IAA Producing *Enterobacter* sp. I-3 as a Potent Bio-herbicide Candidate for Weed Control: A Special Reference with Lettuce Growth Inhibition

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Abstract Development of bio-herbicides is an emerging method to weed management in agricultural field. Very few studies were conducted on identification of microbial bio-herbicides to weed control. The present study was aimed to isolate and identify the effective bio-herbicide potential bacterium from soil and assess their role on plant growth inhibition. Three-hundred and one rhizobacteria were isolated from agriculture field soil samples collected from various parts of Republic of Korea. Two bacterial strains, I-4-5 and I-3 were significantly reduced the seedling growth of radish when compared to their controls. The highest rate of seedling growth inhibition was observed in I-3 bacterial isolate treatment in lettuce and radish. The mechanism of an effective bio-herbicide I-3 to plant growth inhibition was determined by analyzing IAA in their culture medium. IAA biosynthesis pathway of *Enterobacter* sp. I-3 was identified as tryptophan-dependent pathway and its production was increased due to addition of tryptophan in culture medium as quantified by using GC–MS SIM. In an in vitro study revealed that I-3 bacterial culture exudate combined with tryptophan significantly decreased leaf length, leaf width, root length and increased the number of lateral roots of lettuce. Indeed, the genomic DNA of I-3 bacterium was isolated and 16S

rDNA was sequenced to find out the name of the bacterium. Based on phylogenetic analysis, I-3 isolate was identified and named into *Enterobacter* sp. I-3. The results of this study suggest that the utilization of *Enterobacter* sp. I-3 to crop field can be act as a potential bio-herbicide against weed growth.

Keywords *Enterobacter* sp. I-3 · Lettuce · Indole-3-acetic acid · Plant growth inhibition

Introduction

As concerns about wellbeing and awareness against agricultural product safety is increasing not only in Korea but it is a major concern in all over the world. The investigation on residual agricultural chemicals in foods showed that 31.75 % out of 6,590 food products were identified as unsuitable for human consumption due to the maximum residue limit (MRL) of pesticides [1]. The majority of green vegetables such as napa cabbage, lettuce, crown daisy, horseradish, winter grown cabbage, mustard had the exceeded level of pesticides. Triazole-type compounds have been widely used in agriculture and cause to health hazard and environmental pollutions [2]. Diniconazole, a triazole compound is reported as a plant growth inhibitor which inhibits the growth of napa cabbage, grass and phalaenopsis and, while hexaconazole also inhibits the plant growth of rice. These chemicals block gibberellin (GA) biosynthesis which leads to affect the plant growth. On the other hand, trinexapac-ethyl is an herbicide, inhibits GA biosynthesis and has been used to limit the shoot growth [3]. Production of safety food is threatened by exceeding agricultural chemicals when spraying the plant growth retardants. For that reason, as an effort to produce

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safe agricultural products and to reduce the use of plant growth retardants, fungicides, pesticides and herbicides, an environmental friendly method such as microbial pesticides and microbial fertilizers is being studied to control pest and retard the plant growth.

There are countless numbers of microorganisms in varied environments and most of the microorganisms are used to increase the productivity of crop plants by symbiotic association. The soil microbes including bacteria and fungi directly or indirectly involve in growth and development of plants. However, the two kinds of bacteria, plant growth promoting or inhibiting bacteria exist in soil. A numbers of bacteria promote plant growth by nitrogen fixation in rhizosphere [4]. Phosphate solubilizing bacteria which hydrolyze organic and inorganic phosphorus from insoluble compounds help to enhance the plant growth [5]. Phytohormones such as auxin, cytokinin, gibberellin produced by bacteria expressed a positive effect on plant growth [4]. Moreover, the plant beneficial bacteria protect plants by secreting antibiotics, hydrogen cyanide and siderophore which inhibit the growth of pathogenic organisms. Some of bacteria inhibit the growth of plants in soil. Plant growth inhibition of bacteria is caused by various mechanisms. Volatile materials arise from bacterial culture inhibit the plant growth and pathogens [6–8]. For instant, some species of *Pseudomonas* and *Chromobacterium* genus have a negative effect on plant growth due to the production of hydrogen cyanide. However, a non-volatile compound, hydrocinnamic acid produced by *Bacillus subtilis* IJ-31 showed an inhibition pepper seeding growth with low amount of active gibberellin [9]. Several rhizobacteria produce indole-acetic acid (IAA) [10]. Utilization of microorganisms to control weeds is an alternative method to chemical herbicides and this approach has been used successfully to control the growth of different weeds [11]. The establishment of deleterious rhizosphere bacteria (DRB) in weeds rhizospheres would be helpful to weed control management in crop fields and more economical than application of chemical herbicides [12]. Therefore, in this study, we isolated a bacterium from soil and tested their inhibitory effects on plant growth by hormonal secretion. The objectives of this study were to isolate and identify a potent bacterium for plant growth inhibition and to determine their inhibitory mechanism by analysing IAA.

Materials and Methods

Collection, Isolation and Preservation of Bacteria from Soil

To screen the plant growth inhibiting or bio-herbicidal activity of bacterium, soil samples were collected from

farm land of Republic of Korea and, 5 g of soil was transferred to sterile 0.85 % NaCl added flask and gently had shaken to dissolve the soil. The obtained soil suspensions were serially diluted with sterile distilled water and 0.1 ml aliquots were inoculated on petriplates containing autoclaved Luria–Bertani media agar (LB; Merck Co., Germany), for culturing bacteria. This study was used to identify the bacteria for inhibiting plant growth. The petriplates of bacterial cultures were incubated for 24 h at 30 °C and, the grown bacterial colonies were differentiated by their morphology and colours. The individual colonies were separately cultured on LB medium and stored in 50 % glycerol at –20 °C for long time preservation.

Screening of Plant Growth Inhibiting Bacteria

Chinese radish and lettuce seeds were purchased from Hungnong Co. and Danong Co. (Korea) respectively, and surface sterilized with 5 % sodium hypochlorite and thoroughly washed with distilled water. Four bacterial strains (I-1-2, I-2-3, I-3 and I-4-5) were selected from LB agar medium petri-dish and Chinese radish seeds were pre-treated with ten times dilution of bacterial culture broth (30 °C, 200 rpm) and inoculated in petri-dish and then cultured at 25 ± 2 °C in an aseptic environment for 3 days. The distilled water and LB medium application were used as control for this experiment. The length of shoot and root and fresh weigh of plants was measured. In a green-house study, Chinese radish and lettuce seeds were sown on autoclaved horticulture soil and bacterial isolates I-3 and I-4-5 were inoculated on seeds containing trays. The growth inhibition of bacterial isolates were determined by measuring the height and fresh weight of plants, length, width and number of leaves at 7 days old plants.

Identification and Phylogenetic Analysis of I-3

The bacterial isolate, I-3 was cultured on LB medium for 3 days at 30 °C and culture medium was removed after centrifugation. The collected pellet was used to extract the genomic DNA to identify the bacterial isolate I-3 on the basis of partial 16S ribosomal DNA (rDNA) sequence as per the method described by Kang et al. [4]. The forward primer (518F: CCAGCAGCCGCGTAATACG) and reverse primer (800R: TACCAGGGTATCTAATCC) were used to sequence the 16S rDNA. To know the nucleotide sequence homology of this bacterial isolate, the obtained I-3 sequences were submitted to the BLAST search program (<http://www.ncbi.nlm.nih.gov/BLAST/>). The closely identical sequences obtained from BLAST search results were aligned through CLUSTAL W and their phylogenetic relation was detected on neighbor-joining tree constructed by using MEGA software.

In vivo Study on Plant Growth Inhibition by IAA Producing I-3

For determination of optimal medium to IAA production in bacterial culture, I-3 strain was inoculated on LB medium supplemented with varying concentrations of tryptophan (0.5, 1, 2 and 3 g). It was incubated at 30 °C on a shaking incubator and IAA was quantified at 3, 5 and 7 days, after inoculation. The bacterial strain, I-3 cultured with LB and tryptophan supplemented medium were centrifuged at 10,000 rpm for 20 min at 4 °C as separately, and the supernatant was filtered through 0.45 µm cellulose acetate filter (DISMIC®; Frisette ApS, Knebel, Denmark). The filtrates were acidified into pH 2.8 by using HCl and 40 µl [D⁵]-IAA was added and it was extracted three times with 4 ml ethyl acetate. The ethyl acetate extracts were then combined together and evaporated under vacuum at 45 °C in a rotary evaporator. The organic layer was vacuum dried and added with 60 % methanol (MeOH), while the pH was adjusted to 8.0 ± 0.3 using 2 N NH₄OH and passed through a reverse-phase C18 column. The methanol fractions were prepared by dissolving the residue in 1 ml of methanol and adding 1.5 ml ethereal diazomethane. The methylated samples were re-dissolved in ethyl acetate before the IAA analysis by GC–MS with SIM (6,890 N network GC system, and 5,973 network mass selective detector; Agilent, Palo Alto, CA, USA). The experiments were repeated three times.

Seeds of lettuce were sterilized with sodium hypochlorite (2.5 %) for 30 min, and thoroughly rinsed with sterile double distilled water (DDW). The seeds were inoculated on MS medium solidified with agar as served as control and different kinds of media were prepared to compare the lettuce plant growth inhibition such as MS + 1 % LB medium, MS + 1 % I-3 bacterial cultured in LB medium supplemented with 3 % tryptophan, MS + IAA 10^{−6} M and MS + IAA 10^{−4} M in square dish plates (23 cm × 23 cm × 1.5 cm). The inoculated plates were kept at 25 °C in light on 14 h and 20 °C in dark on 10 h per day in an aseptic culture room. The leaf length, leaf width, leaf number, root length and lateral roots numbers were measured after 2 weeks.

Statistical Analysis

The data obtained from the experiments were analysed and calculated the mean of ten replicates at three times. As the experimental design is completely randomized and data for each experiment were analyzed using Sigma Plot Software (10.0) and analysis of variance (ANOVA) was used to compare the statistical difference based on Duncan's multiple range test (DMRT), at significance level of $p \leq 0.05$.

Results

Effect of PGIR on Chinese Radish and Lettuce Plant Growth

Three-hundred and one bacterial strains were isolated from the soil samples. The isolated bacterial colonies were separated based on morphology and inoculated on fresh media and glycerol for long time preservation. Among 301 strains, the four bacterial strains I-1-2, I-2-3, I-3 and I-4-5 were tested to know their effect on Chinese radish seedling growth. The bacterial isolates treated seeds showed lower rate of shoot length, root length and fresh weight than bacteria untreated seedlings (Table 1; Supplementary Fig. 1). A significant reduction of shoot length was found in seeds treated with I-3 (0.30 mm) and I-4-5 (1.04 mm). The inhibitory effect of medium composition (LB) was observed in length of shoots and roots. The higher rate of root length and fresh weight was also detected in I-3 and I-4-5 bacteria treated seedlings than other bacterial treatments. I-3 and I-4-5 bacterial isolates induced growth inhibition were further determined by measuring aerial parts of Chinese radish at 7 days (Table 2; Supplementary Fig. 2). In a comparison with controls, plant height, leaf length, leaf width and fresh weight of leaves were decreased in plants inoculated with I-3 and I-4-5 bacteria. The plant height and leaf width were significantly higher in LB medium supplemented soil than their controls and there were no significant difference between numbers of leaves in plants interacted with or without LB medium, I-3 and I-4-5. A severe inhibitory effect of Chinese plant growth was found in I-3 associated plants. To confirm the significant role of I-3 on plant growth inhibition, I-3 and I-4-5 were tested on lettuce (Table 2; Supplementary Fig. 3). The results of this study showed that I-3 and I-4-5 stains significantly inhibited plant height, leaf length, leaf width and fresh weight of leaves in lettuce. However, the inhibition role of I-3 was higher than I-4-5 bacterium on lettuce plant growth.

Table 1 Assessment of bacterial strains I-1-2, I-2-3, I-3 and I-4-5 for their growth inhibitory effect on Chinese radish seedling

Treatment	SL (mm)	RL (mm)	FW (mg)
Con	2.68 ^a	3.20 ^a	11.05 ^a
LB	2.21 ^{bc}	0.98 ^d	11.02 ^a
I-1-2	2.32 ^b	1.41 ^b	9.58 ^b
I-2-3	2.20 ^{bc}	1.10 ^c	10.62 ^{ab}
I-3	0.30 ^d	0.22 ^f	2.38 ^d
I-4-5	1.04 ^c	0.54 ^e	7.79 ^c

Means followed by the same letter in the column are not significantly different ($p \leq 0.05$) as determined by DMRT

SL shoot length, RL root length, FW fresh weight

Table 2 Effect of I-3 and I-4-5 strains on growth of Chinese radish and lettuce plants

Chinese radish						Lettuce			
Treatment	PH (cm)	LL (cm)	LW (cm)	LN	LFW (g)	PH (cm)	LL (cm)	LW (cm)	LFW (g)
Con	6.70 ^b	8.99 ^a	3.72 ^b	6.55 ^a	11.19 ^a	6.31 ^a	7.31 ^a	3.92 ^a	12.17 ^a
LB	7.10 ^a	8.75 ^a	4.05 ^a	6.35 ^a	11.36 ^a	6.53 ^a	7.42 ^a	3.84 ^a	11.80 ^a
I-3	5.18 ^d	6.66 ^c	3.08 ^c	6.15 ^a	6.21 ^c	4.04 ^b	5.71 ^b	3.43 ^b	7.21 ^b
I-4-5	5.53 ^c	7.18 ^b	3.52 ^b	6.35 ^a	7.01 ^b	4.64 ^b	5.60 ^b	3.40 ^b	7.55 ^b

Means followed by the same letter in the column are not significantly different ($p \leq 0.05$) as determined by DMRT

PH plant height, LL leaf length, LW leaf weight, LN leaf numbers, LFW leaf fresh weight

Identification and Phylogenetic Analysis of I-3 Bacterium

To know the name of a potential plant growth inhibiting bacterial isolate I-3, the genomic DNA of I-3 was extracted and 16 rDNA was sequenced. The phylogenetic analysis was carried out on I-3 bacterial 16S rDNA sequence (Fig. 1). Sequences of other genera were also used to determine the actual relationship among participating candidates in the group. The result of BLAST search revealed that bacterial strain I-3 had maximum sequence homology with *Enterobacter* sp. and was named as *Enterobacter* sp. I-3. The sequence was submitted to NCBI GenBank and was assigned accession number KJ956038.

Influence of Tryptophan Composition on IAA Production in I-3 Bacterial Culture

The root elongation and stunted shoot length of lettuce revealed that I-3 bacterium might be produced the root elongation hormone, IAA during their interactions. In a spectrophotometric study of IAA analysis showed that I-3 bacterium secreted higher concentration of IAA than I-4-5

strain. The IAA production was optimized in I-3 culture by addition of IAA precursor, tryptophan at the rate of 0.5, 1, 2 and 3 g (Fig. 2). The low quantity of IAA was detected in I-3 strain cultured without tryptophan. The significantly higher concentration of IAA was observed in 2 and 3 g of tryptophan supplemented medium. The IAA production rate is gradually increased from 0 to 7 days in tryptophan added medium inoculated with I-3. The maximum rate of IAA was observed in I-3 bacterium cultured with 3 g tryptophan supplemented medium at 7 days.

Inhibitory Effect of *Enterobacter* sp. I-3 on Plant Growth under In vitro and In vivo Conditions

The growth inhibition of lettuce by bacterial culture was compared with two different concentrations of IAA, tryptophan and LB medium (Table 3; Supplementary Fig. 4). The plants grown under LB medium showed an increased level of leaf length, leaf width, length of root and numbers of lateral roots than their controls. The high concentration of IAA (10^{-6} M) supplemented medium significantly enhanced the lettuce leaf length, leaf width, root length, and reduced the number of lateral roots, while low

Fig. 1 Neighbor-joining phylogenetic tree based on the sequence obtained from 16S rDNA of I-3 and related bacteria. Percentage of confidence levels generated from 1,000 bootstrap trees is indicated in each node

Fig. 2 IAA production in I-3 culture medium contained 0.5, 1, 2 and 3 g of tryptophan

Table 3 Effect of *Enterobacter* sp. I-3 on leaf and root growth

Treatment	LL (mm)	LW (mm)	LN	RL (mm)	NLR
MS	32.3 ^{bc}	12.0 ^c	5.0 ^a	62.7 ^b	11.7 ^c
MS + LB	36.3 ^b	13.0 ^b	5.0 ^a	90.0 ^a	13.3 ^b
MS + I-3	36.0 ^b	13.3 ^b	5.0 ^a	47.3 ^c	11.0 ^c
MS + I-3 + Trp	27.3 ^c	9.0 ^d	5.0 ^a	26.0 ^d	17.0 ^a
MS + IAA 10 ⁻⁶	41.3 ^a	14.0 ^a	5.0 ^a	88.7 ^a	10.0 ^d
MS + IAA 10 ⁻⁴	29.0 ^c	8.7 ^d	5.0 ^a	41.0 ^c	16.7 ^a

Means followed by the same letter in the column are not significantly different ($p \leq 0.05$) as determined by DMRT

MS MS medium, MS + LB MS and LB media, MS + I-3 MS medium with I-3 culture, MS + I-3 + Try MS medium supplemented with I-3 culture and tryptophan, MS + IAA 10⁻⁶ MS medium added with IAA 10⁻⁶ M, MS + IAA 10⁻⁴ MS medium added with IAA 10⁻⁴ M, LL leaf length, LW leaf width, LN leaf numbers, RL root length, NLR number of lateral roots

concentration of IAA (10⁻⁴ M) drastically inhibited the leaf length, leaf width, root length and promoted the number of lateral roots when compared to other treatments and controls. The bacterial isolate, I-3 culture exudate combined with tryptophan significantly decreased leaf length, leaf width, root length and increased the number of lateral roots, which showed the prominent inhibition in lettuce growth.

Discussion

Deleterious root associated bacteria induces harmful effects on plants, which can be utilized as bio-herbicides to control weeds [12]. In current study, four (I-1-2, I-2-3, I-3 and I-4-5) of the 301 bacterial isolates screened for plant growth inhibition effect on weeds and, significantly higher deleterious effect on growth and biomass of Chinese radish was found in

I-3 and I-4-5 isolates interacted seedlings. In a further screening of I-3 and I-4-5 strains against plant growth under environmental controlled conditions showed that I-3 strain was more effective to decrease the plant height, leaf length, leaf width and biomass than I-4-5 strain. The response of plant growth is varied between different bacterial inoculations. Recently, Barghouthi and Salman [13] showed severe, lesser and no inhibitory effect of array of bacteria on broomrape plant growth. The number of DRBs belongs to the genus of *Aeromonas*, *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Burkholderia*, *Chryseomonas*, *Enterobacter*, *Microbacterium*, *Pseudomonas* and *Xanthomonas* were reported by several studies [12–14]. On the other hand, the deleterious effect of bacterium on plants can be species or cultivars specific [15]. For this reason, we tested the bacterial isolate I-3 and I-4-5 on lettuce to confirm their deleterious effect. The similar kind of results, as like stunted growth of Chinese radish was obtained in I-3 and I-4-5 bacterial treatments. The prominent inhibition of Chinese radish and lettuce plant height and leaf characters by I-3 strain without any disease symptoms suggests that I-3 bacterium can able to control the wide range of weed growth. Furthermore, I-3 strain was identified as *Enterobacter* sp. I-3 after sequencing 16 s rDNA and phylogenetic analysis.

The suppression of plant growth during the interaction of rhizosphere microorganisms is linked to the secretion of secondary metabolites from microorganisms [16]. A reason of plant growth inhibiting effect of *Enterobacter* sp. I-3 was hypothesized that it could secrete the higher concentration of IAA. Auxin biosynthesis in microbes and their interaction with crop plants were widely reported [10, 17]. Plants response to exogenous IAA differs due to the concentration perceived by tissues. The optimal levels of IAA were accountable to improve plant growth and, the elevated level of IAA caused negative effect to plants [18]. For instant, Xie et al. [19] demonstrated the role of

Pseudomonas putida produced IAA on plant growth and suggested that the low quantity of IAA increased the plant growth and while, more accumulation of IAA played a major role to inhibit the root growth. Moreover, it stimulates the aminocyclopropane-1-carboxylate (ACC) synthase, which involves for increasing the amount of ethylene from their biosynthesis [20].

Interestingly, significant amount of IAA was detected in *Enterobacter sp.* I-3 culture due to the presence of an increased activity of tryptophan deaminase, an enzyme produces IAA from tryptophan [21]. To increase their production, different concentration of tryptophan, a precursor for IAA biosynthesis was added in culture medium. The higher production of IAA was obtained in 3.0 g tryptophan added I-3 culture medium. In a similar previous study documented that *Enterobacter taylorae*, a DRB isolate significantly inhibited the growth (90.5 %) of bindweed (*Convolvulus arvensis* L.) when cultured in high concentration of tryptophan (10^{-5} M) than non-treated control [14]. The subsequent plant growth study findings showed that *Enterobacter sp.* I-3 significantly inhibited the root growth and areal part of lettuce in tryptophan added MS medium than their controls and IAA amended media. I-3 isolate induced reduction in root growth due to their production of secondary metabolites such as IAA at optimal medium components and aggressive colonization to lettuce root surfaces, which might altered the root physiology. Sarwar and Kremer [14] strongly suggested that proving tryptophan to auxin-secreting DRB to enrich the toxicity against weed seedlings would be a concrete method for biological weed control.

Conclusions

The results of this present investigation showed that IAA producing *Enterobacter sp.* I-3 interacted with lettuce plants and might be enhanced the ethylene synthesis to inhibit lettuce plant growth. Moreover, the obtained significant results of this study allow us to suggest the *Enterobacter sp.* I-3 application to agricultural fields can be an alternative to agrochemicals for inhibiting weed growth.

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