Effects of Growth Regulators Produced by *Methylobacterium radiotolerans* Ed5-9 and Crude Antimicrobial Agents Extracted from *Streptomyces* TM32 on Tissue Culture of *Gymnema inodorum* (Lour.) Decne.

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Abstract

Since the demand for medicinal plant in pharmaceutical industries as plant based raw materials is becoming greater in quantity, the propagation of large number of plant in relatively short period of time can be produced by tissue technique. However, one of the most serious problems of plant cell and tissue culture is microbial contamination. The effectiveness of crude plant growth promoting substances extracted from the isolates *Methylobacterium radiotolerans* ED5-9 (MIAA) and the *Streptomyces* TM32 (SCE) for plant growth and development on tissue culture of the *G. inodorum*, a medicinal plant widely used in Northern Thailand, was therefore investigated. After incubation for 4 weeks, cooperation of the MIAA and SCE in basal liquid medium condition showed significantly ($p < 0.05$) higher increase in the length of shoot tip of explants than those semi-solid medium. High levels of IAA from both the *M. radiotolerans* ED5-9 and the *Streptomyces* TM32 caused reduction of shoot growth and root development when compared with the control treatment consisted of the MIAA alone. The measurement of greenness of leaves (SPAD readings) showed lower values from the liquid medium than that of the semi-solid treatments. The active antioxidant activity and total phenolic content accumulated in the plantlets of *G. inodorum* in semi-solid cultured plantlets were at a higher level than those in liquid treatment. Moreover, all these of SCE treatments, which contained crude antifungal agents, had no microbial contamination appearance during incubation. This study suggested that beneficial effects of the growth promoting substances producing, particularly IAA less than 0.01 mg/L, from both the *M. radiotolerans* ED5-9 and the *Streptomyces* TM32 or even from the *M. radiotolerans* ED5-9 alone might have potential for use as bio-fertilize or anti-microbial agent on micro-propagated Chiang Da, *G. inodorum*, in the semi-solid medium condition.

Keywords: *Gymnema inodorum*; *Methylobacterium radiotolerans*; plant growth promoting substances; *Streptomyces*; tissue culture

Introduction

G. inodorum (Lour.) Decne, Asclepiadaceae, known as Chiang Da, is one of the reputed medicinal plant widely used in Northern Thai food, has an advantage that the taste is sweet and not bitter (Shimizu et al., 1997). It is also realized for not only on health beneficial effect, but also on healing rheumatic arthritis, diabetes mellitus, and gout which is due to high containing of polyphenols as the major antioxidant in its leaves and young shoots (Klungsupya et al., 2008) and the inhibitory effect of triterpenoids consisted of gymnemic acids that can decrease blood glucose (Shimizu et al., 1997, 2001). Since the medicinally active part of the *G. inodorum* is not related to taste modifying action and is useful for controlling blood sugar (Shimizu et al., 1997), therefore Thai Chiang Da herb has paid attention to be produced as herbal tea in the industry other than the use for Thai cuisine as vegetable.

Plant tissue culture techniques are often used for propagation of medicinal plant species that give high concentration of secondary pharmacological metabolites and are genetically identical. These techniques can produce the propagation of large number of plants in relatively short period of time. However, one of the most

serious problems of plant cell and tissue culture is microbial contamination (Leifert and Cassells 2001). Most of contaminated bacteria escape from the initial process of surface sterilization (Van Dan Houwe and Swennen 2000) and perpetuate being hidden during growth on plant multiplication media, but will become visible after subsequent sub culturing (Cassells, 1991, 2001). Endophytic bacterial contamination cannot be completely removed with any surface sterilization techniques, thus it requires antibiotic treatment (Mathias et al., 1987) which could be an effective way to reduce the loss of plantlets by microbial contamination. The elimination of the contamination without any phytotoxic effect on the banana explants has been successful by employing the antibiotics, such as amoxicillin, augmentin, cefotaxime, and rifampicin, in the medium (Sabale et al., 2015). In yam cultures, although, phytotoxicity (necrosis) may not be noticed between the first two weeks of antibiotic application but it can occur after extended exposure to the combination of antibiotics (Mbah and Wakil, 2012). Some plant beneficial microorganisms are, therefore, of interest for safety and lower the cost of application in plant tissue culture than synthetic antibiotics since some metabolites produced from plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978) or plant growth-promoting bacteria (PGPB) (Bashan & Holguin, 1998) can encourage plant growth, increase yield, reduce pathogen infection, and reduce plant stress, without causing apparent disease (Compant et al., 2010). The PGPR such as Actinomycetes is an ultimate source of the antibiotic, especially the bacteria of the genus *Streptomyces*, which has been discovered that most of all antibiotics used in the present come from these kinds of bacteria. *Streptomyces* sp. Strain Ap1 isolated from soil around the roots of *Argania spinosa* L. has been found to produce isochainin substance (Bouizgarne et al., 2006), while aranciamycin anhydride compounds are released from *Streptomyces* sp. Tü 6384 isolated from the rhizophere of a pine tree (Nachtigall et al., 2010). The finding on interesting results of Nakaew et al. (2015) that *Streptomyces* TM32 related to *Streptomyces sioyaensis* can display distinctive antagonistic activity against the fungus, *Rigidoporus* sp., known to cause white root rot disease, can cause in decreasing latex productivity and mortality of rubber trees. Among the PGPB, bacterial endophytes, colonizing internal plant tissue for at least part of their life cycle, isolated from various plants with the capacity to synthesize antibiotics and substances like hormones of plant, such as indole-3-acetic acid (IAA), cytokinins (CKs), and gibberellins (Gas), can affect not only growth and development of plant (Saleh-Lakha and Glick, 2006) but also stimulate the plant defense system (Navarro et al., 2006). A plant-associated pink-colored methylobacteria or pink pigmented facultative methylotrophs (PPFMs) from the genus *Methylobacterium* is found to be one of bacterial endophytes that have a greater potential to synthesize and release IAA as secondary metabolites (Ivanova et al., 2001) which improve rice growth in tissue culture condition (Parimala Devi et al., 2010). The positive results in testing tissues of barley regenerates and meristematic potato plants also show for the presence of *Methylobacterium* (10^4-10^5 cells/plant) in passages that followed inoculation (Shirokikh et al., 2007). From our previous study, the PPFMs isolate which capable of producing a phytohormone, IAA, has been isolated from *Murdannia loriformis*. (Hassk.) R. Rao & Kammathy leaves and the most effective isolate is ED5-9, identified later as similar to a strain of *Methylobacterium radiotolerans* JCM 2831(T) by partial 16S rDNA gene sequencing (Sarin et al., 2013). The *M. radiotolerans* ED5-9 has also been investigated *in vitro* condition for growth promoting in some plants, such as *Rauvolfia serpentina* (Bauthet et al., 2014), *Murdannia loriformis*, and *Gymnema inodorum* (Chidburee et al., 2015) by addition of its fermentation broth into the plant media. The TM32 isolate can also synthesize plant hormones, such as IAA. Nevertheless, there is no report available on the effectiveness of cooperative use of the isolate *M. radiotolerans* ED5-9 and *Streptomyces* TM32 for plant

growth and development in tissue culture. However the environmental stresses in plant tissue culture, such as less light exposure and sinking in the medium, may cause chlorophyll loss due to stress in plants (Hendry and Price, 1993).

Thus, this research was aimed to investigate the effect of plant growth regulators produced by the isolate *M. radiotolerans* ED5-9 in combination with crude antimicrobial agents extracted from fermentation broth of the isolate *Streptomyces* TM32 on tissue culture of *Gymnema inodorum* in both semi-solid and liquid medium conditions.

Methods and Materials

Preparation of cell-free fermentation broth of the isolate *M. radiotolerans* ED5-9

M. radiotolerans ED5-9 was grown by inoculating in 100 mL liquid nutrient medium (LNM) containing of 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 1% methanol (v/v) filtered sterile (0.45μm; Millipore Corporation, Benford, MA) in a 250 mL Erlenmeyer flask and the media was adjusted to pH 5.5. The culture was incubated under the shaking conditions of 150 rpm at 30°C for 60 h then centrifuged at 10,000 g at 4°C for 15 min. In order to increase the IAA concentration, the supernatant was evaporated at 40° C, 50 rpm, 7.2 kPa and reduced to a half scale of the starting volume using rotary evaporator (Rotavapor® R-205, Büchi Labortechnik AG) then filtered to sterile through the filter paper (0.45μm). The supernatant was stored at 4°C prior to use. The bacterial IAA concentration in the supernatant of the *M. radiotolerans* Ed5-9 (MIAA) was measured by colorimetric method (Glickmann and Dessaux 1995). One millilitre of the supernatant was mixed with 2 mL of Salkowski's reagent. The mixture reagent was incubated in the dark at ambient temperature for 30 min before measuring the absorbance at 530 nm. The concentration of each sample was calculated from a standard plot of pure IAA (Sigma).

Preparation of crude extract from fermentation broth of the isolate *Streptomyces* TM32

Strain TM32 was grown on Hickey-Tresner (HT) agar (Atlas, 1996) at 30°C for 7 days. Then, its colonies on HT agar were punctured using cork borer of diameter 0.8 cm, and 5 pieces of agar blocks that contained its active growth were inoculated in 200 mL of International *Streptomyces* Project 2 (ISP2) medium (Atlas, 1996) and incubated at ambient temperature (~25°C) with shaking reciprocally at 120 rpm for 10 days. The culture broth was then added with 400 mL ethyl acetate and incubated at ambient temperature with shaking every 15 min for 1 h. The supernatant containing the interested substances was transferred for evaporation of ethyl acetate residue using the rotary evaporator at 40°C till the remaining volume was approximately lower than 5 mL. The remaining extracted solution was consequently evaporated under the airflow in the fume hood till dry. This dried crude extract of the *Streptomyces* TM32 (SCE) was re-suspended with dimethyl sulfoxide (DSMO) (Nakaew et al., 2015) and used for further studies.

Evaluation of growth promoting activity in plant tissue culture

Preparation of explants and clean culture

Field grown plants of *G. inodorum* cultivar code 4 collected from the Agricultural Technology Research Institute; RMUTL, Thailand were used as a source of the explants to prepare the clean culture for both plantlets

induction experiments. The shoot tip explants were soaked in solution of 15% Clorox for 10 min. Then, the explants were washed with sterile distilled water for 5 min three times and inoculated in the modified Murashige and Skoog (1962), MS, semi-solid medium containing 6-benzyl amino purine (BAP) 4 mg/L. The culture were incubated at the temperature of 25 ± 2°C and 3000 Lux Illumination comprising 16 h photoperiod provided by cool fluorescent light in order to induce plant regeneration and maintained for 4 weeks. After proper initiation occurred they were sub cultured on the MS(1962) media containing BAP 2 mg/L and naphthalene acetic acid (NAA) 0.5 mg/L and then used as the clean culture for the trials.

Experimental trials

The shoot tips (0.5*0.5 cm) of the clean culture were used as the explants for the plantlets induction. The explants were surface-disinfected as previous described. Then they were transferred to both semi-solid and liquid MS(1962) media containing with the *M. radiotolerans* ED5-9 concentrated supernatant of 0.0067 mg/L (Chidburee et al., 2015) and various concentrations the crude extract from *Streptomyces* TM32, including T_1;0.0067 mg/L MIAA + 0.02 mg/L SCE, T_2; 0.0067 mg/L MIAA + 0.04 mg/L SCE, T_3; 0.0067 mg/L MIAA + 0.08 mg/L SCE, and T_4;0.0067 mg/L MIAA (control). The cultures were incubated at 25 ± 2°C and 3000 Lux Illumination comprising 16 h photoperiod. There were 18 tested bottles per treatment with one explant per bottle. The regenerated plantlets under dark and light growth conditions as described before were observed and recorded for growth parameters at weekly interval for 4 wks. The 4 weeks old harvestable fresh plantlets (1 mg) was also extracted by 50 mL of methanol, to measure antioxidant activity which determined by 2,2-dipheyl-1-picrylhydrazyl (DPPH) free radical scavenging assay (Mensor et al., 2001; Floegel et al., 2011) and to quantify total phenol substances of plant materials which evaluated by Folin-Ciocalteu's phenol reagent (Zoecklein et al., 1995; Kim et al., 2003). The leaf greenness values (SPAD) of the regenerated plantlets was measured by chlorophyll meter (Minolta SPAD - 502, Japan).

Statistical analyses

The 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 (LSD) by using a Minitab version 17 program.

Results

Inclusion of the MIAA and SCE in both basal medium conditions increased the length of shoot tip of explants. There were significant differences in the shoot length among treatments after incubation for 3 weeks, which a comparison of all SCE containing treatments and no SCE revealed a significant effect, especially in the liquid medium condition (Table 1). Increasing the SCE from 0.02 to 0.08 mg/L decreased the growth rate of shoot, while addition of the MIAA alone (T_4) showed the highest shoot length and the growth rate of shoot when grown in both the liquid and semi-solid medium. In the fourth week, the shoot grown in the liquid medium had higher the growth rate of shoot than in the semi-solid medium by 3 to 4 times. Their subsequent root and leaves development of the Chiang Da explants showed to be slightly significant affected after grown in the different growth media and medium conditions for 4 weeks (Table 2 and Fig 1). All of semi-solid medium treated with the SCE gained better root germination, number of roots per plant and root length than the liquid medium.

However, increasing the concentrations of the SCE decreased the root germination and root length, but increased in the number of roots per plant when the Chiang Da explants were cultured, particularly, in the semi-solid medium. The relative chlorophyll concentration was roughly calculated in a leaf by assessing the differential transmittance of light by the SPAD meter, a useful instrument for nondestructive determination. The result showed that the leaf greenness (SPAD readings) of leaves showed lower values in the liquid medium than the semi-solid treatments (Table 2). Although increasing of the SCE amounts in the medium reduced the shoot development, but all of the SCE treatments had no fungal contamination appearance, while the liquid medium control, the MIAA alone (T_4), had 5.6% of both fungal and bacterial contamination (Table 3).

Table 1 Shoot tip development of the Chiang Da explants when grown in different growth media and medium conditions for 4 weeks. Each data point is derived from 18 replicates.

Treatment	Medium condition	Shoot length (cm.)				Growth rate (cm./ week)
		Week 1	Week 2	Week 3	Week 4	
T_1	semi-solid	1.61±0.06	1.52±0.05	1.48±0.09bc	1.91±0.08c	0.075
	liquid	1.39±0.03	1.39±0.05	2.17±0.12a	2.43±0.07b	0.260
T_2	semi-solid	1.45±0.04	1.42±0.09	1.57±0.08bc	1.79±0.07cd	0.085
	liquid	1.63±0.17	2.21±0.70	2.14±0.09a	2.58±0.06b	0.238
T_3	semi-solid	1.38±0.03	2.13±0.70	1.38±0.06c	1.60±0.04d	0.055
	liquid	1.48±0.06	1.59±0.06	2.09±0.09a	2.39±0.09b	0.228
T_4	semi-solid	1.56±0.05	1.60±0.05	1.63±0.08b	1.88±0.07c	0.080
	liquid	1.57±0.08	2.41±0.86	2.24±0.09a	2.86±0.11a	0.323
$LSD_{0.05}$		NS	NS	*	*	nd

Means in the same column followed by the same letter are not significantly different.

* Significant at $p < 0.05$, NS = not significant, nd= not determine

Table 2 Root and leaves development of the Chiang Da explants when grown in different growth media and medium conditions after 4 weeks. Each data point is derived from 18 replicates.

Treatment	Medium condition	Root			Leaves	
		Germination (%)	Number of roots per plant	Root length (cm.)	Number of leaves per plant	Leaf greenness values (SPAD unit)
T_1	semi-solid	77.80±10.10ab	3.21±0.43ab	0.77±0.15abc	4.11±0.31	26.48±3.16ab
	liquid	50.00±12.10bc	2.89±0.48ab	1.04±0.16ab	4.29±0.22	14.67±2.38cd
T_2	semi-solid	55.60±12.10abc	3.10±0.67ab	0.26±0.03c	4.17±0.28	20.89±2.61bc
	liquid	61.10±11.80ab	2.33±1.33ab	0.77±0.32abc	4.22±0.17	15.04±2.08cd
T_3	semi-solid	27.80±10.90cd	4.09±0.44a	1.13±0.29a	3.11±0.25	26.32±2.04ab
	liquid	16.67±16.67d	1.60±0.40b	0.28±0.05c	4.17±0.34	10.88±1.52d
T_4	semi-solid	83.33±9.04a	2.07±0.36b	0.58±0.15abc	4.33±0.33	29.68±2.77a
	liquid	77.80±10.10ab	3.14±0.44ab	0.27±0.06c	4.72±0.23	22.35±3.12b
$LSD_{0.05}$		*	*	*	NS	*

Means in the same column followed by the same letter are not significantly different.

* Significant at $p < 0.05$, NS = not significant

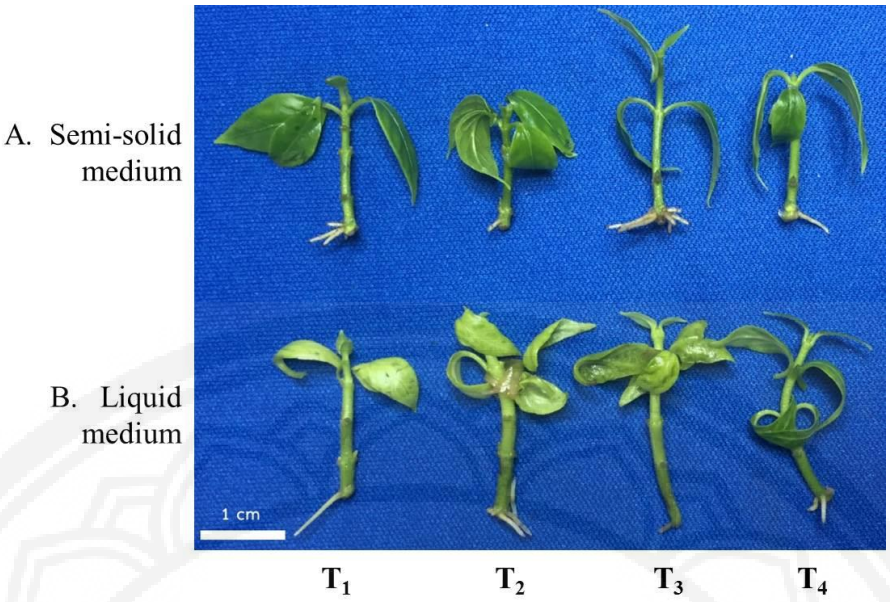

Figure 1 Morphology of the Chiang Da plantlets after grown in different growth media and medium conditions for 4 weeks.

Table 3 Percentage of microbial contamination (out of 18 plantlets) of the Chiang Da plantlet after grown in different growth media and medium conditions for 4 weeks.

Treatment	Medium condition	Microbial contamination (%)	
		Fungi	bacteria
T_1	semi-solid	0.00	0.00
	liquid	0.00	0.00
T_2	semi-solid	0.00	0.00
	liquid	0.00	5.56
T_3	semi-solid	0.00	0.00
	liquid	0.00	0.00
T_4	semi-solid	0.00	0.00
	liquid	5.56	5.56

The results revealed the fresh weight biomass of plantlet grown in the solid culture medium was less as compared to that in the liquid culture medium (Table 4). Increasing the SCE from 0.02 to 0.08 mg/L decreased the fresh weight yield, while addition of the MIAA alone (T_4) exhibited the highest explant yield when grown in both the liquid and semi-solid medium. Nevertheless, determination of the active antioxidant activity and total phenolic content present in the plantlets of *G. inodorum* extracted and measured through DPPH and Folin-Ciocalteu analysis showed that the accumulation of active phenolic compound in the semi-solid cultured plantlets were at a higher levels than those in the liquid treatment (Table 4).

Table 4 Fresh weight, antioxidant activity and total phenolic content of the Chiang Da plantlets after grown in different growth media and medium conditions for 4 weeks. Each data point is derived from 18 replicates.

Treatment	Medium condition	Fresh weight (g/explant)	Antioxidant activity (mg/ 100 g fresh weight)	Total phenolic content (mg/ g fresh weight)
T_1	semi-solid	0.090±0.008d	43.29±1.07b	0.89±0.02b
	liquid	0.144±0.022bc	24.03±3.88c	0.48±0.05c
T_2	semi-solid	0.066±0.006d	31.45±7.85bc	0.85±0.11b
	liquid	0.159±0.019b	17.15±4.78c	0.43±0.03c
T_3	semi-solid	0.058±0.004d	29.54±6.59bc	0.85±0.14b
	liquid	0.097±0.013cd	17.07±3.18c	0.40±0.02c
T_4	semi-solid	0.072±0.005d	63.99±8.67a	1.59±0.05a
	liquid	0.294±0.032a	27.54±2.17bc	0.49±0.03c
$LSD_{0.05}$		*	*	*

Means in the same column followed by the same letter are not significantly different.

* Significant at $p < 0.05$

Discussion

The different growth on shoot tip development of the Chiang Da explants when grown in media and medium conditions may be due to no concentration of agar to a provide adequate contact between plant tissue and the medium and better diffusion of medium constituents and availability of water, resulting in better growth (Suthar et al., 2011). As far as to our knowledge, that auxin can stimulate the existing growth of meristem and shoot tip of plants, that a low concentration of auxin (0.0001 – 0.01 mg/L) in concurrence with high levels of cytokinin is often useful and required when shoot multiplication, although in some cases it is sufficient to have only cytokinin. At the cellular level, the confluence action of auxin and cytokinin control cell division, each of which performs to influence different stages of the cell cycle. DNA replication is influenced by the auxin, while cytokinin seems to play some control over the occurrences moving to a stage of mitosis (George et al., 2008). The auxin concentrations higher than 0.01 mg/L are often inhibitory due to an increase in production of ethylene. The adaptation in the levels of auxin and cytokinin are often required when initiation of root development, where root development is generally reached by treatment with auxin alone (George et al., 2008). The *M. radiotolerans* ED5-9 has also been reported to produce cytokinins in an amount of 0.54 μg/mL of culture media after incubation for 5 days (Prombunchachai, 2015). Therefore, high level of auxin, IAA, from the *M. radiotolerans* ED5-9 tested in this study (0.0067 mg/L) and from the *Streptomyces* TM32 (54.00 mg/L of culture media after incubation for 7 days) reported by Nakaew et al. (2015) including the tested shoot tip explants which itself can produce auxin, and can diffuse to roots, may cause reduction of shoot growth and root germination when compare to the control treatment which contained less concentration of IAA. The influence of IAA on rooting has been further supported by Umamaheswari et al. (2014), the number of roots and biomass can decrease after extending the duration of culture of explants in medium with higher concentrations of IAA due to osmotic stress (Min et al., 2007).

This experiment was designed for different concentration of crude substances extracted from microbial fermentation study as a mean to create a variation of % N attained in the culture medium. Less greenness values of leaves in this study may be thus affected by % N of culture medium and by light exposure according to

submerging of plantlets in the medium. According to N concentration in leaf correlates with chlorophyll concentration because it mostly involves in construction of N-containing enzymes and other organic compounds so that foliar N can be estimated by the SPAD meter, and it can be used as a rapid diagnostic device (Chapman and Barreto, 1997). The environmental stresses, such as less light exposure and sinking in the medium, may cause chlorophyll loss due to stress in plants (Hendry and Price, 1993). The accumulation of active phenolic compound in the semi-solid cultured plantlets were at a higher level than those in liquid treatment, which closely associated with the antioxidant activity and phenolic content obtained in plants through optimization of farm plantation has been investigated (Sritontip et al., 2016). Thus, as promising alternatives for the production of valuable secondary metabolites, plant tissues culture is widely accepted. The *Streptomyces* TM32 has shown a typical antagonistic activity against the fungus, in which the fermentation broth of the isolate having the effective dose for 50 percent (ED_{50}) of 2.61 m/L equivalent to 1.19 g/L of metalaxyl after incubation for 7 days and it was also able to produce chitinase as a cellulolytic enzyme (Nakaew et al., 2015), and therefore there were no fungal contamination appeared in the SCE treatments. However the antifungal activity of the isolate TM32 might not only due to the action of chitinase but also the mixture of diverse bioactive compounds that remain in its crude culture fluid such as antibiotics, antifungal agents, siderophore, etc.

Conclusion and Suggestion

In this study the regenerated shoots were, however, subjected to rooting and growth on MS medium in the presence of any growth promoting substance extracted from microbial fermentation broth. The growth promoting substances produced from both combination of the *M. radiotolerans* ED5-9 and the *Streptomyces* TM32 or from the *M. radiotolerans* ED5-9 alone, therefore, showed beneficial effects on micro-propagated Chiang Da, *G. inodorum*, plantlets and this may have a potential for use in the commercial production of a bio-fertilize or anti-microbial agent for Chiang Da, since the desire for pharmaceutical industries as plant based raw materials is increasing.

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