# Effect of Fermentation Broth from Indole-3-Acetic Acid (IAA) Producing *Methylobacterium radiotolerans* ED5-9 on the Growth and Development of *Murdannia loriformis* (Hassk.) Rolla Rao & Kammathy under *In vitro* Condition

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**Abstract**

The possible role of indole-3-acetic acid (IAA) produced from *Methylobacterium radiotolerans* ED5-9 has been studied in the tissue culture of *Murdannia loriformis* for 4 weeks. The concentration of IAA in liquid nutrient medium (LNM) of *M. radiotolerans* ED5-9 culture was 3.36 ± 0.20 μg/ml. This supernatant was mixed with Murashige and skoog (MS) medium in varying volumes (1, 2 and 4 ml/l) and compared with MS medium supplemented with IAA for the tissue culture of *M. loriformis*. Results show that bacterial IAA can promote plant growth in terms of increasing number of shoots, roots and leaves as well increases in root length and dry weight more than chemically synthesized IAA. Root formation and increase in root length was found to be highest in treatments having 2 ml/l bacterial supernatant in 0.8% agar medium with an average of 11.00 ± 4.60 roots per explants and 2.10 ± 0.70 cm, respectively. In addition, Total phenolic compounds and antioxidant activity were found in quantity as 0.36 ± 0.46 and 4.14 ± 0.52 mg/g fresh weight, respectively. As the concentration of IAA in supernatant from *M. radiotolerans* ED5-9 was increased, the number of roots also increased.

**Keywords:** indole-3-acetic acid (IAA), *Methylobacterium radiotolerans* ED5-9, *Murdannia loriformis*, Tissue culture

## Introduction

Tissue culture is a technique of plant propagation using plant cells, tissues and organs cultured on artificial medium under aseptic condition to produce plantlets in large number with better health status but having the same genetic material in short time (Debnath, Malik, & Bisen, 2006). The artificial medium being used in tissue culture contains nutrients and plant hormones such as auxin or indole-3-acetic acid (IAA) which is important and necessary in controlling physiological processes such as cell division, cell enlargement and cell differentiation (Neumann, Kumar, & Imani, 2009). Moreover, the ratio between auxin and cytokinin is associated with controlling the growth in which auxin acts as a primary factor in the control of growth and morphology of roots and cytokinin affects secondary metabolite formation (Rao & Ravishankar, 2002; Arroo et al., 1995).

The synthesis of the hormone IAA is not restricted to plants. It is also found to be synthesized by various bacterial species such as the group of pink-pigmented facultative methylotrophs (PPFMs) which belongs to the genus *Methylobacterium*. These bacteria are Gram negative, rod shaped and require oxygen for cellular respiration (Green, 2006). This group of bacteria are most likely to be found living in the phyllosphere regions of plants, both on the surfaces (epiphytes) and in the internal tissues (endophytes), having a symbiotic relationship with the plant. These bacteria consume methanol being released from the plants and use it as a carbon and energy source for their own growth. As a result of metabolism, some plant hormones such as auxin and cytokinin are released by the bacteria and can stimulate the growth of plants (Kutschera, 2007). Earlier

studies have shown that the use of *Methylobacterium* sp. D10 cells can stimulate morphogenesis of callus and shoots of soft wheat (*Triticum aestivum*) and the regenerated plants display bright green leaves and a well developed root system. Similarly, *Methylobacterium* sp. NPFM-SB3 was found to promote formation and growth of lateral roots in rice seedlings (*Oryza sativa* L. Cv CO-43) (Kalyaeva et al., 2003; Senthilkumar, Madhaiyan, Sundaram, & Kannaiyan, 2007). In addition, the supernatant produced from the bacterium *Halomonas desiderata* RE1 mixed in Murashige and Skoog (MS) medium can promote elongation of shoots of *Brassica oleracea* L. more than the control group (Ali & Hasnain, 2007). The study of Mostafa & Alhamd (2011) found that IAA have effect to increase production of secondary metabolites (such as total chlorophyll content, antioxidant and total phenolic compound) in *Balanites aegyptiaca* plants. Similarly, quantity of total phenolic compound and antioxidant activity increases in *Canscora decussata* (Roxb.) Roem. & Schult (Kousalya & Bai, 2016).

The ability of IAA from the supernatant of bacterial culture medium to promote growth of plants *in vitro* is interesting. In this study, supernatant from *Methylobacterium* was used in plant tissue culture. Previously, *Methylobacterium radiotolerans* ED5-9 was isolated from leaves of *Murdannia loriformis*, a medicinal plant thought by Thai people to protect and cure cancer, inhibit cancer cells and improve the quality of life for patients after chemotherapy (Jiratchariyakul, Okabe, Moongkarndi, & Frahm, 1998; Jiratchariyakul et al., 2006; Sarin, Prombunchachai, Nakaew, & Chidburee, 2013). Therefore, the main objective of this study was to investigate the possibility of using the supernatant containing IAA in varying concentrations to promote growth and development in the tissue culture of *M. radiotolerans* ED5-9 compared with the use of synthetic IAA at the same concentration. The results of this study can be used in the future in developing a suitable method to induce plant growth and replace the use of chemically synthesized hormones in plant tissue culture.

## Methods and Materials

### Bacterial strains and culture conditions

*M. radiotolerans* ED5-9 in this study was isolated from leaves of *M. loriformis* (Sarin, Prombunchachai, Nakaew, & Chidburee, 2013). The bacterium was cultured in liquid nutrient medium (LNM) with composition of 2 g/l $KH_2PO_4$, 2 g/l $(NH_4)_2SO_4$, 0.125 g/l $MgSO_4.7H_2O$, 0.5 g/l NaCl, 0.002 g/l $FeSO_4.7H_2O$, and 0.1 g/l yeast extract. After sterilization, methanol was mixed at 1% (vol/vol) in the medium and then incubated in an incubator shaker at the rate of 180 rpm at 30°C for 60 hr (Shirokikh, Shupletsova, & Shirokikh, 2007). Subsequently, the medium from the culture was centrifuged at 8,000 g at 4°C for 15 min and the supernatant was kept for estimation of IAA concentration and used in the experiments on plant tissue culture.

### Estimation of IAA of *M. radiotolerans* ED5-9

The quantity of IAA in supernatant was detected by colorimetric measurement using spectrophotometer. One milliliter of supernatant was mixed with 2 ml Salkowski's reagent which consisted of 4.5 g/l $FeCl_3$ in 10.8 M $H_2SO_4$ and incubated at room temperature in the dark for 30 min before measuring the absorbance at 530 nm (Glickmann & Dessaux, 1995). The concentration of IAA in the supernatant was calculated based on absorbance values compared with a standard curve. The presence of IAA was confirmed using high performance liquid chromatography (HPLC). The supernatant was filtered with 0.45 millipore and analyzed

using HPLC (Model Shimadzu LC-10AD) equipped with column Prodigy 5U OD53 100A (250 x 4.6 mm). Methanol/water/acetic acid (40:60:1) was used as mobile phase at a flow rate of 1 ml/min. Eluates were detected at 220 nm at 30°C. Authentic IAA (Sigma) was used as standard (Ali & Hasnain, 2007).

**Plant material and culture media**

The surface of *M. loriformis* shoots were sterilized with 70% ethanol for 30 sec and 0.6% NaClO solution for 30 min then washed with sterile water 4-5 times and incubated on Murashige and Skoog (MS) medium (Murashige & Skoog, 1962). with 2 mg/l of benzyladenine (BA) under a light intensity of 42 mol/$m^2$/s for photosynthetic active radiation at 25 ± 2°C with a 16-hr-light photoperiod for 3 weeks until the shoots completely developed. Subsequently, the leaves and the shoots of *M. loriformis* were cut to about 0.5 x 0.5 cm. The cut tissues were then cultured on MS medium containing varied volume or concentration of supernatant from bacterial culture medium. The experiment was laid out in a completely randomized design (CRD) with 6 treatments and each treatment included 5 replications as follows: (T1), (T2) and (T3) MS medium with the bacterial supernatant (BS) in volume 1, 2 and 4 ml/l, respectively, (T4) 0.8% agar medium with 2 ml/l of BS, (T5) MS medium with synthetic IAA at a concentration of 6.7 µg/l, and (T6) MS medium as control. All treatments were cultured under a light intensity of 42 mol/$m^2$/s for photosynthetic active radiation at 25±2°C with a 16-h-light photoperiod for 4 weeks. The changes in growth parameters (number of shoots, roots and leaves, the roots length and the dry weight); physiological parameters (pigment content) and the quantity of secondary metabolites (total phenolic content and antioxidant) were recorded in all treatments.

**Preparation of methanolic extracts**

The *M. loriformis* explants grown for 4 weeks were thoroughly crushed in a mortar. A few drops of methanol were added during crushing. Finally the total volume was adjusted to 50 ml. This was used for the analysis of pigment content, total phenolic compounds and antioxidant activity. The same method was done in all treatments (Pongsathorn, Duangporn, Sireethon, & Pornchanok, 2012).

**Determination of pigment content**

The detection of the quantity of pigments such as chlorophyll a, chlorophyll b and carotenoids in the plant samples was determined using the absorbance values of methanol extract at wavelengths of 470, 653 and 666 nm. The quantity of pigment in the extract was then calculated using the following equation (Lichtenthale & Buschman, 2001; Pongsathorn et al., 2012):

$$Chl_a = 15.65A_{666} - 7.340A_{653}$$
$$Chl_b = 27.05A_{653} - 11.21A_{666}$$
$$C_{x+c} = (1000A_{470} - 2.860Chla - 129.2Chlb)/245$$

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

**Determination of total phenolic compounds**

Total phenolic compounds in the plant samples were estimated using the Folin-Ciocalteu method. One milliliter of the methanolic extract was mixed with 9 ml deionized water and 1 ml Folin-Ciocalteu reagent. The mixture was allowed to stand in the dark for 5 min at room temperature. Subsequently, the mixture 10 ml

of 7% $Na_2CO_3$ solution was added and the total volume was adjusted to 25 ml using deionized water. The mixture was then allowed to stand for 30 min in the dark at room temperature before measuring the absorbance value at wavelength 750 nm. The quantity of total phenolic compounds in the form of gallic acid equivalent was calculated in comparison with the standard curve for gallic acid (Pongsathorn et al., 2012).

**Determination of DPPH free radical scavenging activity**

The activity of antioxidant was determined by the reduction of the amount of 1, 1-diphenyl-2-picrylhydrazyl (DPPH). Fifty microliter of the methanolic extract was mixed with 2 ml 0.06 mM DPPH. The mixture was allowed to stand for 30 min in the dark at room temperature. Subsequently, the absorbance of the mixture was measured at wavelength 517 nm. The decrease in DPPH was calculated by comparing with the standard curve of DPPH concentration. The percentage of inhibition which showed scavenging activity was calculated using the following equation (Pongsathorn et al., 2012):

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

Where, $A_{control}$ is absorbance of the control (DPPH solution without sample) and $A_{sample}$ is absorbance of the test sample extract. Scavenging activity was compared with synthetic antioxidant: trolox (analog of vitamin E) used as positive control. Moreover, the activity of antioxidant was reported in terms of IC50 as the concentration of extract to inhibit free radical formation by 50% (Hsh, 2006).

**Statistical analysis**

All treatments contain 5 replicates. All data were analyzed using SPSS v16.0 for Windows. The analysis of variance was done and differences among treatment means were determined using the Least Significant Difference (LSD) comparison methods ($p \leq 0.05$).

## Results

**Estimation of IAA**

The concentration of IAA in the supernatant from the culture of *M. radiotolerans* ED5-9 was 3.36 $\pm$ 0.20 $\mu$g/ml. by The presence of IAA compound in the supernatant was confirmed using HPLC. Figure 1 shows the presence of a peak of IAA standard and the test sample which occurred at the same retention time.

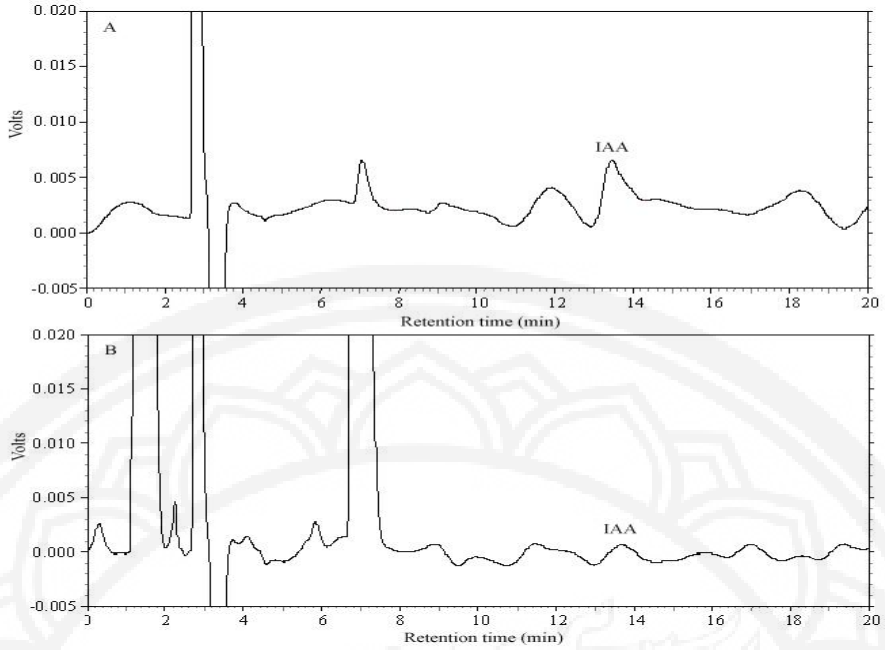


**Figure 1** HPLC analysis of bacterial supernatant compared with IAA standard solution. A) IAA standard solution; B) bacterial supernatant

**Effect of bacterial IAA on the growth parameters of *M. loriformis* explants**

The supernatant from bacterial culture medium, with an initial concentration of 3.36 ± 0.20 mg/l IAA, was mixed in MS medium at 1, 2 and 4 ml/l. This made the final concentration of IAA in the medium to be 3.4, 6.7 and 13.4 µg/l, respectively. For the control, MS medium was added with synthetic IAA at a concentration of 6.7 µg/l for comparison. Although the size of shoots and leaves of the tissue cultured plants were small after 4 weeks, it was observed that all treatments were similar in terms of regeneration of the shoots and roots as well as the plants having bright green leaves. The regeneration of roots and subsequent increase in root length of *M. loriformis* was observed in all treatments containing IAA (from bacteria and chemically synthesized) but not found in the medium devoid of IAA.

The change in the different growth parameters is shown in Table 1. The growth and development of *M. loriformis* explants in comparing T2 with T5, the plants in T2 have higher numbers of shoots, roots and leaves, and greater root length and dry weight than T5. The highest number of shoots and leaves was observed in T1 with an average of 3.40 ± 2.30 shoots per explants and 8.00 ± 7.10 leaves per explants, respectively. The highest number of roots and root length was observed in T4 with an average of 11.00 ± 4.60 roots per explants and 2.10 ± 0.70 cm, respectively. The highest average dry weight (0.0200 ± 0.0069 mg) was observed in T6.

**Table 1** The characteristic of *M. loriformis* explants 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 | 3.40 ± 2.30 | 8.00 ± 7.10 | 3.00 ± 1.40 | 0.90 ± 0.10 | 0.0169 ± 0.0082 |
| T2 | 2.60 ± 1.10 | 3.00 ± 1.70 | 7.50 ± 0.70 | 0.90 ± 0.10 | 0.0169 ± 0.0034 |
| T3 | 2.50 ± 1.70 | 4.33 ± 1.90 | 8.00 ± 0.00 | 1.00 ± 0.00 | 0.0179 ± 0.0087 |
| T4 | 3.25 ± 1.00 | 4.33 ± 2.10 | 11.00 ± 4.60 | 2.10 ± 0.70 | 0.0172 ± 0.0053 |
| T5 | 1.00 ± 0.00 | 2.00 ± 0.00 | 4.00 ± 0.00 | 0.50 ± 0.00 | 0.0128 ± 0.0039 |
| T6 | 2.00 ± 1.00 | 1.33 ± 0.60 | - | - | 0.0200 ± 0.0069 |

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

**Effect of bacterial IAA on the physiological parameter of *M. loriformis* explants**

The tissue cultured *M. loriformis* had shown both physical and physiological changes. The changes in physiological characteristics and the quantity of secondary metabolites such as pigments, total phenolic compounds and antioxidant activity is shown in Tables 2 and 3. The study was found that the concentration of pigments in *M. loriformis* treated with bacterial and synthetic IAA showed no significant difference except in the amount of chlorophyll a. (Table 2) The highest concentration of chlorophylls a and b was observed in T6 at 9.12 ± 0.85 and 8.56 ± 3.71 mg/g fresh weight, respectively. Moreover, T5 gave the highest average on the quantity of carotenoids at 1.58 ± 0.37 mg/g fresh weight.

**Table 2** The pigment content of *M. loriformis* explants after cultivation for 4 weeks

| Treatment | Chlorophyll content (mg/g fresh weight) | | |
|---|---|---|---|
| | Chl a | Chl b | Carotenoids |
| T1 | $6.20^{b}$ ± 1.08 | 3.34 ± 1.89 | 1.21 ± 0.77 |
| T2 | $5.13^{b}$ ± 0.47 | 4.76 ± 1.05 | 1.14 ± 0.34 |
| T3 | $5.22^{b}$ ± 1.04 | 4.19 ± 3.10 | 1.01 ± 0.95 |
| T4 | $4.43^{b}$ ± 2.05 | 6.36 ± 1.27 | 0.21 ± 1.03 |
| T5 | $6.48^{b}$ ± 2.27 | 4.70 ± 1.13 | 1.58 ± 0.37 |
| T6 | $9.12^{a}$ ± 0.85 | 8.56 ± 3.71 | 1.34 ± 0.56 |

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

Means with different letter (a-b) differ statistically significance ($p < 0.05$).

The quantity of secondary metabolites such as total phenolic compounds and antioxidant activity of the each treatment is shown in Table 3. There was a significant difference among treatments in terms of these two parameters. The treatment wherein chemically synthesized IAA was used (T5) got the highest average quantity of total phenolic compounds and most antioxidant activity at 0.62 ± 0.40 and 4.83 ± 0.54 mg/g fresh weight, respectively, which were more than the averages in the treatments where supernatant from bacterial culture containing IAA were used. T3 was found to have greatest antioxidant activity having an IC50 value of 2.30 ± 0.64 mg/g fresh weight.

**Table 3** Total phenolic content and antioxidant activity of *M. loriformis* explants after cultivation for 4 weeks

| Treatment | Total Phenolic compounds (mg/g fresh weight) | Antioxidant activity (mg/g fresh weight) | IC50 (mg/g fresh weight) |
|---|---|---|---|
| T1 | $0.55^{ab} \pm 0.46$ | $4.00^{bc} \pm 0.00$ | $2.72 \pm 0.98$ |
| T2 | $0.23^{cd} \pm 0.32$ | $3.77^{c} \pm 0.00$ | $2.78 \pm 0.06$ |
| T3 | $0.15^{d} \pm 0.19$ | $4.04^{bc} \pm 0.41$ | $2.30 \pm 0.64$ |
| T4 | $0.36^{bc} \pm 0.46$ | $4.14^{bc} \pm 0.52$ | $3.74 \pm 1.16$ |
| T5 | $0.62^{a} \pm 0.40$ | $4.83^{a} \pm 0.54$ | $2.67 \pm 0.52$ |
| T6 | $0.30^{cd} \pm 0.20$ | $4.58^{ab} \pm 0.33$ | $2.81 \pm 0.50$ |

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

Means with different letter (a-b) differ statistically significance ($p < 0.05$).

## Discussion

This study presented to effect of fermentation broth of indole-3-acetic acid producing *M. radiotolerans* ED5-9 on the growth and development of *M. loriformis* in tissue culture. In general, the IAA producing bacteria in genus *Methylobacterium* was synthesized via indole-3-pyruvic acid pathway by uses L-tryptophan as precursor but some time the synthesis maybe via L-tryptophan independent pathway (Doronina, Ivanova, & Trotsenko, 2002). The concentration of IAA in fermentation broth produced from *M. radiotolerans* ED5-9 was similar to *M. radiotolerans* VKM B-2144 (=JCM 2831) in the study of Ivanova, Doronina, & Trotsenko (2001) which reported that the bacteria can produce IAA concentration at 3.00 μg/ml. The results indicate the capability of *M. radiotolerans* VKM B-2144 (=JCM 2831) to produce IAA. Interestingly, *M. radiotolerans* ED5-9 was able to systhesize IAA without L-tryptophan as the precursor. The previous study of Omer et al. (2004) reported that *Methylobacterium* isolated from *Trifolium repens* and *T. pretence* can be able to produce IAA in culture medium without L-tryptophan supplements. The previous study about bacterial IAA synthesis in the culture medium without L-tryptophan has been reported. Prinsen et al. (1993) reported that *Azotobacter brasilense* can be able to produce IAA substance via tryptophan-independent pathway. Therefore, in case of the culture medium without L-tryptophan supplements of *M. radiotolerans* ED5-9 might be synthesized IAA via tryptophan-independent pathway similar to *A. brasilense*.

The results of the growth and development of *M. loriformis* explants in tissue culture was an indication of the necessity of IAA in the culture medium wherein the IAA stimulates the formation of roots both in tissue culture and stem cuttings (Davies, 2004). In addition, it was observed that the supernatant of bacterial culture medium containing IAA was more efficient in promoting root formation of *M. radiotolerans* ED5-9 than the chemically synthesized IAA. The result of this study is similar the results obtained using bacterial IAA from *Pseudomonas aeruginosa* which stimulated increase in length of roots and shoots in cowpea more than the control by ~2.6 and ~1.1 fold, respectively and also similar to the results of another study wherein bacterial IAA from *Halomonas desiderata* RE 1 promoted root formation of mungbean (*Vigna radiata*) both in terms of number and length (Ali & Hasnain, 2007; Sarsirekha, Shivakumar, & Sullia, 2012).

The variation on the quantity of pigments in *M. loriformis* was not due to the effect of IAA treatment but rather was brought about by other factors associated with the culture process such as temperature, humidity, pH and nutrient availability which affected the growth and development the of plants (Andrews, Sprent, Raven, &

Eady, 1999). However, the quantity of chlorophyll a, chlorophyll b and carotenoids correlates to chloroplast development whereas total phenolic compound which is one group of antioxidant compounds correlates to phenylpropanoid pathway which controlled with cytokinin production (Singh et al., 2003; Devi et al., 2010; Kousalya & Bai, 2016). The influence of the supernatant with IAA from bacteria on the quantity of secondary metabolites is not clear owing to the similarities in all treatment although IAA is known to affect many physiological processes in plants but the control of growth depends on the ratio between auxin and cytokinin, wherein auxin acts as primary factor in controlling the growth and morphology of root while cytokinin affects secondary metabolites synthesis (Arroo et al., 1995).

## Conclusion

This study demonstrates the potential of the supernatant from the culture medium of *M. radiotolerans* ED5-9 which contains IAA to promote growth and development of *M. loriformis* explants in plant tissue culture. There was an efficient stimulation of root formation, increased root length as well as increase in the number of shoots, leaves and dry weight by using bacterial IAA which have more potential effect than chemically synthesized IAA as shown in T2 and T4. It was also observed that the increasing of the number of roots and root length of *M. loriformis* was dependent on the concentration of the supernatant manner in MS medium. Due to the affectivity of IAA from *M. radiotolerans* ED5-9 it can be used to stimulate root formation and increase the root length in plant tissue culture replacing chemically synthesized IAA.

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