Effects of Gel Rooting Medium Containing *Methylobacterium radiotolerans* Ed5-9 and *Streptomyces* TM32 Fermentation Broth on Cutting Propagation of *Gymnema inodorum* (Lour.) Decne.

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Abstract

As plant cuttings have no roots, they are easily damaged and dehydrate when planted in growth medium. Better ways of protecting plants against environmental stress and increasing the sustainability of crop production after cutting propagation are needed. The aim of this study was to investigate the effects of alginate beads that contained *Methylobacterium radiotolerans* ED5-9 and *Streptomyces* TM32 fermentation broth as the growing medium on the cutting propagation of a Thai herbs, Chaing da, *Gymnema inodorum*. After 4 weeks of cultivation, the plant cuttings tested with alginate bead supplemented with both bacterial fermentations gave a higher percentage of survival than did those treated without bead (T10). The T1, 2.60 ml/l of *Streptomyces* TM32 and 0.25 mg/l of *M. radiotolerans* ED5-9 fermentation broths blended with 3% alginate treatment, presented the highest percentage of survival (90.48±6.56%), rooting (50.00±22.40%) and number of leaves per plant (5.17±0.54). These promising results were obtained when compared with the non-alginate and the synthetic IAA treatments. Therefore, using a gelling biocompatibility technique with bacterial fermentation broth was able to improve organic cutting propagation that was vulnerable to dehydration and infection by pathogens.

Keywords: *Methylobacterium radiotolerans*, *Streptomyces*, fermentation broth, *Gymnema inodorum*, gel rooting medium, cutting propagation
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1. Introduction

Plant cutting, a simple cloning method which allows commercial growers to clone a certain plant to ensure consistency throughout their crops and can be effective depending on various factors such as type of cutting, plant species, cutting time and environment. Plant cuttings have no root systems, therefore, they are likely to die from dehydration if the conditions are not appropriate [1]. There are ways of improving the growth of cutting propagations. For example, a number of moist media are used, including but not limited to soil, perlite, vermiculite, coir, rock wool, expanded clay pellets, compost [2], and even water given the right conditions. Moreover, the roots of the cutting will usually follow the wound after dipping the cutting into accelerating hormone or anti-fungal agents. The rooting hormones, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA), which are also known as auxins, can be applied to the stems of root cuttings to increase the number of adventitious roots [1]. However, in organic production, the synthetic forms of auxins such as IAA, IBA and NAA are often not permitted, and thus alternative hormones/stimulators or techniques are employed to improve rooting success rate and quality. There are natural and organic sources that can successfully provide a rooting boost to freshly taken cuttings such as honey [3], coconut water [4], willow extract [5] and even bacterial phytohormone [6].

Among the plant growth-promoting bacteria (PGPB), bacterial endophytes can be isolated from various plants that have the capacity to synthesize antibiotics and substances like plant hormones. Bacterial hormones, such as indole-3-acetic acid (IAA), cytokinins (CKs) and gibberellins (Gas), can affect not only growth and development of plant [6] but also stimulate the plant defense system [7]. Pink pigmented facultative methylotrophs (PPFMs) from the genus *Methylobacterium* were found to be plant growth-promoting bacterial endophytes that had great potential to synthesize and release IAA as a secondary metabolite [8, 9]. From our previous study, PPFMs isolate with the ability to produce phytohormone, IAA, was isolated from *Murdannia loriformis* (Hassk.) R. Rao & Kammathy leaves and the most effective isolate ED5-9 was identified later by partial 16S rDNA gene sequencing as a strain of *Methylobacterium radiotolerans* JCM 2831(T) [10]. Further work using *M. radiotolerans* ED5-9 has been done *in vitro* conditions for growth promotion in some plants, such as *Rauvolfia serpentina* [11], *Murdannia loriformis* and *Gymnema inodorum* [12], by addition of its fermentation broth into the growing plant media. Furthermore, the phytohormone synthesizing actinobacteria isolate *Streptomyces* TM32, which was related to *Streptomyces sioyaensis* discovered by Nakaew *et al*. [13], exhibited antagonistic activity against the fungus, *Rigidoporus* sp., which is known to cause white root rot disease of rubber trees, with an effective dose (ED_{50}) of fermentation broth at 2.60 ml/l. There is also a promising result examined by Sarin *et al.* [14] on the application of the isolate *M. radiotolerans* ED5-9 fermentation broth in combination with crude antimicrobial agents extracted from fermentation broth of the isolate *Streptomyces* TM32 on tissue culture of *Gymnema inodorum* (Lour.) Decne., Asclepiadaceae, known as Chiang Da (Thai name). This plant is one of the reputed medicinal plants that can decrease blood glucose [15, 16] and is widely used in Northern Thai cuisine as a local vegetable and in commercial herb tea. Therefore, an efficient method of asexual propagation via cuttings of vegetative Chiang Da is needed for the production in large quantity.

Rooting hormone, in gel form, has become the popular form of rooting hormones, because there is no preparation needed and it can be applied right to the plants, with no extra step involved [17]. It easily adheres and covers the cut surface completely, and it is the least likely to get washed away when the plants are watered. Gel IBA, a commercial hormone, has been favorable for rooting native olive cutting [18]. In particular, alginate hydrogels, which are naturally occurring anionic polymers typically obtained from brown seaweed, have been attractive to numerous applications due to their biocompatibility, low toxicity, relatively low cost, and mild gelation by addition of divalent cations such as Ca^{2+} [19]. These gels provide a physiologically moist microenvironment.

All molecules, from small to macromolecules, can also be released from alginate gels being used as growing medium, depending on the cross-linker types and cross-linking methods, which can also be specifically designed to support plant growth and supply roots with necessities such as hormones, water, air, and nutrients. Therefore, the aim of this study was to investigate the effects of fermentation broth of *M. radiotolerans* ED5-9 and *Streptomyces* TM32 blended in alginate as growing medium on the cutting propagation of *Gymnema inodorum* as a means to protect plants against environmental stress and to increase the sustainability of crop production.

2. Materials and Methods

2.1 Preparation of cell-free fermentation broth of the isolate *Methylobacterium radiotolerans* ED5-9

Methylobacterium radiotolerans ED5-9 was cultured in 100 ml liquid nutrient medium (LNM) containing 2 g/l KH_2PO_4, 2g/l $(NH_4)_2SO_4$, 0.125 g/l $MgSO_4.7H_2O$, 0.5 g/l NaCl, 0.002 g/l $FeSO_4.7H_2O$, 0.1 g/l yeast extract and filtered sterile 1% methanol (v/v) in a 250 ml Erlenmeyer flask, and the media was adjusted to pH 5.5. After incubation under the shaking conditions of 150 rpm at 30°C for 60 h, the culture was centrifuged at 10,000 g at 4°C for 15 min. In order to increase the IAA concentration, the supernatant was then evaporated at 40°C, 50 rpm, 7.2 kPa until reaching a half scale of the starting volume in a rotary evaporator (Rotavapor® R-205, Büchi Labortechnik AG) and filtered in order to preserve sterility through a filter paper (0.45 µm). The supernatant was stored at 4°C prior to use. The bacterial IAA concentration in the supernatant of *M. radiotolerans* Ed5-9 (MIAA) was quantified by colorimetric method [20]. A mixture containing 1 ml of the supernatant and 2 ml of Salkowski's reagent was incubated in the dark at ambient temperature for 30 min before its absorbance at 530 nm was measured. The calculated concentration of each sample was achieved from a standard plot of synthetic IAA (Sigma) concentrations ranging from 0.0-30.0 mg/l.

2.2 Preparation of fermentation broth of the isolate *Streptomyces* TM32

Streptomyces TM32 was cultured on Hickey-Tresner (HT) agar [21] at 30°C for 7 days. Its colonies on HT agar were then cut with cork-borers of 0.8 cm diameter, and 5 pieces of its active growth agar were inoculated in 200 ml of International *Streptomyces* Project 2 (ISP2) medium [21]. The culture was incubated at ambient temperature (~25°C) with shaking reciprocally at 120 rpm for 7 days and was later centrifuged at 10,000 g at 4°C for 15 min. *Streptomyces* TM32 supernatant was sterilized by autoclaving and kept at 4°C prior to study and was used at the ED_{50} volume of 2.60 ml/l against fungus, *Rigidoporus* sp., discovered by Nakaew *et al*. [13] to prevent fungal disease infection on the cutting stem. The actinobacterial IAA concentration was also estimated as described before.

2.3 Evaluation of growth promoting activity in plant cuttings

2.3.1 Preparation of plant cuttings

As the production of uniform, vigorous and adequate stem tip cutting stages for rooting were required for testing experiments, a clean culture was prepared by tissue culture technique. Field grown plants of *Gymnema inodorum* cultivar code 4 collected from the Agricultural Technology Research Institute, Rajamangala University of Technology Lanna (RMUTL), Thailand, were used as a source of the explants. The shoot tip explants were first soaked in a solution of 15% Clorox for

10 min and washed three times with sterile distilled water for 5 min. They were then incubated in the modified semi-solid Murashige and Skoog (MS) medium [22] containing 6-benzyl amino purine (BAP) 4 mg/l. The cultures were kept under a light intensity of 42 mol/m^2/s at 25±2°C with a 16-h-light photoperiod and 8-h-dark period for 4 weeks in order to induce plant regeneration. After proper initiation, they were cut and placed onto the new MS media containing BAP 2 mg/l and naphthalene acetic acid (NAA) 0.5 mg/l and used later as the clean culture for the stem tip cutting trials after growing for 4 weeks.

2.3.2 Preparation of alginate rooting medium

Sodium alginate (Sigma) gel (3%) fortified with various concentrations of bacterial fermentation broth or synthetic IAA according to experimental treatment design (Table 1) was prepared in sterile distilled water and was then gently added, drop by drop, through a sterile cut tip syringe into 100 mM $CaCl_2$ solution to make a bead of about 1 cm in size. The resulting alginate beads were allowed to stand for 12 h for hardening and were then autoclaved and kept in the dark at room temperature (25±2 ºC).

Table 1. Treatment design for *G. inodorum* cutting trials

Treatment	*Streptomyces* TM32 fermentation broth* (ml/l)	MIAA (mg/l)	Synthetic IAA (mg/l)
T1	2.60	0.25	-
T2	2.60	0.50	-
T3	2.60	1.00	-
T4	2.60	2.00	-
T5	2.60	-	0.50
T6	-	0.50	-
T7	2.60	-	-
T8	-	-	0.50
T9	-	-	-
T10 (without alginate bead)	-	-	-

*The volume of ED_{50} against *Rigidoporus* sp. discovered by Nakaew *et al*. [13], which contained 0.01 mg/l of IAA.

2.3.3 Experimental trials

A stem tip cutting (2 cm in length) of the *G. inodorum* clean culture was stabbed and placed into the alginate at a depth of about 3-5 mm, and the bead was then planted in a small pot (3.5 cm in diameter and 5 cm. in height) containing moist sterile peat moss. Fifteen replicates per treatment with one stem tip cutting per pot as described in Table 1 were performed. All treatments were left in a dish that was filled with the water under the light intensity of 42 mol/m^2/s for photosynthetic active radiation at 25±2°C with a 16-h-light photoperiod and 8-h-dark period for 4 weeks. Survival rates, stem tip height, leaf numbers and alginate bead morphology were observed and recorded weekly. The four-week-old harvestable fresh plants were measured for the leaf greenness values (SPAD unit) by a chlorophyll meter (Minolta SPAD -502, Japan).

2.4 Statistical analyses

Randomized Complete Block Design (RCBD) was applied for the experimental design. Mean values and the standard error of mean were calculated. Results of the measurements were subjected to analysis of variance (ANOVA) and significance at the p value < 0.05 was tested by Fisher's Least Significant Difference (LSD) by using a Minitab version 17 program.

3. Results and Discussion

After 4 weeks of cultivation, most treatments of plant cuttings with alginate beads gave a higher survival percentage than those treated without beads (T10), except for the T7, which contained only the *Streptomyces* TM32 fermentation broth. The T1, T2 and T4 presented the highest percentages of 90.48% as shown in Figure 1. However, all treatments including T1 to T4 treatments, which contained the fermentation broth of both bacteria, with the exception of T7 were not significantly different from T10.

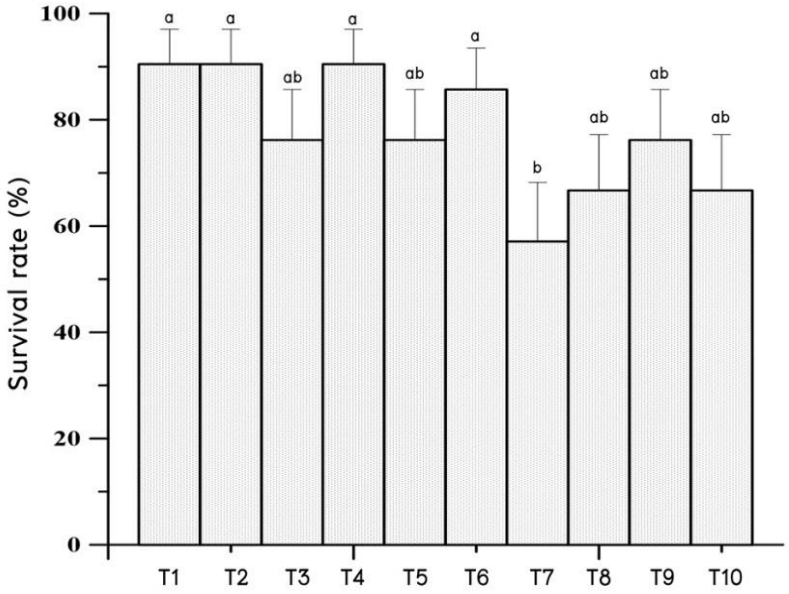

Figure 1. Effect of different concentrations of fermentation broth of *M. radiotolerans* Ed5-9 (MIAA) on survival rate of *G. inodorum*. Means followed by the same letter on the bar chart are not significantly different at p <0.05, n = 15, based on Fisher's Least Significant (LSD).

The highest rooting (50.00±22.40%), number of roots per plant and root length were obtained from the T1 test (Table 2). Although the plant cuttings tested in the T2, T7, T8 and T9 survived, no obvious roots had formed. There were only very small knobs appearing at the end of stems (Figure 2). However, cuttings still have both water and carbon stored, which are available for their growth. This detached part of the plant remains physiologically active, allowing mitotic activity and new rooting. Generally, rooting occurs within 3 to 4 weeks, but some plants take longer. The cuttings that were encouraged to form new roots may have done so due to IAA in the alginate beads;

they then grew by absorbing the minerals and nutrients contained in fermentation broth within alginate medium. Although the tested T1 showed the highest number of leaves per plant, the result was not significantly different from T2, T3, T4, T7, T8, and T10 (Table 2). On the other hand, the height of stem tip of T1was shorter (2.88±0.28 cm) after planting for 4 weeks (Table 3). The T4 and T5 gave the stem tip heights of 3.15±0.16 and 3.16±0.10 cm, respectively, which were higher than other treatments, however, there was no significant difference in stem tip height among the treatments. The alginate bead treatments that contained both bacterial fermentation broths (T1-T4) might have provided minerals and nutrients for the cuttings via their new roots, resulting in better growth of the cuttings than the growth of those treated without bacterial fermentation broth (T10). In line with this observation, the better growth of tested treatments with alginate bead (T1-T4) also showed higher chlorophyll content than the tested treatment without alginate bead of the T10 (Figure 3) and especially T2 and T4 were significantly different from T8 and T10. The alginate beads gradually degraded over the 4 weeks (Figure 4), and the cutting plants continued to grow in the peat moss. There was also no fungal contamination in the treatments.

Table 2. Effect of different concentrations of fermentation broth of *M. radiotolerans* Ed5-9 (MIAA) on rooting percentage, number of roots, root length and number of leaves of *G. inodorum* cuttings (Mean±SE ; n = 15)

Treatment	Rooting (%)	No. of root per plant	Root length (cm)	No. of leaves per plant
T1	50.00±22.40^{ns}	2.67±1.20^{ns}	1.47±0.73^{ns}	5.17±0.54^{a}
T2	nr	nr	Nr	3.83±0.65^{ab}
T3	16.70±16.70^{ns}	1.00±0.00^{ns}	0.41±0.00^{ns}	4.25±1.31^{ab}
T4	33.30±21.10^{ns}	2.50±1.50^{ns}	0.55±0.38^{ns}	3.50±0.5^{ab}
T5	16.70±16.70^{ns}	1.00±0.00^{ns}	0.24±0.00^{ns}	3.00±0.52^{b}
T6	16.70±16.70^{ns}	2.00±0.00^{ns}	0.34±0.00^{ns}	2.67±0.67^{b}
T7	nr	nr	nr	3.33±0.33^{ab}
T8	nr	nr	nr	3.25±1.11^{ab}
T9	nr	nr	nr	2.67±0.67^{b}
T10	16.70±16.70^{ns}	2.00±0.00^{ns}	2.23±0.00^{ns}	4.25±1.03^{ab}

nr = no obvious root

ns = not significantly different between treatments. Means followed by the same letter are not significantly different at p <0.05, based on Fisher's Least Significant (LSD).

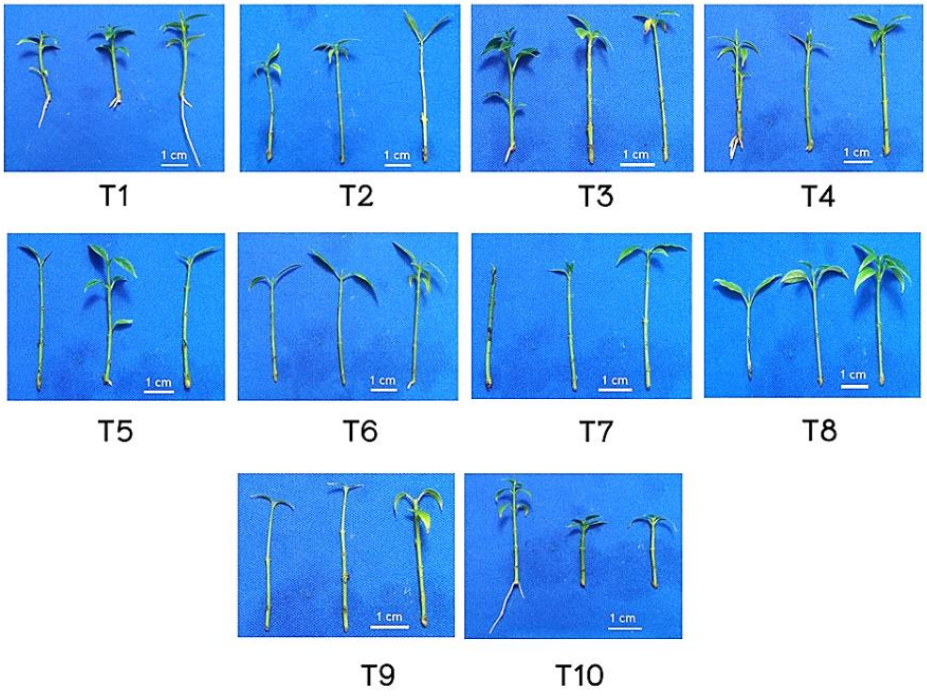

Figure 2. Morphology of *G. inodorum* cuttings after growing in different treatments for 4 weeks

Table 3. Stem tip height of *G. inodorum* cuttings measured at weekly intervals after planting in various treatments (Mean±SE ; n=15)

Treatments	Stem tip height (cm)			
	1st weeks	2nd weeks	3rd weeks	4th weeks
T1	2.74±0.20	2.76±0.21	2.82±0.28	2.88±0.28
T2	2.64±0.27	2.64±0.27	2.64±0.27	2.64±0.27
T3	2.46±0.19	2.46±0.19	2.46±0.19	2.48±0.16
T4	3.06±0.12	3.06±0.12	3.10±0.12	3.15±0.16
T5	3.04±0.08	3.04±0.08	3.04±0.08	3.16±0.09
T6	3.00±0.07	3.00±0.07	3.00±0.07	3.02±0.08
T7	2.82±0.16	2.82±0.16	2.82±0.16	2.82±0.16
T8	2.48±0.16	2.48±0.16	2.48±0.16	2.48±0.16
T9	2.62±0.22	2.62±0.22	2.62±0.22	2.62±0.22
T10	2.78±0.10	2.80±0.11	2.82±0.12	2.86±0.16

There is no significantly difference between treatments at p <0.05

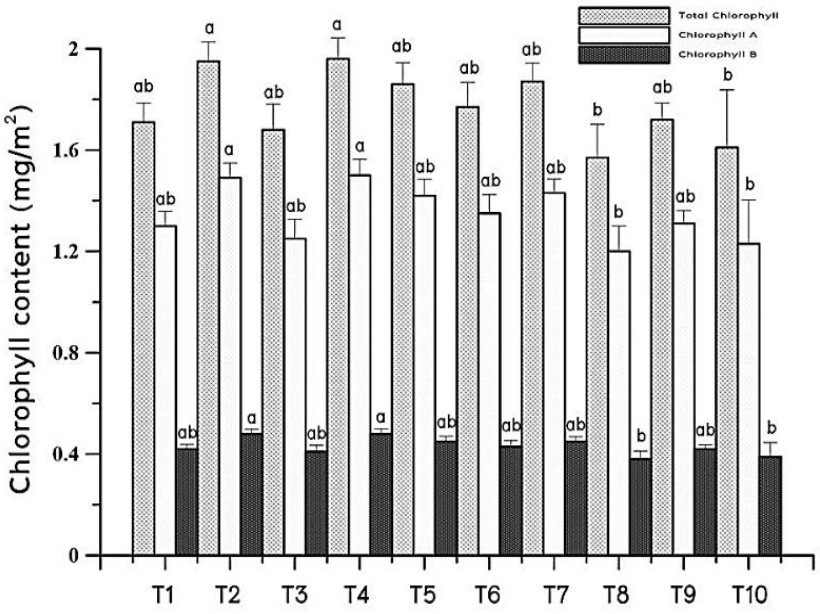

Figure 3. Leaf chlorophyll content of *G. inodorum* stem tip cuttings after planting at various treatments for 4 weeks

Figure 4. Weekly observation of alginate beads

Nowadays, alginate is known to be linear copolymers containing blocks of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues. The G-blocks of alginate are believed to participate in intermolecular cross-linking with divalent cations such as Ca^{2+}, used in this study to form hydrogels [23, 24]. Therefore, the rate of IAA hormone and other useful substance release from the fermentation broth gel depends on the physical properties and the stability of the gel.

The proper percentage of alginate in hydrogel affects the diffusion of water and nutrient from alginate bead to plant. Better diffusion in the gel medium and availability of water result in the better growth and plant survival. It has been suggested that gel bead obtained by using 3% of alginate

developed in 100 mM $CaCl_2$ solution was able to improve *in vitro* growth of pomegranate (*Punica granatum* L.) nodal segments [25] and *Cucumis sativus* L. shoot tips [26]. Since Thimann and Koepfli [27] found that synthetic preparations of IAA had root-forming properties, they also demonstrated that it could be used in stimulating root formation on cuttings. The influence of IAA on rooting had been reported by Shibaoka [28], who showed that an increased number of roots in cuttings of adzuki bean [*Vigna angularis* (Willd.) Ohwi & H. Ohashi var. *angularis* (syn. *Azukia angularis* (Willd.) Ohwi) seedlings was obtained when IAA at a high concentration was applied during the first 24 h. However, in this research, the lowest concentration of the natural IAA from *M. radiotolerans* ED5-9 (0.25 mg/l), in cooperation with the fermentation broth from *Streptomyces* TM32 (2.60 ml/l) (T1), was able to enhance rooting percentage better than other treatments. The *M. radiotolerans* ED5-9 used in this study has also been reported to produce cytokinins in an amount of 0.54 μg/ml of culture media after incubation for 5 days [29]. Application of alginate beads for the encapsulation of *Mentha arvensis* nodal segments gave the highest rate of shoot formation (80%) when observed in MS medium containing 2.0 mg/l cytokinins (BAP) and 0.2 mg/l auxin (NAA) [30]. The result showed that the percentage of rooting was reduced when the concentration of MIAA was increased in medium in which the high concentrations of IAA might cause osmotic stress [31]. Moreover, the tested *Streptomyces* TM32 could also produce an IAA concentration of 4.26 mg/l in culture media after incubation for 7 days. The existing growth of root meristem and shoot tip of plants can be stimulated at a low concentration of auxin (0.0001-0.01 mg/l), and auxin concentrations higher than 0.01 mg/l are often inhibitory due to an increase in production of ethylene [32]. The effectiveness of this application in promoting rooting on the stem cuttings may also depend on adequate absorption by plant tissue and depth of treatment where the absorption of supplement substance solution can run down the epidermis and be absorbed through the cut part [33]. Furthermore, this experiment was designed for different concentrations of microbial fermentation study as a means to create a variation of chemical substances attained in the culture medium. The growth of cuttings in this study could be thus affected by nutrient constitution of culture medium. Most of the results related with alginate bead treatments in this preliminary experiment that were not significantly different from the treatment without alginate bead may have been due to all the factors described above. Therefore, further study of the optimum size of alginate bead and some conditions needs to be done.

4. Conclusions

A blended fermentation broth of M. *radiotolerans* Ed5-9 (MIAA) 0.25 mg/l with the fermentation broth of *Streptomyces* TM32 2.60 ml/l could be used to promote rooting and growth of the *G. inodorum* cuttings. Since the aim of this research was to grow *G. inodorum* cuttings in alginate blended with bacterial fermentation broth containing phytohormones such as IAA, antibiotics and some nutrients in order to promote rooting medium and then let them continue growing in peat moss in order to insure root compatibility from the beginning, it can be concluded that promising results were obtained when compared with the non-alginate and synthetic IAA treatments. Thus, the utilization of bacterial fermentation broth with gelling biocompatibility technique can improve organic cutting propagations that are susceptible to fungal pathogens and are vulnerable to dehydration.

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