



Effect of *Methylobacterium radiotolerans* ED5-9 with Capability of Producing Indole-3-Acetic Acid (IAA) and 1-Aminocyclopropane-1-Carboxylic Acid Deaminase on the Growth and Development of *Murdannia loriformis* (Hassk.) Rolla Rao & Kammathy under *In Vitro* Condition

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Abstract

This study aimed to evaluate the production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzyme from endophytic *Methylobacterium radiotolerans* ED5-9, and to investigate the effects of *M. radiotolerans* ED5-9 on *Murdannia loriformis*, under tissue culture condition. *M. radiotolerans* ED5-9 was isolated from leaves of *M. loriformis* and cultured *in vitro*. It produced indole-3-acetic acid (IAA) with a concentration of 3.36 ± 0.20 $\mu\text{g/ml}$ after incubation for 60 hr. Activity of ACC deaminase enzyme was observed at 365.05 ± 90.51 nmol of α -ketobutyrate/mg of protein/h. To test the effects of inoculation, the experiment was carried on by immersion of the explants of *M. loriformis* into *M. radiotolerans* ED5-9 suspension for 1, 3 and 5 min, and subsequently cultured on the MS medium in which 2 mg/l of IAA substance was used as the control. The results showed that duration time of the immersed explants in *M. radiotolerans* ED5-9 suspension for 5 min had the highest average number of shoots (6.00 ± 1.00 shoots per explants), root length (0.71 ± 0.26 cm) and dry weight (46.00 ± 5.40 mg). However, the above results of growth were similar to the growth of *M. loriformis* explants in the control. The results indicated the possible utilizing of *M. radiotolerans* ED5-9 can produce ACC deaminase enzyme and IAA to enhance growth and development of the *M. loriformis* explants under the *in vitro* condition.

Keywords: *Methylobacterium radiotolerans*, *Murdannia loriformis*, indole-3-acetic acid, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, *in vitro*

Introduction

The genus *Methylobacterium* is Gram-negative, aerobic and rod-shaped bacteria that requires oxygen for respiration system. They can utilize C1-compounds such as methanol, formaldehyde and formate as carbon and energy sources for growth as well as promoting plant growth. (Green, 2006) *Methylobacterium* can be naturally found, especially in various parts of plants, for example, in the surfaces (epiphytes) and the internal tissues (endophytes). (Green, 2006) These bacteria commonly colonize the phyllosphere as a phytosymbionts. They consume methanol released

during plant growth and also use this methanol as a carbon and energy source for their own growth to synthesize plant hormones, such as auxin and cytokinin. (Tsavkelova, Klimova, Cherdyntseva, & Netrusov, 2006) Therefore, *Methylobacterium*, which is regarded as a plant hormone producing bacteria, has the property to increase the development of various physiological characterizations of plants; like branching, seedling vigour, seed germination, seedling growth, root differentiation and photosynthetic activity by enhancing the chlorophyll concentration. (Dileepkumar & Dube, 1992; Holland & Polacco, 1994; Holland, 1997; Cervantes, Graham & Andrade, 2005) In addition, this microbe is able to generate 1-aminocyclopropane-1-carboxylic



acid (ACC) deaminase enzyme that involves and balances ethylene hormone in plants tissues. (Madhaiyan, Poonguzhali, Ryu, & Sa, 2006) Bacterial ACC deaminase can decrease the levels of ACC that come from the stimulation of growth in the root by auxin or indole-3-acetic acid (IAA). (Penrose & Glick, 2001) The reduction of ACC levels results in lower ethylene hormone levels, thus influencing root elongation and plant growth. The lower levels of ethylene hormone are, the higher enhancement of root initiation, growth and root elongation. The converse results to root were observed with higher levels of ethylene. (Glick, Penrose, & Li, 1998; Glick et al., 2007)

The relationship between beneficial bacteria and plants in promoting plant development and differentiation has been reported under both *in vivo* and *in vitro* conditions. An efficient technique used to generate many plantlets in a short period of time is incubating plants in artificial medium under sterile condition, absence of plant hormones, and presence of some beneficial bacteria, that is, *Azospirillum brasilense* Sp245 or *Methylobacterium* sp. D10. (Debnath, Malik, & Bisen, 2006; Russo et al., 2008; Neumann, Kumar, & Imani, 2009) These beneficial bacteria could promote plant growth, induction of callus formation, stimulation a well developed root system of plant (Kalyaeva et al., 2003; Russo et al., 2008; Neumann et al., 2009), formation of shoot, accumulation of pigment (chlorophyll a, chlorophyll b and carotenoids) (Devi, Sundaram, & Poorniammal, 2010) by stimulating formation of shoot and roots. Furthermore, some beneficial bacteria such as plant growth-promoting rhizosphere (PGPR) can promote synthesis of phenolic compounds and secondary metabolites, for example, in case of chickpea (*Cicer arietinum*) which could produce phenolic compound after spraying with PGPR. (Singh, Sarma, & Singh, 2003)

Previous studies were reported the ability of *M. radiotolerans* ED5-9 which were isolated from leaves of *Murdannia loriformis* (Hassk.) Rolla Rao & Kammathy, one of the herbs used to prevent cancer in Thailand, to produce IAA in medium without L-tryptophan supplement. (Jiratchariyakul, Okabe, Moongkamdi, & Frahm, 1998; Jiratchariyakul et al., 2006; Sarin, Prombunchachai, Nakaew, & Chidburee, 2013). The characteristic of *M. radiotolerans* ED5-9 that can produce IAA was similar to the other beneficial bacteria. This strain also dwells in associate plants without further damage. Since many advantages of *M. radiotolerans* have been reported, this species was provoked interest from many researchers to extend their investigation. Therefore, the present study focused on the cultivation of *M. radiotolerans* ED5-9 with *M. loriformis* under *in vitro* conditions in order to evaluate the physiological and morphological results of *M. loriformis*, and also to study additional features of *M. radiotolerans* ED5-9 on production of ACC deaminase.

Methods and Materials

Bacterial strains and culture conditions

M. radiotolerans ED5-9, which can produce IAA in medium without L-tryptophan supplement, was isolated from leaves of *M. loriformis*. (Sarin et al., 2013) *M. radiotolerans* ED5-9 was cultured in a 250 ml Erlenmeyer flask containing 100 ml liquid nutrient medium (LNM) with 2 g/l KH_2PO_4 , 2g/l $(\text{NH}_4)_2\text{SO}_4$, 0.125 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/l NaCl, 0.002 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.1 g/l yeast extract. The LNM medium was sterilized by autoclaving at 121°C for 15 min and set aside to cool before adding of 1% (v/v) filter (Millipore, 0.45µm) sterilized methanol. The culture was incubated in an incubator shaker under the shaking rate of 180 rpm at 30°C for 60 hrs and then it was centrifuged at 8,000 g at 4°C for 15 min. (Shirokikh,



Shupletsova, & Shirokikh, 2007; Sarin et al., 2013) The supernatant from centrifugation was stored at 4°C for analysis of bacterial IAA concentration, while the bacterial cells were washed twice in sterile 30 mM MgSO₄.7H₂O. Cell concentration was adjusted to approximately 10⁹ cell/ml using sterile 30 mM MgSO₄.7H₂O before inoculation into explants. (Madhaiyan, Poonguzhali, & Sa, 2007)

Estimation of plant growth promoting characteristics of *M. radiotolerans* ED5-9

Determination of IAA

One millilitre of supernatant was mixed in a test tube with 2 ml of Salkowski's reagent composed of 4.5 g/l FeCl₃ in 10.8 M H₂SO₄. The mixture reagent was incubated at room temperature in the dark for 30 min before measuring the absorbance at 530 nm by spectrophotometer (Pharmacia Biotech Novaspec II). (Glickmann & Dessaux, 1995) The absorbance value was compared with the standard curve of IAA concentration. The IAA compound in supernatant was also detected by high performance liquid chromatography (HPLC). The supernatant was filtered with 0.45 millipore filter and analyzed by HPLC (Shimadzu LC-10AD) equipped with column Prodigy 5U OD53 100A (250 x 4.6 mm). Methanol/water/acetic acid (40:60:1) were used as mobile phase at a flow rate of 1 ml/min. Elutes were detected at 220 nm. Authentic IAA was used as standard. (Ali & Hasnain, 2007)

ACC deaminase assay

Activity of ACC deaminase enzyme was determined from the amount of α-ketobutyrate which was the by product from the hydrolyzed ACC. Bacteria were incubated in 15 ml of glycerol peptone (GP) broth in incubator shaker at 180 rpm at 30°C for 60 hr until their growth was late log phase up to stationary phase. Then, it was centrifuged with 8,000 g at 4°C for 15 min. Bacterial cells were washed with sterile DF minimal salts medium and resuspended in 7.5 ml sterile DF minimal salts

medium to which 45 μl of 0.5M ACC was added as nitrogen source. The culture was incubated in incubator shaker at 180 rpm at 30°C for 24 hr before centrifugation. Bacterial cells were used to measure the activity of ACC deaminase enzyme by detecting the quantity of α-ketobutyrate at the wavelength of 540 nm and compared with the standard curve of α-ketobutyrate concentration. (Penrose & Glick, 2003)

Effect of *M. radiotolerans* ED5-9 on the growth of *M. loriformis* explants

Source of plant explants and experimental design

The compact callus explant of *M. loriformis* in this experiment was cleaned for culture, incubated on Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) to which 2 mg/l benzyladenine (BA) was added under light intensity at 42 mol/m²/s for photosynthetic active radiation at 25±2°C with a 16-h-light photoperiod for 4 weeks until the explants completely developed. The leaves and shoots were cut out from cleaned cultures. The size of cut plants were about 0.5 x 0.5 cm. The experiment was laid out in a completely randomized design (CRD) divided into 4 treatments as follows: (T1) explants imbibed in bacterial suspension (BS) for 1 min, (T2) explants imbibed with BS for 3 min, (T3) explants imbibed with BS for 5 min and (T4) explants not treated with BS (control). Then, T1-T3 were cultured on MS media while T4 was cultured on MS media supplemented with IAA at 2 mg/l. All treatments were cultured on shelf under light intensity 42 mol/m²/s for photosynthetic active radiation at 25±2°C with a 16-h-light photoperiod for 4 weeks.

The growth and development of *M. loriformis* explants in tissue culture in each treatment were carefully observed. Changes in growth (number of shoots, roots and leaves, the roots length and the dry weight), physiology (pigment content) and the quantity of secondary metabolites (total phenolic



content and antioxidant activity) were monitored and recorded after 4 weeks.

Preparation of methanolic explants extracts (Pongsathorn, Duangporn, Sireethon, & Pornchanok, 2012)

The methanolic extract was prepared from the explants aged 4 weeks. One gram of explant was crushed thoroughly using mortar and a few drops of 95% methanol were added. The volume was adjusted using 95% methanol until the total volume of 50 ml.

$$\begin{aligned} \text{Chl}_a &= 15.65A_{666} - 7.340A_{653} \\ \text{Chl}_b &= 27.05A_{653} - 11.21A_{666} \\ C_{x+c} &= (1000A_{470} - 2.860\text{Chl}_a - 129.2\text{Chl}_b) / 245 \end{aligned}$$

Where, Chl_a = the chlorophyll a content, Chl_b = the chlorophyll b content and C_{x+c} = the carotenoid content. A_{470} = the absorbance at 470 nm, A_{653} = the absorbance at 653 nm and A_{666} = the absorbance at 666 nm.

Determination of total phenolic content (Pongsathorn et al., 2012)

The total phenolic compound was determined using Folin-Ciocalteu method. One milliliter of methanolic extract was carefully mixed with 9 ml of deionized water and 1 ml of Folin-Ciocalteu reagents and then allowed to stand for 5 min. Subsequently, ten milliliter of 7% Na_2CO_3 solution was added into the mixture. The volume was then adjusted using deionized water to total volume of 25 ml. The mixture was incubated at room temperature for 30 min in the dark before measuring the absorbance at

The explant extracts were used to determine some chemical properties of *M. loriformis*.

Determination of pigment content (Lichtenthale & Buschman, 2001; Pongsathorn et al., 2012)

The pigments including chlorophyll a, chlorophyll b and carotenoids were quantified by measuring the absorbance at wavelength 470, 653 and 666 nm, respectively. The pigment content was calculated using the absorbance value by the following equation:

750 nm. Gallic acid equivalent was used to estimate the total phenolic compounds. The concentration of gallic acid in each sample was determined by comparison with the gallic acid standard curve.

Determination of DPPH free radical scavenging activity (Pongsathorn et al., 2012)

Antioxidant activity was determined using free radical scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH). Fifty micro liter of methanolic extract was mixed with 2 ml of 0.06 mM DPPH and incubated at room temperature for 30 min in the dark. Then, the reduction of the DPPH free radical was measured using absorbance at 517 nm and calculated by standard curve of the concentration of DPPH solutions. Scavenging activity was calculated as percentage inhibition by using the following equation:

$$\text{Inhibition (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where, A_{control} = absorbance of the control (DPPH solution without sample) and A_{sample} = absorbance of the test sample extract. Scavenging activity was compared with positive control using synthetic antioxidant: trolox (analog of vitamin E). The

antioxidant activity of each sample was reported in terms of IC50 as the concentration of extract to inhibit free radical formation at 50% with the calculation from the percentage of inhibition curve. (Hsh, 2006)



Statistical analysis

All treatments in the tissue culture contained 3 replicates and each replicate contained 3 explants. Data on each of treatments were analyzed using SPSS v16.0. Least Significant Difference (LSD) comparison methods at significant level at 0.05 were employed to analyse variance procedure and difference among treatment means.

Results

Estimation of plant growth promoting characteristics of *M. radiotolerans* ED5-9

The amount of IAA produced by *M. radiotolerans* ED5-9 in LNM medium was measured using colorimetric measurement applying Salkowski's

reagent as a specific reagent which reacts to indole compounds. The concentration of IAA in liquid medium was $3.36 \pm 0.20 \mu\text{g/ml}$. HPLC analysis for the IAA detection showed that the peak of the IAA standard and the culture filtrate were presented at the same retention time (Figure 1) which shows that *M. radiotolerans* ED5-9 produces IAA in fermented broth. The activity of ACC deaminase enzyme to utilize ACC of *M. radiotolerans* ED5-9 in the cell free extract was gauged by analyzing the level of α -ketobutyrate as product of ACC hydrolysis. The activity of ACC deaminase enzyme was $365.05 \pm 90.51 \text{ nmol } \alpha\text{-ketobutyrate/mg of protein/h}$.

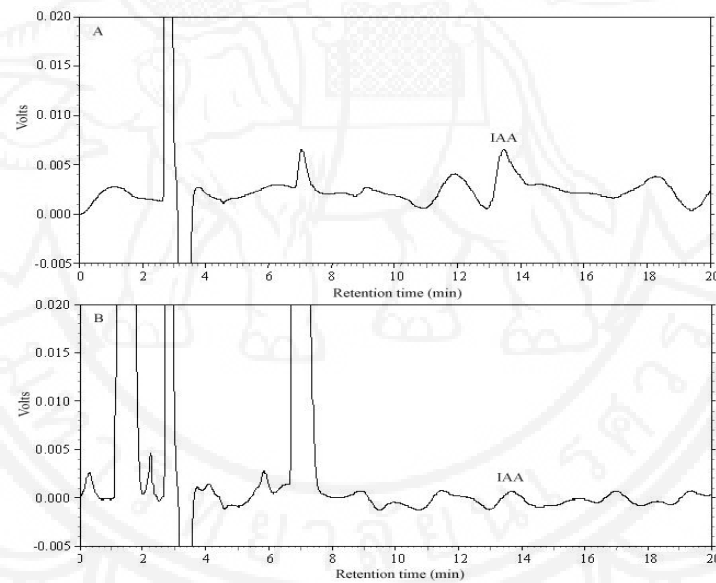


Figure 1 The comparison of HPLC analysis between IAA standard solution (A) and the bacterial supernatant (B) Effect of *M. radiotolerans* ED5-9 inoculation on the growth of *M. loriformis* explants

The *M. loriformis* explants in the each treatment had grown and formed small sized shoots, roots and leaves. The number of shoots, roots and leaves, the length of root and the dry weight of the *M. loriformis* explants in the each treatment were shown in Table 1. *M. radiotolerans* ED5-9 was able to promote the growth of *M. loriformis* explants similarly to the synthetic IAA hormone. The various parameters of growth in the each treatment did not differ

significantly. The number of shoots, the length of roots and the dry weight of explants in T3 had the highest average of 6.00 ± 1.00 shoots per explant, $0.71 \pm 0.26 \text{ cm}$ and $46.00 \pm 5.40 \text{ mg}$, respectively. In addition, the number of leaves in T1 had the highest average at 5.94 ± 1.23 leaves per explant and the number of roots in T4 had the highest average at 13.67 ± 6.11 roots per explant.



Table 1 The characteristic of *M. loriformis* explants imbibed with *M. radiotolerans* ED5-9 after cultivation for 4 weeks

| Treatments | No. of Shoots/explant (shoots) | No. of Leaves/explant (leaves) | No. of Roots/explant (roots) | Length of Root/explant (cm) | Dry weight (mg) |
|------------------|--------------------------------|--------------------------------|------------------------------|-----------------------------|-----------------|
| T1(1min+MS) | 4.22 ± 2.12 | 5.94 ± 1.23 | 7.56 ± 0.96 | 0.64 ± 0.05 | 35.50 ± 10.10 |
| T2(3min+MS) | 3.89 ± 0.15 | 4.28 ± 2.10 | 8.17 ± 2.02 | 0.46 ± 0.14 | 30.60 ± 5.10 |
| T3(5min+MS) | 6.00 ± 1.00 | 5.89 ± 0.84 | 12.00 ± 3.46 | 0.71 ± 0.26 | 46.00 ± 5.40 |
| T4(MS+2mg/l IAA) | 3.11 ± 1.39 | 4.00 ± 2.83 | 13.67 ± 6.11 | 0.64 ± 0.05 | 36.50 ± 4.40 |

Each value is presented as mean ± standard deviation (SD)

Pigment content of extracts

The pigment content of 4 weeks old tissue cultured *M. loriformis* was determined. The quantities of pigments: chlorophyll a, chlorophyll b and carotenoids do not differ significantly as shown in

Table 2. The treatment 1 had the highest average quantity of chlorophyll a and chlorophyll b at 5.45 ± 1.80 and 4.40 ± 1.02 mg/g fresh weight. However, T3 had the highest average quantity of carotenoids at 1.11 ± 0.45 mg/g fresh weight.

Table 2 The pigment content of *M. loriformis* explants imbibed with *M. radiotolerans* ED5-9 after cultivation for 4 weeks

| Treatments | Chlorophyll content (mg/g fresh weight) | | |
|------------------|---|---------------|-------------|
| | Chlorophyll a | Chlorophyll b | Carotenoids |
| T1(1min+MS) | 5.45 ± 1.80 | 4.40 ± 1.02 | 0.84 ± 0.40 |
| T2(3min+MS) | 4.88 ± 0.51 | 3.67 ± 0.52 | 0.89 ± 0.08 |
| T3(5min+MS) | 5.37 ± 1.07 | 3.37 ± 1.05 | 1.11 ± 0.45 |
| T4(MS+2mg/l IAA) | 4.29 ± 0.73 | 3.07 ± 1.74 | 0.83 ± 0.26 |

Each value is presented as mean ± standard deviation (SD)

Total phenolic content of extracts

The total phenolic compounds, as secondary metabolites produced during the growth of plants, are classified to belong to a large group of antioxidant agents which act as free radical terminators. The synthetic IAA hormone and *M. radiotolerans* ED5-9 were able to stimulate the production of phenolic compounds in tissues of *M. loriformis* under tissue culture conditions as shown in Table 3. The quantity of total phenolic compounds in the each treatment, however does not differ significantly although T2 had the highest average in terms of the quantity of total phenolic compounds at 0.93 ± 0.10 mg/g fresh weight.

Antioxidant activity

The antioxidant activity of the methanolic extracts from *M. loriformis* in the each treatment was able to reduce the concentration of stable free radical of DPPH. Activity of antioxidants with similar values in each treatment does not differ significantly as show in Table 3. The efficiency of antioxidant was shown to have IC50 values, indicating that the concentration in the sample could reduce free radical down to 50%. The most efficient antioxidant in the extracts showed lower IC50 values as shown in Table 3. T4 had the most efficient antioxidant having the lowest average IC50 values among all treatments at 6.62 ± 0.71 mg/g fresh weight, although, no significant difference was observe among treatments.



Table 3 Total phenolic content and antioxidant activity of *M. loriformis* explants imbibed with *M. radiotolerans* ED5-9 after cultivation for 4 weeks

| Treatment | Total phenolic content (mg/g fresh weight) | Antioxidant activity (mg/g fresh weight) | IC50 (mg/g fresh weight) |
|------------------|---|---|-----------------------------|
| T1(1min+MS) | 0.71 ± 0.17 | 1.45 ± 0.03 | 9.68 ± 4.66 |
| T2(3min+MS) | 0.93 ± 0.10 | 1.49 ± 0.02 | 8.17 ± 0.18 |
| T3(5min+MS) | 0.68 ± 0.23 | 1.48 ± 0.05 | 7.22 ± 1.37 |
| T4(MS+2mg/l IAA) | 0.53 ± 0.10 | 1.47 ± 0.02 | 6.62 ± 0.71 |

Each value is presented as mean ± standard deviation (SD)

Discussion

This study determined the effect of *M. radiotolerans* ED5-9 in stimulating the growth and development of *M. loriformis* under *in vitro* condition. The level of IAA and ACC deaminase enzyme was also observed in *M. radiotolerans* ED5-9. The concentration of IAA detected in this study was similar to Ivanova, Doronina and Trotsenko (2001) who reported that *M. radiotolerans* VKM B-2144 (=JCM 2831) can produce IAA concentration at 3.00 µg/ml. The property of IAA and ACC deaminase enzyme production is the characteristic of beneficial bacteria. So, *M. radiotolerans* ED5-9 was regarded as one of the beneficial strains. In general, the IAA was synthesized by bacteria in genus *Methylobacterium* which used L-tryptophan as a precursor via indole-3-pyruvic acid pathway. (Doronina, Ivanova, & Trotsenko, 2002) Interestingly, in this study, *M. radiotolerans* ED5-9 can synthesize IAA substance in the culture medium without L-tryptophan supplements. The previous study of Prinsen, Costacurta, Michiels, Vanderleyden, and Onckelen (1993) reported that *Azotobacter brasilense* can produce IAA substance in case of L-tryptophan lacking via tryptophan-independent pathway. Therefore, the IAA synthesis from *M. radiotolerans* ED5-9 in case of the culture medium without L-tryptophan supplements in this study result might be synthesized IAA substance via tryptophan-independent pathway similar to A.

brasilense. In addition, the utilizing of ACC from *M. radiotolerans* ED5-9 revealed the presence of ACC deaminase enzyme concordant to the other strain, like *M. radiotolerans* COLR1. Chinnadurai, Balachandar and Sundaram (2009) reported that *M. radiotolerans* COLR1 which was isolated from phyllosphere of rice has activity of ACC deaminase enzyme at 339.39 ± 5.53 nmol α -ketobutyrate/mg of protein/h. This enzyme can also reduce the ethylene levels in tissues of plant, Therefore length of shoots, length of root and plant biomass of rice and tomato were observed to be higher than the control. (Chinnadurai, Balachander, & Sundaram, 2009)

The growth and development of plant explants in tissue culture were indicated by the alteration of morphology (number of shoots, roots and leaves, the root length and the dry weight), physiology (chlorophyll), and accumulation of secondary metabolites. The growth and development of plant explants not only depend on nutrients but also were influenced by balance of plant hormone within tissue, especially the ratio between auxin and cytokinin. Auxin is a primary factor controlling the growth and morphology of roots while cytokinin to effects to secondary metabolites formation. (Arroo et al., 1995; Rao & Ravishankar, 2002) This experiment inoculated explants with *M. radiotolerans* ED5-9 resulting in auxin and ACC deaminase enzyme production, directly affecting growth and development of morphogenetic structures. This development might result from the interruption of *M.*



radiotolerans ED5-9 in balancing the system of endogenous plant hormones in the explants. The present result is similar to previous research (Shirokikh, Shupletsova, & Shirokikh, 2007) that used *Methylobacterium* sp. to produce auxin and stimulate growth of root by applying to callus of potato. (Shirokikh et al., 2007) Theoretically, the quantity of chlorophyll a, chlorophyll b and carotenoids correlates to chloroplast development whereas total phenolic compound correlates to phenylpropanoid pathway. However, chlorophyll a, chlorophyll b, carotenoids and phenolic compound link with cytokinin production. (Singh et al., 2003; Devi et al., 2010) The result found that the production of chlorophyll a, chlorophyll b, carotenoids and phenolic compound from both control and inoculating treatment do not differ, indicating that both treatments are not influenced by cytokinin.

Conclusion

M. radiotolerans ED5-9 had an ability to produce ACC deaminase enzyme and IAA to promote morphological growth and development of *M. loriformis* in tissue culture better than synthetic IAA hormone. However, inoculation and synthetic IAA produced similar amounts of pigment and total phenolic compounds. In addition, the property of ACC deaminase enzyme was expected to encourage the vigor of seedlings and resistance to adverse conditions. The positive results of *M. radiotolerans* ED5-9, which have the ability to live with plants, on the growth and development of explants of *M. loriformis* in tissue culture, are an alternative to possibility of the application in the plant tissue culture.

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