sulfate and the anthracene in the dry soil sample was extracted via Soxhlet apparatus. Then the volume of the anthracene extract was reduced to 1 ml by rotary evaporator and the extract was subject to gas chromatography – flame ionization detector (Shimadzu, Model GC-2014) for anthracene analysis. The GC column was a DB-5MS (Agilent Technologies, USA) (30 x 0.25 mm x 0.25 μm).

2.6 Statistical analysis

One-way ANOVA and Least Square Difference (LSD) were used for variance analysis and pairwise comparison. Microsoft Excel was used to analyze these data.

3. Results and Discussion

3.1 Growth of sunn hemp grown in anthracene-contaminated soil

Based on the results from the 36th day of the experiment, anthracene seemed to be toxic to the shoot growth of sunn hemp. The shoot length and shoot fresh weight of sunn hemp grown in uncontaminated soil were higher than the shoot length and shoot fresh weight of sunn hemp grown in anthracene-contaminated soil without receiving *Streptomyces* sp. St1 (Table 1, Figure 2A, 2B). The shoot length, shoot fresh weight and shoot dry weight of sunn hemp grown in uncontaminated soil were 43.4±1.8 cm, 2.09±0.23 g, and 0.38±0.03 g, respectively. The shoot length, shoot fresh weight and shoot dry weight of sunn hemp grown in anthracene-contaminated soil without receiving *Streptomyces* sp. St1 were 34.4±1.4 cm, 0.56±0.06 g and 0.27±0.05 g, respectively. Moreover, the shoot length, shoot fresh weight and shoot weight of sunn hemp grown in anthracene-contaminated soil when receiving free cells+spores of *Streptomyces* sp. St1 had less growth than that grown in

anthracene-contaminated soil without receiving any *Streptomyces* sp. St1; however, the sunn hemp did not die and the plant health looked better than that grown in anthracene-contaminated soil without receiving any *Streptomyces* sp. St1 (Figure 2C). Actually, free cells+spores of *Streptomyces* sp. St1 should promote the growth of sunn hemp, but the reasons for this observation are not known. Immobilized cells+spores of *Streptomyces* sp. St1 in rice straw and coconut husk could increase the shoot length, shoot fresh weight and shoot dry weight of sunn hemp grown in anthracene-contaminated soil compared to that grown in anthracene-contaminated soil without receiving any *Streptomyces* sp. St1 (Table 1, Figure 2D, 2E). The shoot length, shoot fresh weight and shoot dry weight of sunn hemp grown in anthracene-contaminated soil that received immobilized cells+spores of rice straw or coconut husk were around 41.2-44.4 cm, 2.81-3.45 g, and 0.44-0.53 g, respectively. Only sunn hemp grown in uncontaminated soil and anthracene-contaminated soil when receiving immobilized cells+spores of *Streptomyces* sp. St1 flowered within 36 days.

The results in Table 1 and Figure 3 showed that the root length, root dry weight and number of nodules per plant of sunn hemp grown in uncontaminated soil were not significantly different from those grown in anthracene-contaminated soil with or without receiving cells+spores of *Streptomyces* sp. St1 at 36 d of the experiment. The root length and root dry weight of sunn hemp were around 6.5±0.9 to 10.9±2.0 and 0.05±0.01 to 0.14±0.03 g, respectively. However, the root fresh weight of sunn hemp grown in anthracene-contaminated soil without receiving any *Streptomyces* sp. St1 had less growth (0.15 g) than that grown in non-contaminated soil (0.47 g). Root morphology of sunn hemp grown in anthracene-contaminated soil without receiving *Streptomyces* sp. St1 seemed to be less branching when compared with the roots of sunn hemp from the other treatment. Direct exposure of sunn hemp roots to anthracene contaminated soil may increase the anthracene toxicity to their roots. Anthracene has also been reported to decrease the root biomass of lettuce and radish (Wieczorek & Wieczorek, 2007).

Figure 2. Growth of sunn hemp (36 days of experiment) in uncontaminated soil (A), anthracene-contaminated soil (B), anthracene-contaminated soil with free cells+spores of *Streptomyces* sp. St1 (C) and immobilized cells+spores of *Streptomyces* sp. St1 in rice straw (D) or coconut husk (E)

Table 1. Shoot and root growth of suun hemp grown in anthracene-contaminated soil without or with free cells+spores or immobilized cells+spores of *Streptomyces* sp. St1.

Treatment	Day 12th			Day 36th				
	Length (cm)	Fresh weight (g)	Dry weight (g)	Length (cm)	Fresh weight (g)	Dry weight (g)	Nodules per plant	Flowering (%)
Shoot growth								
without anthracene	10.6 ± 0.7ab	0.26 ± 0.02b	0.02 ± 0.00b	43.4 ± 1.8a	2.09 ± 0.23b	0.38 ± 0.03b	-	71.4
anthracene	12.0 ± 1.4ab	0.43 ± 0.04a	0.04 ± 0.00a	34.4 ± 1.4b	0.56 ± 0.06c	0.27 ± 0.05bc	-	0
anthracene and free cells+spores	7.28 ± 0.6c	0.24 ± 0.02bc	0.01 ± 0.00b	20.6 ± 2.1c	1.00 ± 0.19c	0.14 ± 0.02c	-	0
anthracene and immobilized in rice straw	12.6 ± 1.1a	0.33 ± 0.02b	0.02 ± 0.00b	41.2 ± 2.4ab	2.81 ± 0.50ab	0.44 ± 0.05ab	-	42.8
anthracene and immobilized in coconut husk	9.12 ± 1.0bc	0.16 ± 0.02c	0.01 ± 0.00b	44.4 ± 3.7a	3.45 ± 0.40a	0.53 ± 0.07a	-	42.8
Root growth								
without anthracene	3.0 ± 0.3a	0.04 ± 0.01ab	0.003 ± 0.001a	10.9 ± 2.0a	0.47 ± 0.08a	0.14 ± 0.03a	4.2 ± 0.5a	-
anthracene	3.2 ± 0.6a	0.05 ± 0.01a	0.003 ± 0.001a	6.5 ± 0.9a	0.15 ± 0.01b	0.10 ± 0.05a	12.4 ± 2.9a	-
anthracene and free cells+spores	2.0 ± 0.6a	0.01 ± 0.00b	0.001 ± 0.000a	10.8 ± 2.4a	0.26 ± 0.04b	0.05 ± 0.01a	15.0 ± 7.0a	-
anthracene and immobilized in rice straw	2.0 ± 0.4a	0.05 ± 0.01a	0.004 ± 0.001a	7.8 ± 1.6a	0.46 ± 0.08a	0.06 ± 0.01a	12.4 ± 2.0a	-
anthracene and immobilized in coconut husk	1.60 ± 0.2a	0.02 ± 0.01b	0.002 ± 0.001a	8.6 ± 0.0a	0.55 ± 0.05a	0.09 ± 0.01a	10.8 ± 1.8a	-

Different lower case letter denoted significantly different ($P<0.05$) between treatment in the same column.

Anthracene has been reported to be toxic on photosynthetic pigment of soybean through a reduction in the quantum yield of photosystem II and damage to the oxygen evolving complex (Tomar, Sharma, & Jajoo, 2015). Surprisingly, the application of *Streptomyces* sp. St1 benefited the total chlorophyll content in sunn hemp leaves on day 36 of the experiment. The results showed that all chlorophyll content in sunn hemp leaves grown in anthracene-contaminated soil without receiving *Streptomyces* sp. St1 on day 36 was lower than all chlorophyll contents in the leaves of sunn hemp grown in uncontaminated soil (Table 2). Inoculation with free or immobilized cells+spores of *Streptomyces* sp. St1 tended to increase all chlorophyll contents in leaves of sunn hemp grown in anthracene-contaminated soil compared to that without receiving *Streptomyces* sp. St1 as free cells+spores or immobilized cells+spores (Table 2). Chlorophyll a, chlorophyll b and total chlorophyll contents in leaves of sunn hemp grown in anthracene-contaminated soil on day 36 of the experiment were around 29.1-31.4, 19.0-50.0 and 49.5-79.2 mg/ml in soil that received free cells+spores, rice straw immobilized cells+spores and coconut husk immobilized cells+spores, respectively.

Based on the root growth of sunn hemp grown in anthracene-contaminated soil with and without receiving *Streptomyces* sp. St1 in this study, IAA production by this plant growth promoting bacteria may stimulate root elongation and develops root branching. IAA could promote plant growth through increase the endophytic bacteria colonization at the plant surface (Mehmood *et al.*, 2018). However, IAA production by *Streptomyces* sp. St1 in the presence of anthracene in the soil was not determined in this study. Thus, there are other mechanisms that may promote plant growth. For example, the ability to produce antibiotic or extracellular enzymes in *Streptomyces* sp., which could protect plants from phytopathogens and providing plant nutrients (Vurukonda, Giovanardi, & Stefani, 2018). Phosphate solubilizing activity also increased the phosphate availability to plants and then promotes plant growth in unfavorable condition (Kalayu, 2019).

The results in this study revealed that inoculation of anthracene-contaminated soil with immobilized cells+spores of *Streptomyces* sp. St1 could promote the growth of sunn hemp to a greater extent than free cells+spores. This may be explained by immobilized cells+spores of *Streptomyces* sp. St1 with rice straw or coconut husk maintaining viable cells of *Streptomyces* sp. St1 better than the inoculation of free cells+spores into anthracene-contaminated soil (Table 3). In general, cell immobilization could make a protective microenvironment for microbial cells through increasing cell tolerance to abiotic stress and the carrier material can act as a surface for bacterial cell adsorption. The accumulation of bacterial cells on the surface of the carrier can promote the growth of bacteria in a biofilm (Zhang *et al.*, 2016). Meanwhile, the application of free cells into the environment led to the direct cell exposure to abiotic stress, which resulted in a loss of cell viability. Based on our results, the plant growth promoting activity of *Streptomyces* sp. St1 seemed to depend on cell immobilization and IAA production activity in *Streptomyces* sp. St1, which has been reported to a maximum of about 20 d of incubation (Somtrakoon *et al.*, 2019). Table 3 shows that the number of viable cells of total bacteria which similar to *Streptomyces* sp. from planted soil inoculated with immobilized cells+spores in rice straw and coconut husk on day 36 of the experiment was higher than that of soil inoculated with free cells+spores. This trend was also observed in unplanted soil with anthracene contamination. The results in this study indicated that decreasing the viable

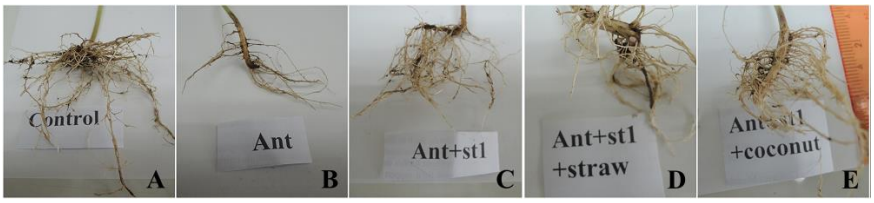

Figure 3. Root of sunn hemp (36 days of experiment) grown in uncontaminated soil (A), anthracene-contaminated soil (B), anthracene-contaminated soil with free cells+spores of *Streptomyces* sp. St1 (C) and immobilized cells+spores of *Streptomyces* sp. St1 in rice straw (D) or coconut husk (E).

Table 2. Chlorophyll content in leaves of sun hemp grown in anthracene-contaminated soil without or with free cells+spores or immobilized cells+spores of *Streptomyces* sp. St1.

Treatment	Day 12th			Day 36th		
	Chlorophyll a (mg/ml)	Chlorophyll b (mg/ml)	Total chlorophyll (mg/ml)	Chlorophyll a (mg/ml)	Chlorophyll b (mg/ml)	Total chlorophyll (mg/ml)
without anthracene	23.7 ± 4.0a	11.4 ± 1.8a	35.1 ± 5.8a	31.2 ± 0.8a	28.1 ± 6.3b	59.4 ± 6.5b
anthracene	29.0 ± 3.0a	14.5 ± 2.0a	43.6 ± 5.0a	22.8 ± 1.2b	12.0 ± 1.0c	34.8 ± 2.0c
anthracene and free cells+spores	8.1 ± 2.4b	4.8 ± 1.6b	13.0 ± 4.0b	30.5 ± 1.4a	19.0 ± 2.9bc	49.5 ± 4.0b
anthracene and immobilized in rice straw	23.9 ± 2.8a	11.0 ± 1.6a	34.9 ± 4.4a	31.4 ± 0.5a	43.0 ± 4.5a	74.4 ± 4.0a
anthracene and immobilized in coconut husk	8.0 ± 1.3b	4.7 ± 0.9b	12.7 ± 2.2b	29.1 ± 1.1a	50.0 ± 3.1a	79.2 ± 4.2a

Different lower case letter denoted significantly different ($P<0.05$) between treatment in the same column.

Table 3. Amount of anthracene remaining, accumulation of anthracene in sunn hemp biomass and number of *Streptomyces* sp. St1 in unplanted and planted soil

Treatment	Anthracene remaining (mg/kg dry soil)		Anthracene accumulation (day 36th) (mg/kg plant)		Number of total bacteria which similar to *Streptomyces* sp. (log cfu/g dry soil)	
	Day 12th	Day 36th	Shoot	Root	Day 12th	Day 36th
Unplanted soil						
anthracene	256.8 ± 43.3a	122.0 ± 13.9a	-	-	-	-
anthracene and free cells+spores	262.9 ± 50.7a	125.7 ± 12.0a	-	-	4.6 ± 0.3	N.D.
anthracene and immobilized in rice straw	247.9 ± 68.5a	123.6 ± 29.0a	-	-	4.9 ± 0.3	3.5 ± 1.0
anthracene and immobilized in coconut husk	93.7 ± 9.6a	66.8 ± 29.7a	-	-	5.1 ± 0.3	3.0 ± 1.0
Planted soil						
anthracene	124.7 ± 23.2a	93.2 ± 17.8a	1.7 ± 1.7	B.D.	-	-
anthracene and free cells+spores	141.5 ± 68.5a	117.9 ± 54.1a	20.4 ± 0.0	26.8	5.0 ± 0.2	2.2 ± 0.1
anthracene and immobilized in rice straw	124.6 ± 46.5a	93.0 ± 42.2a	74.4 ± 69.6	122.2	4.8 ± 0.1	4.4 ± 0.3
anthracene and immobilized in coconut husk	167.2 ± 45.0a	82.0 ± 14.0a	12.8 ± 1.5	3.4	5.1 ± 0.1	4.7 ± 0.1

Different lower case letter denoted significant difference ($P<0.05$) between the same soil in the same column. B.D. mean bellowed detection limit and N.D. means non-detected.

cell number depended on the duration of the experiment. Moreover, soil planted with sunn hemp plants could maintain viable cell numbers of total bacteria which similar to *Streptomyces* sp. to a greater extent than unplanted soil. This may be as planted soil could provide favorable growth conditions (nutrients, oxygen, and habitats) for the growth and development of bacteria (Alagić, Maluckov, & Radojičić, 2015).

3.2 Anthracene removal from soil

In unplanted anthracene-contaminated soil, the ability of *Streptomyces* sp. St1 to degrade anthracene was tested for 36 days. The results revealed that *Streptomyces* sp. St1 did not improve the anthracene removal from unplanted soil within day 36 of the experiment. There were no significant differences in the amount of anthracene remaining between the unplanted soil without receiving free cells+spores of *Streptomyces* sp. St1 or unplanted soil with receiving free cells+spores or immobilized cells+spores of *Streptomyces* sp. St1. The anthracene remaining in the unplanted soil on day 36 of the experiment with and without receiving free cells+spores of *Streptomyces* sp. St1 was 125.7 mg/kg and 122.0 mg/kg dry soil, respectively. In addition, the amount of anthracene remaining in the unplanted soil that received rice straw immobilized cells+spores was 123.6 mg/kg dry soil (Table 3). Additionally, the immobilized cells+spores of *Streptomyces* sp. St1 with coconut husk into unplanted soil tended to increase anthracene removal from the soil, only 66.8 mg/kg dry soil of anthracene remained on day 36 of the experiment, however, this value is not significant (Table 3). These results emphasized that *Streptomyces* sp. St1 could not degrade anthracene; however, this plant growth promoting bacteria could tolerate 100 mg/kg of anthracene prepared in a half formula of PDA (data not shown). The removal of anthracene from unplanted soil may be from other abiotic degradation processes including chemical degradation and photochemical degradation (Ukiwe, Egereonu, Njoku, Nwoko, & Allinor, 2013).

Also, soil planted with sunn hemp and inoculated with *Streptomyces* sp. St1 did not enhance anthracene removal in this study. The tendency of anthracene removal from soil planted with sunn hemp was similar to unplanted soil on day 36 of the experiment. The amount of anthracene remaining in planted soil without receiving *Streptomyces* sp. St1 was 93.2 mg/kg dry soil. The amount of anthracene remaining in planted soil when receiving free cells+spores or immobilized cells+spores of *Streptomyces* sp. St1 were around 82.0-117.9 mg/kg dry soil (Table 3). There were no significant differences between the amount of anthracene remaining in planted soil and unplanted soil with or without receiving *Streptomyces* sp. St1 on day 36 of the experiment; however, rapid anthracene removal was observed in planted soil because the amount of anthracene remaining in planted soil on day 12 of the experiment was lower than that in unplanted soil (Table 3). Moreover, the accumulation of anthracene in biomass of sunn hemp was negligible in this study. The anthracene accumulation in the biomass of the plants was low when compared to the total amount of anthracene in contaminated soil (Table 3). Generally, the growth of plants in contaminated soil is expected to be useful in phytoremediation due to the fact that the growth of plants could increase the microbial number and microbial activity in soil by producing root exudate. Some of these compounds can act as inducers for hydrocarbon degradation (Phillips, Greer, Farrell, & Germida, 2012; Khan, Afzal, Iqbal, & Khan, 2013).

Several researchers reported that the addition of plant growth promoting bacteria usually promoted the growth of plants and stimulated the removal of petroleum hydrocarbons from contaminated soil (Hong *et al.*, 2011; Hou *et al.*, 2015). In contrast, the planting of sunn hemp did not promote the removal of anthracene from contaminated soil and inoculation of planted soil with free cells+spores or immobilized cells+spores of *Streptomyces* sp. St1 did not promote the removal of anthracene from soil. Most researchers reported that the ability to degrade PAHs usually came from the activity of natural indigenous bacteria that were adapted to degrade PAHs (Hou *et al.*, 2015). However,

the soil used in this study was autoclaved at 121 °C for 15 min before the anthracene-contaminated soil preparation, because this study aimed to investigate the activity of *Streptomyces* sp. St1 to stimulate the removal of anthracene from planted soil. Moist heat from the autoclave could reduce the number of natural indigenous microorganisms already present in the soil. This may result in decreasing the anthracene degrading ability in this soil. Rarely microorganism can survive after autoclaving, thus, the number of surviving microorganisms did not sufficiently promote anthracene-degradation in both unplanted and planted soil in this study. Thus, the only advantage of using immobilized *Streptomyces* sp. St1 in this study is to stimulate the growth of sunn hemp grown in anthracene-contaminated sites.

4. Conclusions

Streptomyces sp. St1 enhanced sunn hemp growth in anthracene-contaminated soil when added into soil as immobilized cells+spores. However, this bacterial inoculation did not enhance the anthracene biodegradation in the soil. The use of anthracene-degrading bacteria with plant growth promoting properties will be a challenge for increasing the phytoremediation capacity in contaminated sites.

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