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Enterobacter sp. I-3, a bio-herbicide inhibits gibberellins biosynthetic pathway and regulates abscisic acid and amino acids synthesis to control plant growth

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ABSTRACT

Very few bacterial species were identified as bio-herbicides for weed control. The present research was focused to elucidate the plant growth retardant properties of *Enterobacter* sp. I-3 during their interaction by determining the changes in endogenous photosynthetic pigments, plant hormones and amino acids. The two bacterial isolates I-4-5 and I-3 were used to select the superior bacterium for controlling weed seeds (*Echinochloa crus-galli* L. and *Portulaca oleracea* L.) germination. The post-inoculation of I-3 (*Enterobacter* sp. I-3) significantly inhibited the weeds seed germination than their controls. The mechanism of bacterium induced plant growth reduction was identified in lettuce treated with I-3 bacterium and compared their effects with known chemical herbicide, trinexapac-ethyl (TE). The treatment of I-3 and TE showed a significant inhibitory effect on shoot length, leaf number, leaf length, leaf width, shoot weight, root weight and chlorophyll content in lettuce seedlings. The endogenous gibberellins (GAs) and abscisic acid (ABA) analysis showed that *Enterobacter* sp. I-3 treated plants had lower levels of GAs (GA_{12}, GA_{19}, GA_{20} and GA_8) and GAs/ABA ratio and then, the higher level of ABA when compared to their controls. Indeed, the individual amino acids ie., aspartic acid, glutamic acid, glycine, threonine, alanine, serine, leucine, isoleucine and tyrosine were declined in TE and I-3 exposed plants. Our results suggest that the utilization of *Enterobacter* sp. I-3 inhibits the GAs pathway and amino acids synthesis in weeds to control their growth can be an alternative to chemical herbicides.

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1. Introduction

Weeds are major challenging problem to get higher yield of crop plants from agricultural fields. Barnyard-grass (*Echinochloa crus-galli* L.) and *Portulaca oleracea* L. are common weeds in crop fields. *E. crus-galli* L causes a reduction of rice yield upto 44–96% (Islam and Kato-Noguchi, 2013) and *P. oleracea* L. has been ranked the eighth most common weed in the world (Moneim et al., 2013).The herbicides and pesticides are used to control the weeds and pest in agricultural lands cause harmful side-effects to plants and contaminates soil, water and deposits in food products (Short and Colborn, 1999). Kim et al. (2013) reported that ~32% of food products out of 6590, including cabbage, lettuce and mustard are unsuitable for consumption in Korea, because of more accumulation of pesticides. Indeed, the prolonged exposure of herbicides to weeds has developed the multiple herbicide resistance weed populations (Culliney, 2005). Application of soil microorganisms to control weeds is an alternative way to herbicides and provides a limited chance of development of bioherbicide resistance weeds and has been successful to control the growth of different weeds (Crump et al., 1999; Omer et al., 2010; Weissmann and Gerhardson, 2001). Rhizobacteria have the potential of bioherbicides and can suppress the plant growth. *Pseudomonas putida*, *Stenotrophomonas maltophilia* and *Enterobacter taylorae* have been reported as bioherbicides (Mazzola et al., 1995). The production of metabolites, hydrogen cyanide, ammonia and dimethyle disulfide from deleterious rhizobacteria (DRB) was absorbed by plants through roots to inhibit the weed growth (Astrom and Gerhardson, 1989). The establishment of DRB in weeds rhizospheres would be helpful to weed control management in crop fields and more economical than application of chemical herbicides (Arshad and Frankenberger, 1991).

An important mechanism of plant growth suppression by DRB is to secrete L-tryptophan derived compounds such as auxin (Sarwar

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and Frankenberger, 1994). In a previous study, we isolated and identified a bio-herbicide bacterium *Enterobacter* sp. I-3 from agricultural soil and observed their plant growth inhibitory effects on lettuce and Chinese radish due to the secretion of indole-3-acteic acid (Park et al., 2015). The subsequent investigation was followed in this study to know the endogenous hormonal and amino acids changes in plants during *Enterobacter* sp. I-3 bacterial interaction to suppress the plant growth. Plant growth retardants block gibberellin (GA) biosynthesis in plants. For instant, trinexapac-ethyl (TE) inhibits GA biosynthesis, which leads to suppress the shoot growth (Pannacci et al., 2004). The endogenous GAs involve in seed germination, stem elongation, leaf expansion and flowering (Achard and Genschik, 2009). The inhibition of GAs pathway resulted to stunted plant growth. Indeed, several physiological processes including ion transport and stomatal opening are regulated by amino acids. Although, proline metabolism has been studied extensively to know the function of amino acids during stress condition, the information of other amino acids metabolism is less (Rai, 2002). Herbicides target the specific inhibition of enzyme activity in amino acid biosynthesis, which are useful to control weed growth (Zulet et al., 2013). The aim of this study was to elucidate the mechanism of a bio-herbicide, *Enterobacter* sp. I-3 to control plant growth by determining the regulation of array of gibberellins and amino acids.

2. Materials and methods

2.1. Bacterial inoculation on weed seeds

In a preliminary study, we showed three hundred and one rhizobacterial colonies from soil samples collected from various parts of Republic of Korea. Of these, two bacteria I-4-5 and I-3 effectively inhibited the plant growth (Park et al., 2015). In this study, I-4-5 and I-3 were used to control the different weed seeds germination. The weed seeds such as *Echinochloa crus-galli* (L.) and *Portulaca oleracea* (L.) were sterilized by using sodium hypochlorite (5%) for 10 min, and washed with sterile distilled water for three times and placed over the two layered filter papers in petriplates. The bacterial isolates I-4-5 and I-3 were separately cultured on LB medium and incubated at 30 °C with 200 rpm on a shaker. After three days, each bacterial culture were ten times diluted and then applied to weed seeds containing filter papers and kept at 28 ± 2 °C under a dark condition. The sterile water was used as a control in this experiment. The bio-herbicide effect of I-4-5 and I-3 was observed by calculating the seed germination upto 7 days at periodic intervals.

2.2. Plant growth and *Enterobacter* sp. I-3 bacterial treatment

I-3 bacterial isolate showed higher inhibition of weed seed germination in above experiment than controls and identified as *Enterobacter* sp. I-3 (Park et al., 2015) by using molecular technique and submitted to GenBank (Accession number KJ956038). Further work was conducted on lettuce plant growth to know their inhibitory mechanism of weed controls. *Enterobacter* sp. I-3 isolate was cultured for 7 days at 30 °C on a shaking incubator at 200 rpm. Lettuce seeds were surface sterilized with sodium hypochlorite (5%) for 10 min, and thoroughly rinsed with autoclaved double distilled water. Seeds were sown in plastic tray (50 holes) containing horticultural soil under controlled greenhouse conditions (30 ± 2 °C). Fifteen days old lettuce seedlings were separately treated with 5 ml I-3 bacterial culture suspension and 200 ppm trinexapac-ethyl (TE), an herbicide to compare the effect of I-3 bacterium on growth inhibition. The experiment was repeated three times. The plant samples were harvested at 3 h, 6 h, 9 h and 12 h post inoculation and freeze dried at −50 °C using ISE Bondiro Freeze dryer and used to phytohormones, proteins and amino acids analysis. The growth attributes i.e., plant height, leaf length, leaf width, leaf numbers, shoot weight, root weight and chlorophyll content (SPAD) were recorded after fifteen days of treatment.

2.3. Abscisic acid extraction and quantification

The endogenous ABA contents were extracted following the method of Qi et al. (1998) and Kamboj et al. (1999). Lettuce samples were extracted with isopropanol (95%), glacial acetic acid (5%) and 20 ng of ABA standard was added with this extract. The obtained filtrate was concentrated by a rotary evaporator and dissolved in 1 N NaOH and washed with methylene chloride. The pH of this extract was adjusted into 3.5 by using HCl and partitioned three times with ethyl acetate and the extract was evaporated and dissolved in phosphate buffer (pH 8.0) and then passed to a polyvinylpolypyrrolidone column and re-adjusted the pH 3.5 and partitioned into ethyl acetate. The dried extract was again dissolved in dichloromethane and transferred to a silica cartridge (Sep-Pak; Water Associates, Milford, Massachusetts, USA) prewashed with dichloromethane. To elute the ABA from the cartridge, diethyl ether and methanol (3:2, v/v) was applied. The ABA extract was dried and methylated by adding diazomethane for GC–MS/SIM (6890N network GC system, and 5973 network mass selective detector; Agilent Technologies, Palo Alto, CA, USA) analysis. For quantification, the Lab-Base (ThermoQuset, Manchester, UK) data system software was used to monitor responses to ions of m/e 162 and 190 for Me-ABA and 166 and 194 for Me-[$^2H_6$]-ABA.

2.4. Gibberellins extraction and quantification

The lyophilized samples were ground to fine powder in a chilled mortar and pestle. The endogenous GA was extracted according to a method followed by Lee et al. (1998). Plant extracts were subjected to chromatographic and mass spectroscopy techniques for the identification and quantification of GAs. The extracts were passed through a Davisil C18 column (90–130 μm; Alltech, Deerfield, IL, USA). The eluent was reduced to near dryness at 40 °C in vacuum. The sample was then dried onto celite and then loaded onto SiO_2 partitioning column (de activated with 20% water) to separate the GAs as a group from more polar impurities. GAs were eluted with 80 ml of 95:5 (v/v) ethyl acetate (EtOAc): hexane saturated with formic acid. This solution was dried at 40 °C in vacuum, re-dissolved in 4 ml of EtOAc, and partitioned three times against 4 ml of 0.1 M phosphate buffer (pH 8.0). Drop-wise addition of 2N NaOH was required during the first partitioning to neutralize residual formic acid. One-gram polyvinylpolypyrrolidone (PVPP) was added to the combined aqueous phases, and this mixture was slurried for 1 h. The pH was reduced to 2.5 with 6N HCl. The extract was partitioned three times against equal volumes of EtOAc. The combined EtOAc fraction was dried in vacuum, and the residue was dissolved in 3 ml of 100% MeOH. This solution was dried on a Savant Automatic Environmental Speedvac (AES2000, Madrid, Spain). The dried samples were subjected to high performance liquid chromatography (HPLC) using a 3.9 × 300 m Bondapak C18 column (Waters Corp., Milford, MA, USA) and eluted at 1.0 ml/min with the following gradient: 0–5 min, isocratic 28% MeOH in 1% aqueous acetic acid; 5–35 min, linear gradient from 28% to 86% MeOH; 35 to 36 min, 86% to 100% MeOH; 36 to 40 min, isocratic 100% MeOH. Forty-eight fractions of 1.0 ml each were collected. The fractions were then prepared for gas chromatography/mass spectrometry (GC/MS) with selected ion monitoring (SIM) system (6890N Network GC System, and 5973 Network Mass Selective Detector; Agilent Technologies, Palo Alto, CA, USA). For each GAs, 1 μl of sample was injected in GC/MS SIM. Full-scan mode (the first trial) and three major ions of the supple-

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