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Original Article

Positive role of 1-aminocyclopropane-1-carboxylate deaminase-producing endophytic *Streptomyces* sp. GMKU 336 on flooding resistance of mung bean

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ABSTRACT

Previously, a plant growth-promoting endophytic *Streptomyces* sp., namely GMKU 336, has been shown to improve salt tolerance in rice through the action of 1-aminocyclopropane-1-carboxylate deaminase (ACCD) by converting a precursor of ethylene, (ACC) in plants into ammonia and α-ketobutyrate and consequently reducing the ethylene level. In this study, strain GMKU 336 and its ACCD-deficient mutant were further investigated regarding flooding tolerance in mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72]. The results clearly demonstrated that mung bean associated with *Streptomyces* sp. GMKU 336 significantly increased plant elongation and biomass, chlorophyll content, leaf area, leaf color and adventitious roots, and reduced the ethylene level under flooding conditions when compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant. It was concluded that ACCD-producing *Streptomyces* sp. GMKU 336 that beneficially improved salt tolerance in rice also enhanced flooding tolerance in mung bean via the action of ACCD.

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Introduction

Mung bean [*Vigna radiata* (L.) Wilczek] is one of the most important crops for human nutritional needs. In tropical and sub-tropical region such as Thailand, heavy rainfall in the rainy season frequently induces short-term flooding in crop fields and soil flooding has long been identified as a major abiotic stress that results in decreased growth and crop production (Ahmed et al., 2002; Kumar et al., 2013; Parent et al., 2008). In recent years, a new approach has been developed to improve stress in plants by treating crop seeds and seedlings with plant growth-promoting bacteria (PGPB) harboring 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD) (Farwell et al., 2007). PGPB convert ACC, a precursor of ethylene in plants, to ammonia and α-ketobutyrate which the bacteria can consume by the action of ACCD (Honma and Shimomura, 1978), thereby lowering the level of stressed ethylene in plants (Glick, 2004). Plants inoculated with ACCD-producing PGPB were markedly more resistant to the deleterious effects of stress ethylene under flooding conditions including *Ocimum sanctum* and tomato (Barnawal et al., 2012; Grichko and Glick, 2001; Saleem et al., 2007).

Recently, endophytic actinomycetes have been recognized as new members of PGPB due to their properties to protect plants from infectious diseases (Misk and Franco, 2011) and their ability to enhance plant growth by carrying several PGP-traits including siderophore production (Rungin et al., 2012), plant hormone production (Kruasuwan and Thamchaipenet, 2016), phosphate solubilization (El-Tarabily et al., 2008) and ACCD production (El-Tarabily, 2008; Qin et al., 2014). However, the role of ACCD-producing endophytic actinomycetes to promote plant growth under flooding stress has been less studied. Recently, the endophytic *Streptomyces* sp. GMKU 336 was employed to facilitate growth of rice under salt-stress conditions by reducing stress ethylene via the action of ACCD through converting a precursor of ethylene, ACC, in plants into ammonia and α-ketobutyrate and consequently reduced ethylene, reactive oxygen species (ROS), Na^+ and the Na^+/K^+ ratio (Jaemsaeng et al., 2018). In this work, *Streptomyces* sp. GMKU 336 was further investigated regarding its ability to enhance the growth of mung bean

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CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] under flooding conditions.

Materials and methods

Endophytic streptomycete strains

Endophytic *Streptomyces* sp. GMKU 336 was isolated from the medicinal plant *Clerodendrum serratum* (L.) Moon (Ak-kee-ta-wan) (Indananda, 2013). Strain GMKU 336 displays ACCD activity at 2.85 ± 0.15 μmol α-ketobutyrate/mg protein/h and harbors PGP-traits including phosphate solubilization and siderophore production but no indole-3-acetic acid (IAA) production (Jaemsaeng et al., 2018). The ACCD-deficient mutant was constructed by insertional inactivation of *acdS* gene (encoding for ACCD) of the wildtype strain and showed no ACCD activity (Jaemsaeng et al., 2018).

Inoculation of mung bean with endophytic streptomycetes

Healthy seeds of mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] were obtained from the Chai Nat Field Crops Research Center, Field Crops Research Institute, Department of Agriculture and Cooperatives, Chai Nat, Thailand. The seeds were surface sterilized by immersion in 70% volume per volume (v/v) ethanol for 1 min, 1% weight per volume (w/v) sodium hypochlorite for 5 min and washed six times with sterile distilled water. Then, sterilized seeds were soaked in sterile distilled water for 4 h in the dark, transferred to a sterile moist chamber and incubated at room temperature in the dark for 2 d for seed germination. Fifteen seedlings were used for each experiment, which was performed in triplicate. The pruned-root dip method (Musson et al., 1995) was used to inoculate the seedlings by cutting 0.5 cm from the root tip using a sterile scalpel. Then, the seedlings were immersed in individual sterile glass beakers containing 1×10^8 spores/mL of *Streptomyces* sp. GMKU 336 or its ACCD-deficient mutant for 4 h. The seedlings were re-located to a pot containing sterile soil for 7 d. Next, mung bean plants were flooded with 13 cm of standing water above the soil surface for 21 d. For the positive control, mung bean seedlings were grown under the same conditions without the flooding treatment. The plants were harvested and examined for shoot/root elongation, biomass and plant physiology. The experiment was conducted in a greenhouse during August to October 2017.

Re-isolation of endophytic streptomycetes

Re-isolation of endophytic streptomycetes from the mung bean plants was performed by modifying the method of Rachniyom et al. (2015). Roots and shoots of mung bean were cut separately into small pieces and rinsed with 0.1% (v/v) Tween 20 for 5 min. Then, plant materials were surface sterilized by soaking in 1% (w/v) sodium hypochlorite for 10 min and then immersing in 70% (v/v) ethanol for 1 min and soaking in 10% (w/v) $NaHCO_3$ solution for 5 min. Next, samples of the surface-sterilized plant material were washed in sterile water three times and crushed in ¼ Ringer's solution (0.9% NaCl, 0.042% KCl, 0.048% $CaCl_2$, 0.02% $NaHCO_3$). The resulting solution was spread onto starch casein agar (SCA; Küster and Williams, 1964) supplemented with 100 mg/mL ampicillin, 2.5 U/mL penicillin G, 50 mg/mL amphotericin B and 50 mg/mL cycloheximide. The final washed solution was spread on the medium to ensure that there was no epiphyte contamination. Colonies of endophytic streptomycetes were observed after incubation at 28 ± 2 °C for 7 d and were randomly selected for analysis using 16S rDNA gene sequencing with primers and PCR conditions as described by Rachniyom et al. (2015).

Determination of survival rate

The survival rate of the mung bean plants under flooding conditions was calculated as a percentage compared with those under the non-flooding conditions. Plants that exhibited rot, wilt and died were recorded as dead, whereas surviving plants were recorded as alive.

Determination of leaf color, leaf area, and chlorophyll content

The leaf color was compared with the standardized four panel International Rice Research Institute (IRRI) leaf color chart (LCC) ranging from yellowish green (No. 2) to dark green (No. 5). Leaf area was measured and calculated using grid paper.

Chlorophyll was extracted using dimethyl sulfoxide (DMSO) as described by Hiscox and Israelstam (1979). Approximately 100 mg of leaf fresh weight from each treatment was ground in liquid nitrogen. The macerated leaves were extracted twice by adding 1.0 mL DMSO and sonicated at 30 hz in a water bath sonicator for 2 min. The extracts were centrifuged and the chlorophyll content of the supernatants was measured at 645 and 663 nm within 20 min after the extraction. The chlorophyll content was calculated using Arnon's equations (Arnon, 1949).

Determination of ethylene

Ethylene production was measured by enclosing whole mung bean plants in a 250 mL sealed glass container containing 50 mL acetylene for 1 h. A gas sample (1 mL) was withdrawn and quantified using gas chromatography at the PGPR Biofertilizer and Aerated Compost Soil Microbiology Research Group, Soil Science Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

Statistical analyses

All data from the experiments were calculated and statistically evaluated on 15 seedlings for each condition from biological and technical triplicates. The data were analyzed using one-way analysis of variance and Duncan's test to determine any significant differences between groups at $p < 0.05$. All statistical analyses were performed using the SPSS 20.0 for Windows software (SPSS Inc.; Chicago, IL, USA).

Results

Effect of 1-aminocyclopropane-1-carboxylate deaminase-producing Streptomyces sp. GMKU 336 inoculated mung bean under flooding conditions

Streptomyces sp. GMKU 336 and its ACCD-deficient mutant were inoculated into mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72]. The growth parameters of mung bean were observed after 21 d of flooding treatment in comparison to the non-flooding treatment (Fig. 1A and B, Table 1). Under non-flooding conditions, *Streptomyces* sp. GMKU 336 significantly enhanced plant elongation in shoot/root lengths and the plant biomass in shoot/root fresh and dry weights when compared to the un-inoculated controls (Table 1). Mung bean inoculated with the ACCD-deficient mutant showed similar plant growth parameters to those of the un-inoculated controls plants (Fig. 1A and B, Table 1).

Under flooding conditions, plant elongation and biomass significantly reduced in all plant treatments (Table 1). However, ACCD-producing *Streptomyces* sp. GMKU 336 inoculated mung bean showed significantly more increment in shoot/root elongation

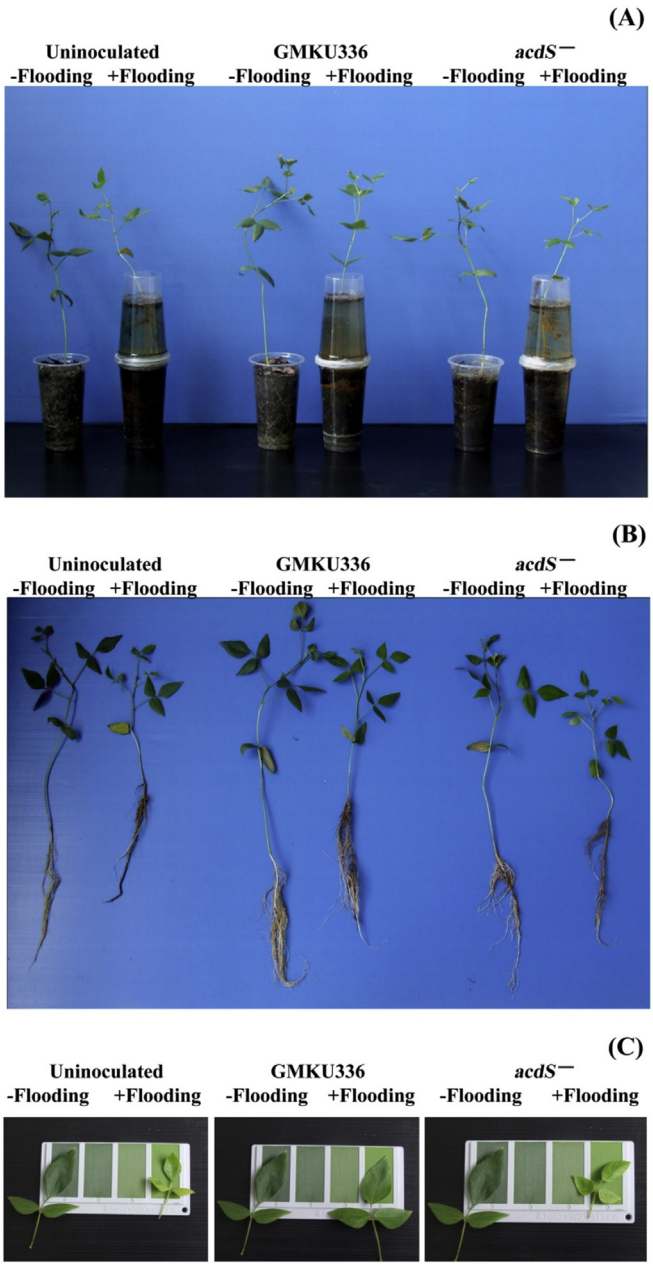

Fig. 1. Effect of 1-aminocyclopropane-1-carboxylate deaminase (ACCD)-producing endophytic *Streptomyces* sp. GMKU 336 on mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] after 21 d of flooding stress: (A) flooding conditions; (B) whole plants, (C) leaf size and color, where Uninoculated = without bacteria treatment; GMKU336 = plant inoculated with *Streptomyces* sp. GMKU 336; acdS^{-} = plant inoculated with ACCD-deficient mutant; −Flooding = control condition; + Flooding = flooding conditions (13 cm flooding).

and biomass (shoot/root fresh and dry weight) compared to the un-inoculated controls (Table 1). Moreover, the development of new adventitious roots was greatly accelerated by flooding stress, particularly in plants associated with strain GMKU 336, which produced more adventitious roots than other treatments (Fig. 1B). In contrast, no development of adventitious roots was observed in the non-flooding controls (Fig. 1B). Furthermore, the survival rate of un-inoculated plants was reduced 20% compared to *Streptomyces* sp. GMKU 336-inoculated plants and those non-flooding treatments (Table 2). ACCD-deficient mutant inoculated plants exhibited similar trends in plant elongation and biomass to the un-inoculated plants in all treatments (Fig. 1B; Table 1). Therefore, ACCD-producing *Streptomyces* sp. GMKU 336 was able to promote the growth of mung bean CN72 with or without flooding stress.

Effect of 1-aminocyclopropane-1-carboxylate deaminase-producing Streptomyces sp. GMKU 336 on leaves and chlorophyll production

The leaf area was markedly reduced under flooding stress in all treatments (Table 2). A significant reduction in the leaf area of un-inoculated plants and those inoculated with the ACCD-deficient mutant was about 2.5-fold when compared to the corresponding non-flooding treatments (Fig. 1C, Table 2). However, leaf area reduction of mung bean associated with *Streptomyces* sp. GMKU 336 was about 1.2-fold lower than in the non-flooding treatment (Fig. 1C, Table 2). Furthermore, the leaf color of mung bean under flooding stress was altered from dark green to yellowish green compared to the non-flooding treatments (Fig. 1C). Conversely, the leaf color of mung bean associated with *Streptomyces* sp. GMKU 336 was greener than the un-inoculated plants and those inoculated with the ACCD-deficient mutant (Fig. 1C).

The chlorophyll content in mung bean was similar in all treatments under normal conditions but was significantly decreased under flooding treatments (Table 2). A significant reduction of 2–2.3 fold of the chlorophyll content was observed in un-inoculated mung bean and those inoculated with the ACCD-deficient mutant compared to the non-flooding controls (Table 2). However, *Streptomyces* sp. GMKU 336-inoculated plants maintained 1.5–1.7 fold higher chlorophyll content than un-inoculated and ACCD-deficient mutant inoculated plants (Table 2). These results suggested that ACCD-producing *Streptomyces* sp. GMKU 336 enhances the leaf area and leaf color as well as the chlorophyll content of mung bean under flooding stress.

Effect of 1-aminocyclopropane-1-carboxylate deaminase-producing Streptomyces sp. GMKU 336 on ethylene production

Under non-flooding conditions, low ethylene levels were determined in all plant treatments (Table 2). The ethylene level was substantially increased 5.3 fold after flooding stress exposure in the un-inoculated mung bean and in mung bean inoculated with the ACCD-deficient mutant compared to the corresponding non-flooding controls. However, *Streptomyces* sp. GMKU 336-inoculated plants had significantly 3-fold lower ethylene levels than the un-inoculated control (Table 2).

Re-isolation of the endophytic streptomycetes from inoculated mung bean

Re-isolation of the wild type and mutant in both flooding and non-flooding treatments was about 1×10^4 colony forming units (CFU)/g root fresh weight (Table 2). Both strains were validated using 16S rRNA gene sequencing (data not shown). In addition, un-inoculated plants did not harbor endophytic *Streptomyces* sp. GMKU 336.

Discussion

ACCD-producing bacteria enhance the growth of a wide range of plants in the presence of various biotic and abiotic stresses, including pathogen damage, flooding, drought, salt and organic and inorganic contaminants (Glick, 2004). Recently, *Streptomyces* sp. GMKU 336 has been reported to promote plant growth under salinity stress via the action of ACCD (Jaemsaeng et al., 2018). This study has contributed further work to define the role of ACCD in plant growth and flooding tolerance of mung bean CN72 [*Vigna*

Table 1
Plant growth and biomass of mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] with and without 1-aminocyclopropane-1-carboxylate deaminase (ACCD)-producing *Streptomyces* sp. GMKU 336 and ACCD-deficient mutant under flooding and non-flooding treatments.

Parameters	Treatments					
	Uninoculated−Flooding	Uninoculated + Flooding	GMKU336−Flooding	GMKU336 + Flooding	acdS$^-$−Flooding	acdS$^-$+ Flooding
Shoot length (cm/plant)	44.3 ± 0.18^b	25.11 ± 0.17^d	46.4 ± 0.23^a	27.19 ± 0.27^c	43.84 ± 0.17^b	23.25 ± 0.25^e
Root length (cm/plant)	18.53 ± 0.16^b	17.07 ± 0.16^d	24.41 ± 0.15^a	18.37 ± 0.15^b	17.87 ± 0.21^c	16.45 ± 0.11^e
Shoot fresh weight (g/plant)	1.82 ± 0.02^b	0.74 ± 0.02^d	1.93 ± 0.02^a	0.82 ± 0.02^c	1.80 ± 0.02^b	0.62 ± 0.02^e
Root fresh weight (g/plant)	0.71 ± 0.01^b	0.44 ± 0.01^d	0.81 ± 0.01^a	0.61 ± 0.01^c	0.69 ± 0.01^b	0.34 ± 0.01^e
Shoot dry weight (g/plant)	0.45 ± 0.01^b	0.17 ± 0.01^d	0.54 ± 0.01^a	0.28 ± 0.01^c	0.43 ± 0.02^b	0.12 ± 0.01^e
Root dry weight (g/plant)	0.17 ± 0.01^b	0.12 ± 0.01^d	0.22 ± 0.01^a	0.14 ± 0.01^c	0.16 ± 0.01^b	0.12 ± 0.01^d

Uninoculated = without bacteria treatment; GMKU336 = plant inoculated with *Streptomyces* sp. GMKU 336; acdS$^-$ = plant inoculated with ACCD-deficient mutant; −Flooding = control conditions; +Flooding = flooding conditions (13 cm flooding).
Values are mean of tree replicates ± SE (n = 15). Different letters indicated statistical differences between treatments (Duncan's test $p < 0.05$).

Table 2
Plant physiological parameters of mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] with and without 1-aminocyclopropane-1-carboxylate deaminase (ACCD)-producing *Streptomyces* sp. GMKU 336 and ACCD-deficient mutant under flooding and non-flooding treatments.

Parameters	Treatments					
	Uninoculated−Flooding	Uninoculated + Flooding	GMKU336−Flooding	GMKU336 + Flooding	acdS$^-$−Flooding	acdS$^-$+ Flooding
Total chlorophyll (mg/g FW)	12.23 ± 0.22^a	6.16 ± 0.27^c	12.19 ± 0.18^a	9.34 ± 0.27^b	12.03 ± 0.17^a	5.39 ± 0.19^c
Leaf area (cm^2/leaf)	13.84 ± 0.90^a	5.77 ± 0.63^c	14.29 ± 0.72^a	11.27 ± 1.03^b	13.57 ± 1.01^a	5.16 ± 0.34^c
Ethylene level (pmol/h/g FW)	1.62 ± 0.12^c	8.58 ± 0.52^a	1.45 ± 0.03^c	2.81 ± 0.11^b	1.50 ± 0.03^c	8.03 ± 0.38^a
Survival rate (%)	100 ± 0.00^a	80 ± 0.00^b	100 ± 0.00^a	100 ± 0.00^a	100 ± 0.00^a	75.5 ± 2.23^c
Endophytic colonization (1×10^4 CFU/g root FW)	ND	ND	4.96 ± 0.08	4.56 ± 0.42	4.65 ± 0.38	4.56 ± 0.43

Uninoculated = without bacteria treatment; GMKU336 = plant inoculated with *Streptomyces* sp. GMKU 336; acdS$^-$ = plant inoculated with ACCD-deficient mutant; −Flooding = control conditions; +Flooding = flooding conditions (13 cm flooding); FW = fresh weight; ND = not detected; CFU = colony forming units.
Values are mean of tree replicates ± SE (n = 15). Different letters indicated statistical differences between treatments (Duncan's test $p < 0.05$).

radiata (L.) Wilczek cv. CN72] by the strain GMKU 336 and its ACCD-deficient mutant.

Streptomyces sp. GMKU 336 has been proved as a true endophyte since it was able to localize and multiply within rice (*Oryza sativa* L. cv. KDML105) without causing any obvious, deleterious effects (Jaemsaeng et al., 2018). In the current work, both *Streptomyces* sp. GMKU 336 and its ACCD-deficient mutant were inoculated into mung bean CN72 and were able to re-isolate from the plants under all conditions. Although, *Streptomyces* sp. GMKU 336 was originally isolated from the medicinal plant *Clerodendrum serratum* (L.) Moon (Indananda, 2013), the results indicated that it has endophytic ability with a wide host range including both monocotyledonous and dicotyledonous plants. The results were in agreement with a previous report on endophytic *Streptomyces* sp. GMKU 3100 originally isolated from rice that was able to neutrally colonize mung bean (Rungin et al., 2012). It clearly indicated that endophytic actinomycetes are nonhost-specific and behave as free-living bacteria. It was suggested that endophytic bacteria may migrate from the rhizosphere to the rhizoplane of their plant hosts and then move to other organs such as roots, stems, leaves and flowers as well as fruits and seeds (Misk and Franco, 2011).

The effects of ACCD-producing *Streptomyces* sp. GMKU 336 and its ACCD-deficient mutant on plant growth were investigated in mung bean CN72 after flooding treatment for 21 d. Under non-flooding conditions, the mung bean plants inoculated with strain GMKU 336 significantly enhanced shoot/root length and biomass when compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant. The results for plant growth were consistent with previous work showing that ACCD-producing *Streptomyces* sp. GMKU 336 has the ability to enhance growth of rice KDML105 (Jaemsaeng et al., 2018). The growth effect has also been found in other ACCD-producing *Streptomyces* for enhanced growth of *Arabidopsis* (Palaniyandi et al., 2013), halophytic *Limonium sinense* (Qin et al., 2014), sugarcane (Kruasuwan and Thamchaipenet, 2016) and tomato (El-Tarabily, 2008).

ACCD-producing *Streptomyces* sp. GMKU 336 inoculated mung bean plants substantially tolerated flooding stress and maintained high shoot and root elongation compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant. The results were in agreement with previous work that an ACCD-producing *Pseudomonas* increased shoot and root growth of tomato (Grichko and Glick, 2001) and canola (Farwell et al., 2007) under flooding conditions.

A decline in photosynthesis under flooding has been reported in mung bean (Ahmed et al., 2002). Here, the leaf chlorophyll content substantially decreased in plants subjected to flooding compared to ones in the non-flooding treatment. However, ACCD-producing *Streptomyces* sp. GMKU 336 reduced this negative effect and maintained a greener leaf color than in the un-inoculated flooding controls. These results were in agreement with previous reports that treatment of ACCD-producing bacteria such as *Achromobacter*, *Herbaspirillum*, *Serratia* and *Ochrobactrum* on *Ocimum sanctum* (Barnawal et al., 2012) and of *Pseudomonas* on tomato (Grichko and Glick, 2001) increased photosynthetic rates which provided some protection against flooding stress.

Morphological adaptation by reducing the leaf area and the induced formation of adventitious roots improved flooding tolerance in mung bean (Ahmed et al., 2002). The current report found that flooding treatment also resulted in a decreased leaf area and affected plant-to-root rot that was observed in un-inoculated plants and those inoculated with the ACCD-deficient mutant. In contrast, mung bean inoculated with ACCD-producing *Streptomyces* sp. GMKU 336 maintained leaf area and increased adventitious root development. Likewise, ACCD-producing *Pseudomonas* clearly stimulated the development of both adventitious roots and the stem in tomato under flooding stress (Grichko and Glick, 2001).

Flooding causes an increase in the accumulation of stress ethylene in plants. Plants inoculated with ACCD-producing PGPB have been documented to show substantial tolerance to flooding stress by lowering stress ethylene (Glick, 2004; Saleem et al., 2007).

In the current work, a significant induction of ethylene production was observed in all plant treatments under flooding stress. Remarkably, mung bean CN72 inoculated with *Streptomyces* sp. GMKU 336 had a lower increment in the ethylene level. These results suggested that the ACCD-producing *Streptomyces* sp. GMKU 336 decreased ethylene production of mung bean via an action of ACCD. Jaemsaeng et al. (2018) recently reported that *Streptomyces* sp. GMKU 336 associated with rice KDML105 that had been exposed to salt stress significantly expressed the *acdS* gene *in vivo* which converted a precursor of ethylene, ACC, in plants into ammonia and α-ketobutyrate, and consequently reduced the levels of ethylene, reactive oxygen species (ROS) and the Na^+ content and Na^+/K^+ ratio. Therefore, it is implied that *Streptomyces* sp. GMKU 336 enhances flooding tolerance in mung bean in the same manner as salt tolerance in rice.

In conclusion, all of the experimental data indicated that ACCD-producing endophytic *Streptomyces* sp. GMKU 336 promoted growth of mung bean CN72 under both normal and flooding conditions. The presence of *Streptomyces* sp. GMKU 336 also enhanced flooding tolerance in mung bean CN72 by decreasing the ethylene production and by increasing the plant growth and biomass, chlorophyll content, leaf area, leaf color and formation of adventitious roots. Noticeably, plants inoculated with the ACCD-deficient mutant exhibited plant growth parameters and physiology in the same manner as the un-inoculated controls. These results clearly indicated the beneficial effects of ACCD-producing endophytic *Streptomyces* sp. GMKU 336 on plant growth promotion under various stress tolerance conditions (salinity and flooding).

Conflict of interest

There is no conflict of interest.

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