Sugarcane Seedling Growth Promotion by Indole Acetic Acid (IAA) Producing *Streptomyces* sp. AS14-2 Isolated from Rhizosphere of Sugarcane and Rice

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Abstract

The present study aimed to screen and identify of indole acetic acid (IAA) producing actinomycetes isolates from the rhizosphere of sugarcane and rice and to evaluate the effects of these isolates on plant growth under pot experiment conditions. Out of eleven IAA producing actinomycete isolates, two isolates include AS13-2 and AS14 showed the highest ability to produce IAA 29.02 and 17.17µg/mL respectively. Based on morphological and 16S rDNA sequence analysis indicated these isolates belong to *Streptomyces* genus. Pot culture experiments revealed that sugarcane seedlings inoculated with the IAA producing *Streptomyces* sp. AS14-2 gave the best enhancement of plant growth and significantly increased shoot lengths, shoot fresh weight, shoot dry weight, root lengths, root fresh weight and root dry weight compared with un-inoculated control treatments. These results suggest that the IAA producing *Streptomyces* sp. AS14-2 could be a promising candidate for utilization in growth improvement in sugarcane crop.

Keywords: Indole acetic acid (IAA), Actinomycetes, Sugarcane

1. Introduction

Sugarcane (*Saccharum offi cinarum* L.) is a major crop cultivated in tropical and sub-tropical countries like Brazil, China, India, Thailand, and Australia (Sindhu *et al*., 2016). In Thailand, sugarcane is the raw material for the sugar and ethanol industry. Cultivation of sugarcane in large quantities caused the use of chemical fertilizers and pesticides. In the recent years, the replacement of chemical inputs with biotechnological products has gathered much attention to many research fields and is a rising alternative to the use of chemicals, irreversible environmental depletion and health concerns for both producers and consumers (Zhao *et al*., 2011).

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Microorganisms that colonize the rhizosphere and plant roots, and enhance plant growth by any mechanism are referred to as plant growth promoting (PGP) microbe. PGP microbe have been reported to promote plant growth by several mechanisms such as the production of siderophores, solubilization of minerals such as phosphorus and potassium, biological nitrogen fixation, and production of the plant growth hormones (Mohite, 2013; Nutaratat *et al*., 2014). There are several PGP microbe inoculants currently commercialized that seem to promote growth through at least one mechanism of the following: suppression of plant disease (Bioprotectants), improved nutrient acquisition (Biofertilizers), or phytohormone production (Biostimulants) (Patel and Patel, 2014). However, the selection of PGP microbe has a possibility of being an alternative to agrochemicals needed to increase the agricultural productivity.

Production of phytohormones, such as auxins or gibberellins, is a direct mechanism that is used by PGR microorganisms. Indole-3-acetic acid (IAA) is one of the most physiologically active auxins which controls many physiological processes including stimulation of cell division, cell elongation, cell differentiation, light and gravitational responses, and regulation of leaf fall and fruit ripening (Meena *et al*., 2015). IAA is a product of L-tryptophan metabolism in microorganisms. Approximately 80% of the rhizosphere bacteria can secrete IAA (Patel and Patel, 2014). Several authors have reported microorganisms included yeast, bacteria and actinomycetes have the ability to produce IAA (Nutaratat *et al*., 2014; Meena *et al*., 2015; Khamna *et al*., 2010)

Actinomycetes are Gram-positive bacteria. They are present in a high proportion of microbial biomass in the plant rhizosphere (Muangham *et al*., 2015) and well known as saprophytic rhizosphere inhabitants. Majority of the PGP actinomycetes carrying IAA trait which is responsible for increased number of adventitious roots, which improves the plants to uptake a large volume of nutrients and absorb water, while increased root exudates in turn benefits the bacteria (Anwar *et al*., 2016). Actinomycetes, particularly genus *Streptomyces*, have been found capable to produce IAA, which isolated from different plants such as *Streptomyces* CMU-H009 isolated rhizosphere soils Thai medicinal (Khamna *et al*., 2010), *Streptomyces ramulosus* strain EUSKR2S82 colonize the *Eucalyptus* roots and increased *Eucalyptus* root length (Himaman *et al*., 2016), *S. aurantiogriseus* VSMGT1014 from rice rhizosphere soils (Harikrishnan *et al*., 2014), and *S. atrovirens* isolated from wheat rhizospheric soil (Abd-Alla *et al*., 2013). This present study aimed to screen and identify IAA producing actinomycetes isolated from the rhizosphere of sugarcane and rice, and to evaluate the plant growth promoting effects of sugarcane seedlings after inoculation in pot experiments.

2. Materials and Methods

2.1 Isolation of actinomycetes from rhizosheric soil

Soil samples were collected from the rhizosphere of sugarcane and rice (15 samples) in Lop Buri Province. Rhizospheric soil was separated from the roots and air-dried at room temperature (28–30°C) for 7 days. Ten grams of air dried soil sample were suspended in 90 ml of normal saline (0.85% of NaCl) and placed on an orbital shaker (at 100 rpm) at room temperature. The soil suspension was heated at 50°C for 6 min for reducing the number of Gram-negative bacteria. Subsequently, 0.1 mL of diluted soil suspension were spread onto starch casein agar plates and incubated at room temperature for 4–10 days (Gopalakrishnan *et al*., 2011; Prapagdee *et al*., 2008). Colonies with different morphological characteristics were picked and streaked onto glucose yeast extract malt extract (GYM) (glucose 10 g/L, malt extract 4 g/L and yeast extract 4 g/L) agar plates for purification. Stock cultures were maintained in 20% (v/v) glycerol at -20°C. The actinomycete isolates were studied spore morphology, substrate and aerial hyphae, pigment production and colony characteristics by observed following growth on oat meal agar plates, incubated at room temperature for 7 days. The microscopic characterization was performed by slide culture method.

2.2 Determination of IAA levels produced by actinomycete isolates

All the actinomycete isolates were screened for their IAA production ability, according to the method of Himaman *et al*. (2016) with some modifications. Two actinomycete discs (5 mm) of 5 days culture on yeast extract malt extract agar plates were inoculated into glucose yeast extract broth (GYE broth) (glucose 10 g/L and yeast extract 10 g/L) containing with 0.2% (w/v) L-tryptophan, on a rotary shaker at 180 rpm for 7 days at ambient temperature (28–30°C). The actinomycete cultures were centrifuged at 6,000 rpm for 10 min. The supernatant (1 mL) was mixed with 2 mL of Salkowski's reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 M $FeCl_3$ solution) and incubated in the dark for 30 min at room temperature. Appearance of a pink color indicated IAA production. Optical density (OD) was read at 535 nm using a spectrophotometer. The amount of IAA produced was estimated by using standard curve of IAA. Production of IAA was determined from three replicates for each isolate of actinomycete.

2.3 Identification of actinomycete isolates

Genomic DNA was extracted from cultured cells following Kumar *et al*. (2010). The 16S rDNA were amplified using a standard PCR protocol with the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3) (Shen *et al*., 2016). The PCR products were subjected to DNA sequencing at Macrogen Inc. Seoul, Korea. The sequences of the 16S rDNA were compared with those from the GenBank

using the BLAST program. Phylogenetic tree was constructed using neighbor-joining method. Bootstrap analysis was performed to estimate statistical stability of the branches in the cluster with 1000 replicates using MEGA version 6.0 program (Gopalakrishnan *et al*., 2011)

2.4 Pot Experiment

Seven day-old actinomycete cultures were tested for their growth promotion activity on sugarcane cultivar Khon Kaen 3. The sugarcane stems were washed in running water to remove environmental dirt and cut into pieces with size about 5 cm to select the best buds. The surfaces of sugarcane cut pieces were sterilized using 10% sodium hypochlorite and soaked into sterile distilled water. Insert the sugarcane cut pieces with bud of sugarcane in to the polythene bags of 10x15 cm filled with 500 g sterilized soil. The following treatments were investigated: (1) un-inoculated control, (2, 3) inoculated with *Streptomyces* isolate (2) AS13-2 and (3) AS14-2. Ten milliliter of each isolate at 10^8 spores/mL was added to the soil. The inoculation was done five times, at 7-day interval. The plants from the control treatment were irrigated only with water. The plant of 5 pots of each treatment was harvested after 35 days and selected parameters such as root length, shoot length, fresh root weight, dry root weight, fresh shoot weight and dry shoot weight were measured and compared with the un-inoculated controls.

2.5 Statistical analysis

Statistical analysis was conducted by using Analysis of Variance (ANOVA) with software IBM SPSS Statistics version 21. Duncan's multiple range test (P=0.05) was calculated to compare the differences between means in each treatment.

3. Results and Discussion

3.1 Actinomycetes from rhizosphere soils

From 15 different sugarcane and rice rhizosphere soil samples, 95 isolate of actinomycetes were isolated. The isolated colonies had a weft of aerial mycelium that either appeared granular, floccose or powdery. Each of the isolates was different morphological colony color included brownish, dark brown, grey, dark grey and yellowish white.

3.2 Determination of IAA levels produced by actinomycete isolates

Qualitative analysis of culture supernatant of 95 actinomycete isolates, only 11 isolates (11.57%) of them were found to have the ability to produce IAA. Data presented in Table 1 indicated production of IAA in a range of 4.76–29.02 µg/mL. Two isolates, AS14-2 and AS13-2 were produced IAA more than 10 µg/mL. Isolate AS14-2 was the best IAA-producer strain of this study and it produced 29.02 µg/mL of IAA at 7 days of incubation. IAA-producing microorganisms are receiving attention as good sources of biofertilizer (Nutaratat *et al*., 2014).

Several reports have shown that different actinomycetes from many crop rhizosphere soils, the IAA production can vary among different species and strains (Mohite, 2013). *S. atrovirens* ASU14 isolated from wheat rhizosphere soil showed an ability to produce IAA and gave 22 µg/mL (Abd-Alla *et al*., 2013), *S. ramulosus* strain EUSKR2S82 isolated from *Eucalyptus* rhizosphere soil gave IAA 28.03 µg/mL (Himaman *et al*., 2016), *Streptomyces* CMU-H009 isolated from *Cymbopogon citratus* rhizosphere soil showed a high ability to produce IAA 143.95 µg/mL (Khamna *et al*., 2010). These results suggested that IAA-producing actinomycetes isolate AS14-2 and AS13-2 could be a promising candidate for utilization in growth improvement of sugarcane crop. The best producer isolate of IAA was chosen for further studies.

Table 1 Qualitative production of IAA by actinomycete isolates

Actinomycete isolates	IAA (µg/mL)
AS13-2	17.17±0.55^{b}
AS14-2	29.02±0.28^{a}
AS18-3	9.57±0.29^{c}
AS18-10	8.75±1.16^{d}
K1-4	6.87±0.28^{e}
K1-5	5.21±0.07^{f}
K2-4	9.16±0.15^{cd}
K2-6	4.76±0.09^{f}
N2-1	6.39±0.23^{e}
N2-3	7.07±0.08^{e}
SM3-1	6.54±0.22^{e}

Note: Data are means ± standard deviations (SD) of three replicates. Means designated with same letters do not differ significantly ($p \leq 0.05$) according to the Duncan's multiple range test

3.3 Identification of selected IAA-producing actinomycetes isolate

The selected IAA-producing actinomycete isolate AS13-2 and AS14-2 could grow well on oat meal agar plates and produced yellow diffusible pigment. Colonies formed on the agar media were large, leathery and similar to those of *Streptomyces* strains. The color of vegetative mycelium in oat meal agar medium was dark yellow and aerial mycelium was white. Aerial mycelium formed spore mass bearing long spore chains of *rectiflexibles* spores. Based on the morphological characterization, the two isolates were classified as a member of the genus *Streptomyces* (Williams *et al*., 1983).

In order to determine the identity of the two IAA producing actinomycetes, their 16S rDNA were sequenced and analyzed. A neighbor-joining dendrogram was generated using the sequence from the two IAA producing actinomycete isolates, AS13-2 and AS14-2 and representative sequences from the databases (Figure 1). The sequences (about 1,400 bp) were aligned with those *Streptomyces* reference species available in the GenBank database, which confirmed that isolate AS13-2 and AS14-2 belong to *Streptomyces* genus. The both of isolate AS13-2 and AS14-2 had maximum sequence similarity (99%) with *Streptomyces cellulosae*, *S. rimosus*, *S. erumpens*, *S. ochraceiscleroticus*, *S. lividus*, *S. cebimarensis*, *S. monomycini*, *S. nigrescens* and *S. albofaciens*. Based on the sequence similarity and the phylogenetic analysis, isolate AS13-2 and AS14-2 were found to resemble to *Streptomyces cebimarensis.*

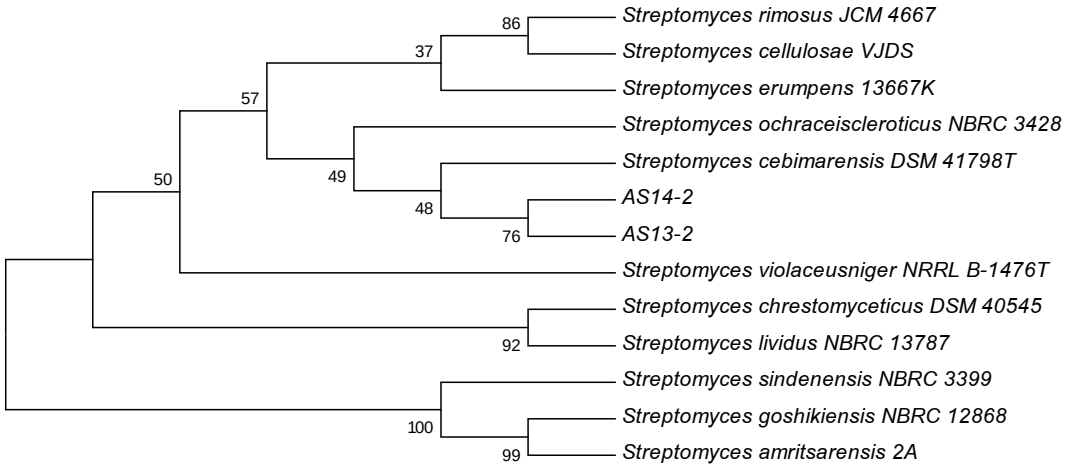

Figure 1 Phylogenetic relationship between the two IAA producing actinomycetes and representative species based on 16S rDNA sequences constructed using the neighbor-joining method. The number at each branch is the percentages of times the group of strains in that branch occurred, based on 1000 cycles in bootstrap analysis.

3.4 Pot experiments

The IAA producing *Streptomyces* sp. AS13-2 and AS14-2 were selected to evaluate growth enhancement of the sugarcane seedlings in pot experiments. Three treatments include sugarcane inoculated 10^8 spores/mL of *Streptomyces* sp. AS13-2, AS14-2 and un-inoculated control were investigated. Thirty-five days seedling sugarcane treated by *Streptomyces* sp. AS14-2 gave statistically significantly ($P \leq 0.05$) enhanced all plant growth promoting parameters; shoot lengths (25.00%), shoot fresh weight (99.00%), shoot dry weight (131.70%), root lengths (9.84%), root fresh weight (128.07%) and root dry weight (48.48%) over the un-inoculated control (Table 2 and Figure 2). And *Streptomyces* sp. AS13-2 enhanced plant growth promoting parameters; shoot fresh weight (44.54%), shoot dry weight (58.53%), root

lengths (9.84%) and root fresh weight (70.61%) over the un-inoculated control, whereas parameter shoot lengths and root dry weight were not significantly different. The results of this study indicate that IAA producing *Streptomyces* sp. AS14-2 treatment can promote sugarcane seedling growth.

IAA-producing microorganisms are known to promote root elongation and plant growth. By producing the plant hormones, microorganisms stimulate plant growth in order to increase the production of plant metabolites which can be beneficial to their growth. The property of synthesizing IAA is considered as the effective tool for screening beneficial microorganisms suggesting that IAA producing bacteria had profound effect on plant growth (Mohite, 2013). Studies carried out by Kruasuwan and Thamchaipenet (2016) showed that IAA producing *Streptomyces* sp. GKU 895 isolated from sugarcane roots measurable increments in root length, shoot fresh weight, and shoot dry weight of sugarcane seedlings over the control. In addition *Streptomyces* sp. GKU 895 produced other plant growth promoting traits such as siderophore production, phosphate solubilization, and antagonistic activities. The inoculation of sugarcane with *Gluconacetobacter diazotrophicus* VI27 strain, which hight IAA production (109.35 µg/ml) showed a significant increase in the number of setts germinated, in the amount of soluble solids, and in the yield of sugarcane juice compared with the control (Beneduzi *et al*., 2013). Thus, it will be useful to investigate further the other plant growth promoting traits of the IAA producing *Streptomyces* sp. AS14-2. More understanding of plant growth promoting traits of these isolates benefits further investigation into environmentally friendly bio fertilizers.

Table 2 Effect of *Streptomyces* sp. AS13-2 and AS14-2 on growth of sugarcane seedlings at 35 days after inoculation

Isolates	Shoot			Root		
	Length (cm)	Fresh weight (g)	Dry weight (g)	Length (cm)	Fresh weight (g)	Dry weight (g)
un-inoculated control	11.10±1.43^{b}	8.06±0.68^{c}	1.23±0.05^{c}	25.40±0.89^{b}	2.28±0.17^{c}	0.33±0.89^{b}
AS13-2	11.50±0.93^{b}	11.65±0.85^{b}	1.95±0.06^{b}	27.90±1.40^{a}	3.89±0.68^{b}	0.39±0.09^{ab}
AS14-2	13.90±0.96^{a}	16.04±0.24^{a}	2.85±0.08^{a}	27.90±0.74^{a}	5.20±0.56^{a}	0.49±0.12^{a}

Note: Data are means ± standard deviations (SD) of five replicates. Means designated with same letters do not differ significantly ($p \leq 0.05$) according to the Duncan's multiple range test

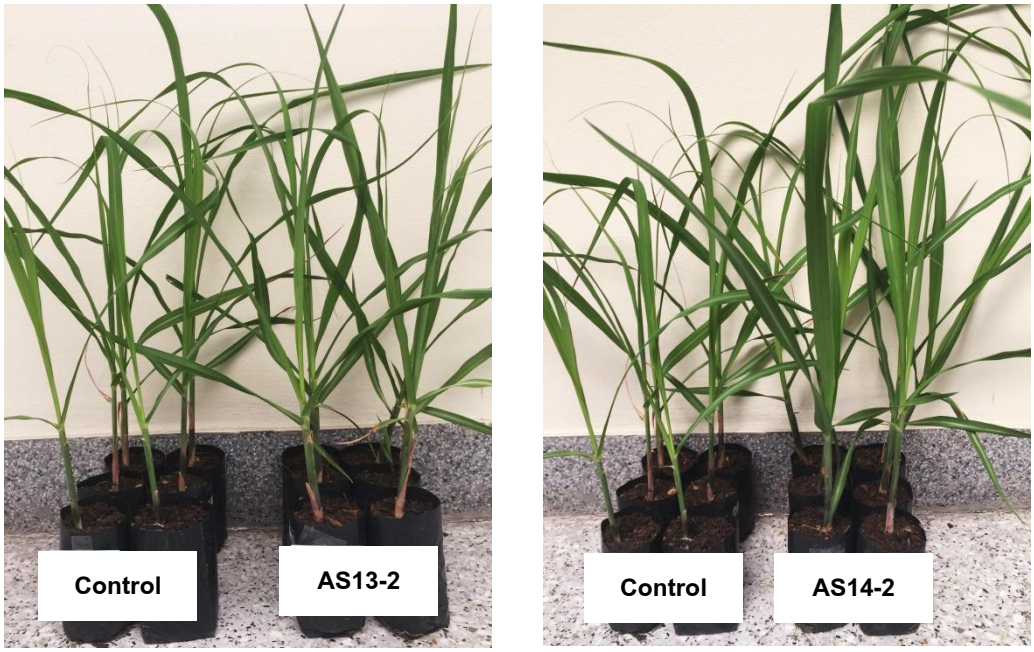

Figure 2 Effects of IAA producing *Streptomyces* sp. AS13-2 (a) and AS14-2 (b) on the growth of sugarcane seedlings at 35 days after inoculation

4. Conclusion

A total of 95 actinomycetes isolated from 15 different the rhizosphere of sugarcane and rice samples were screened for their IAA production capacity and only 11 isolates of them were found to have potential the IAA production ability. The actinomycete isolate AS13-2 and AS14-2 showed the highest ability to produce IAA and the BLAST search results 16S rDNA sequences of these isolates indicated belong to *Streptomyces cebimarensis*. Pot culture experiments revealed that sugarcane seedling inoculated with the IAA producing *Streptomyces* sp. AS14-2 gave the best enhancement of plant growth and significantly increased shoot lengths, shoot fresh weight, shoot dry weight, root lengths, root fresh weight and root dry weight compared with un-inoculated control treatments. IAA producing *Streptomyces* sp. AS14-2 can be regarded as potential for plant growth promoting agent especially for sugarcane plant.

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