Characterization of Plant Probiotic Isolates of Lysinibacillus spp.

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(植物プロバイオティック*Lysinibacillus*属菌株の特性評価)

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General Introduction

World population is increasing in a rapid pace which indicate the necessity of enhancement in food supply to avoid starvation. Specially, increasing the crop production has become very challenging because the cultivable land area is gradually decreasing (FAO, 2020). Therefore, it is predictable that more agrochemicals are going to be used in near future to maintain the food supply. Application of chemical fertilizers can enhance crop yield to a great extent. However, long term and excess use of chemical fertilizers has some side effects also such as soil quality degradation, low water retention ability of soil (Savci, 2012). Moreover, plants cannot uptake all the applied fertilizer instantly, therefore a portion of fertilizer precipitate in the soil and mix with the water bodies due to run off and cause environmental pollution. On the other hand, chemical pesticides also can enhance crop yield by reducing disease caused by several pathogens however, pathogens could become resistant towards pesticides (Hawkins et al., 2019; Sparks and Nauen, 2015). Therefore, the effectiveness of pesticides may also be threatened. Some other technologies have also been adapted such as hybridization, improvement of genetically modified crop. However, these techniques are time consuming and costly (Li, 2020). This scenario refers the need for possible alternatives to solve the problem.

In recent years, plant probiotic bacteria are getting admiration of the scientific communities for their potential of enhancing crop production. Bacteria which can exert beneficial effect on plant such as enhancement of growth and yield, suppression of disease are known as plant probiotic bacteria (Menendez and Garcia-Fraile, 2017). Several mechanisms are involved with the growth promoting potential of plant probiotic bacteria such as nitrogen fixation, Phytohormone biosynthesis (auxins, gibberelings, cytokynins, ethylene and ACC desaminase synthesis), nutrient

solubilization, siderophore production, and biocontrolers (production of plant cell wall degrading enzymes, induced disease suppression, resistance to stresses) (Menendez and Garcia-Fraile, 2017). *Azospirillum*, *Bacillus*, *Burkholderia*, *Paenibacillus*, *Pseudomonas*, and *Rhizobia* are some well reported bacterial genera which can exert above mentioned beneficial effect on plant (Rahman et al., 2018; Glick, 2012).

Lysinibacillus is a new genus which is previously classified as *Bacillus*. Formerly, along with a novel species, two previously classified species, *Bacillus sphaericus* and *Bacillus fusiformis* were reclassified to the new genus *Lysinibacillus* based on the unique peptidoglycan composition, physiology and phylogenetic position based on 16S rRNA gene sequencing (Ahmed et al., 2007). In the cell wall peptidoglycan, lysine and aspartic acid is present as the diagnostic amino acids and representing the A4a (Lys−Asp) type cell wall peptidoglycan (Zhu et al., 2015; Zhu et al., 2014). Due to the presence of the Lys−Asp type of peptidoglycan in the cell wall it was named as *Lysinibacillus*. Diphosphatidylglycerol, phosphatidylglycerol, ninhydrin-positive phosphoglycolipid are major polar lipids and menaquinone MK-7 is the dominant respiratory lipoquinone system of the genus. Iso-C15: 0 is the major cellular fatty acid. The G+C content is 35−38 mol%. They are positive for oxidase and catalase tests whereas negative for the indole and H_2S production, nitrate reduction and β-galactosidase (ONPG) tests. Cells are motile, rod shaped and produce spherical or ellipsoidal endospores (Ahmed et al., 2007). Since the above two species were reclassified as *Lysinibacillus*, another 26 species were identified as the members of this genus which are enlisted in LPSN-bacterio-net (http://www.bacterio.net/lysinibacillus.html) and NCBI (http://www.ncbi.nlm.nih.gov/).

The genus *Lysinibacillus* is widely known for its bio insecticidal properties (Berry, 2012). Along with insecticidal activity, bioremediation ability of the genus is also well acquainted (Velásquez and Dussan 2009; Lozano and Dussán, 2013). As the genus is reclassified from *Bacillus*, the members of the genus contain many of the traits similar as *Bacillus*. Therefore, beside the insecticidal and bioremediation ability they also exhibit some plant growth promoting properties which attracted the interest of researchers, recently. Moreover, they can adapt to a wide range of environment (such as, saline condition, acidic condition, metal contaminated condition, high temperature) and produce endospores, which is very important for any plant growth promoting agent. In field condition, very few numbers of plant growth promoting agents could show their efficacy due to lack of the ability of adaptation in a wide range of environment. Therefore, in this study we investigated the efficacy of *Lysinibacillus* strains in the target of finding a potential plant probiotic bacterial agent.

To achieve the goal, in Chapter 1, we evaluated the plant growth promoting ability of the *Lysinibacillus* strains which were previously isolated and identified in our laboratory.

In Chapter 2, we screened a potential *Lysinibacillus* strain which can suppress devastating seedling diseases caused by different soil borne pathogens.

Chapter 1 Evaluation of the plant growth promoting potential of *Lysinibacillus* spp.

Introduction

In agriculture, large amounts of chemical fertilizers are commonly used to increase crop production and meet the global food demand of the increasing population. In parallel, the public concerns related to the impact of the use of chemical fertilizer on the environment have also increased (Gupta *et al*., 2015). It has been well-documented that the long term and indiscriminate use of chemical fertilizers degrades the soil quality and causes several environmental issues (Savci, 2012). Moreover, the production process of fertilizer involves the utilization of tremendous amounts of energy (accounting for 1.2% of the world′s gross energy needs) and the emission of carbon dioxide (CO_2) and other greenhouse gasses (Woods *et al*., 2010; Ghavam *et al*., 2021). Therefore, it is urgent to implement environment-friendly and effective strategies to reduce the dependency on chemical fertilizers. As one of the possible solutions to this issue, the use of plant biostimulants is gaining significant interest by the scientific communities and agricultural industries considering the potential toward enhancing crop productivity in a sustainable manner (Rouphael and Colla, 2020; Hamid *et al*., 2021). Plant biostimulants are substances or microorganisms that are applied to plants to enhance nutrition efficiency, abiotic stress tolerance, and/or crop quality traits regardless of their nutrient content (du Jardin, 2015). Among the active ingredients of plant biostimulants, plant growth-promoting bacteria (PGPB), living in or on soil, rhizosphere, and plant tissues, have drawn considerable attention. PGPB are known to promote plant growth through diverse direct and indirect mechanisms (Glick 2012; Olanrewaju *et al*., 2017). For decades now, bacteria of diverse genera, such as *Azospirillum*, *Bacillus*, *Burkholderia*, *Paenibacillus*, *Pseudomonas*, and *Rhizobia*, have been identified as PGPB as well as extensively studied in an attempt to develop plant biostimulants for a variety of crops (Govindasamy *et al*.,

2010; Suárez-Moreno *et al*., 2012; Panpatte *et al*., 2016; Cassán *et al*., 2020; Lindström and Mousavi, 2020). Among these PGPB group, *Bacillus* species are one of the most common and effective ingredient of plant biostimulants (Nguyen *et al*. 2019), because this genus can produce endospores that are highly resistant to a wide variety of abiotic environmental stresses, such as dryness, UV radiation, and high temperature, and the preparation of the commercial formulation of plant biostimulants is easier and inexpensive. In order to enrich the portfolio of microbe-based plant biostimulants, it is essential to search for such types of practical PGPB.

Lysinibacillus is a newly classified genus that was previously classified as *Bacillus* (Ahmed *et al*. 2007). Since *Lysinibacillus* species have also demonstrated an ability to form endospores, it is expected to serve as a suitable agent for microbe-based products, including plant biostimulants (Ahsan and Shimizu, 2021). In fact, *L. sphaericus*, which is well known as an entomopathogen, has been formulated as mosquito insecticides and is commercially available worldwide (Berry, 2012). Recent studies have demonstrated that the *Lysinibacillus* species are ubiquitous in nature and that several of them are isolated from plant rhizosphere, phyllosphere, and from inside plant tissues; they possess plant growth-promotion (PGP) associated traits, such as auxin production, phosphate solubilization, siderophore production, and nitrogen fixation (Vendan *et al*., 2010; Trivedi *et al*., 2011; Sharma and Saharan, 2015; Verma *et al*., 2016). Moreover, a few studies have reported that some *Lysinibacillus* strains can promote plant growth (Sahu *et al*., 2018; Shabanamol *et al*., 2018).

Our laboratory has a collection of various type of bacteria isolated from the rhizosphere, plant tissues, and soil, including the strains of the genus *Lysinibacillus*. In this study, we evaluated the PGP activities of our *Lysinibacillus* strains to select a potential plant biostimulant.

Materials and methods

Bacterial strains

Lysinibacillus strains used in this study were chosen from our laboratory's bacterial collection based on their partial 16S rRNA gene sequences. They were listed in Table 1. Strains GIC31, GIC41, GIC51, GIC81, and GIC119 were originally isolated from paddy field soil, while strain GUCS34 was isolated from tea garden soil. Strains T16 and T20 were derived from rhizosphere soil of tomato (*Solanum lycopersicum* L.). Remaining two strains, C75 and W30, were discovered from rhizosphere soil of Chinese chive (*Allium tuberosum* Rottler ex Spreng.) and Welsh onion (*Allium fistulosum* L.), respectively.

For more precise taxonomic classification, the full-length 16S rRNA gene sequences were determined according to the methods of Fu et al. (2020). The 16S rRNA gene sequences of the strains were submitted to the GenBank database. The sequences were analyzed by a global alignment algorithm implemented in the EzBioCloud database (https://www.ezbiocloud.net/) (Yoon et al., 2017) and compared with the sequences of known *Lysinibacillus* species in the same database. A phylogenetic tree was constructed with the neighbor-joining method using MEGA version 7.24.2827 (Kumar et al., 2018).

Inoculum preparation

Lysinibacillus strains were cultured on nutrient broth (NB) (Nissui Pharmaceutical Co., Tokyo, Japan) at 30 °C for 2 days with shaking at 200 rpm. Cells were harvested by centrifugation at 9,900×*g* for 10 min and washed twice with 10mM $MgCl_2{\cdot}6H_2O$. Washed cells were

resuspended in 10mM $MgCl_2 \cdot 6H_2O$ and adjusted to an optical density at 600 nm (OD_{600}) of 0.5 (ca. 10^7 CFU/mL).

Determination of indole-3-acetic acid (IAA) production

Ability of the bacterial strains to produce IAA was examined by the method of Kumar et al. (2012) with slight modifications. In brief, a 30-µL aliquot of cell suspension of each strain was inoculated to 10-mL tryptic soy broth (TSB, Becton, Dickinson and Company Sparks, MD, USA) supplemented with 0.5 mg of L-tryptophan and incubated with shaking at 200 rpm for 2 days at 30 °C. After the incubation, culture broth was centrifuged at 5000×*g* for 10 min. Two mL of supernatant were carefully pipetted out and mixed with 2 mL of Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution) (Gordon and weber, 1951). IAA concentration was measured colorimetrically by comparing OD_{530} value of standard pure IAA with culture supernatant of each bacterial strain.

Assessment of phosphate solubilization ability

The cell suspension of each *Lysinibacillus* strain was spotted onto Pikovskaya′s agar medium supplemented with tricalcium phosphate (Pikovskaya, 1948) and incubated at 30°C for 14 days. The phosphate-solubilizing ability of the bacterial strains was indicated by the appearance of a clear zone around the bacterial colonies. In this experiment, *Flavobacterium* sp. Strain GFA4, which was previously identified as a phosphate-solubilizing bacterium, was cultured on the same medium as the positive control and it was confirmed that this strain produced a clear zone around its colony.

Siderophore production assay

The production of siderophore by bacterial strains was determined using the overlaid chrome azurol S agar (O-CAS) assay (Pérez-Miranda et al., 2007) with slight modifications. In brief, cell suspension of each bacterial strain was spotted onto iron-free King′s B medium and incubated at 30 °C. After 1 week of incubation, chrome azurol S (CAS) agarose (Schwyn and Neilands, 1987) was applied on the King′s B plates and incubated for 30 min. Development of yellow/orange halo in the overlaid agarose around the bacterial colonies was considered as positive for siderophore production.

Assessment of ACC deaminase activity

The ACC deaminase activity was determined according to the method performed by Dell′Amico *et al*. (2005). In short, bacterial strains were grown in TSB media. After centrifugation, a 30-µL aliquot of cell suspension (OD_{600}=0.5) of each strain was transferred to DF media containing 3.0 mM ACC as a sole source of N and incubated with shaking at 200 rpm for 2 days at 30 °C. For control, DF media without any nitrogen source was used. Bacterial growth was measured by spectrophotometer at OD_{600} nm.

Assessment of nitrogen fixation potential

Nitrogen-fixation potential of bacterial strains was assessed by the method of Setten et al. (2013) with slight modifications. In brief, bacterial strains were grown for 24 h in L medium (Setten et al., 2013) containing 7.57 mM $(NH_4)_2SO_4$ at 30 °C with shaking at 200 rpm. After the incubation, bacterial cells were harvested by centrifugation at 5000×*g* for 10 min and washed two times with nitrogen-free L medium. Washed cells were resuspended in nitrogen-free L medium and adjusted to OD_{600} of 0.5. Subsequently, a 50-µL aliquot of cell suspension was inoculated into 5 mL of nitrogen-free L medium in a 15-mL tube and incubated at 30 °C with shaking at 200 rpm

under aerobic (test tube covered with parafilm) and micro-aerobic (test tube with plastic screw caps) conditions. After 48h of incubation, bacterial cell concentration was spectrophotometrically measured at OD_{600}.

Primary screening for plant growth-promoting (PGP) strains in spinach

The effect of *Lysinibacillus* inoculation on the growth of spinach was evaluated to identify potential PGP strains. Spinach seeds (*Spinacia oleracea* L. cv. Banchu-summer-sky) were surface sterilized with 70% (v/v) ethanol for 1 min, followed by 2% sodium hypochlorite for 5 min, then thoroughly rinsed with sterilized distilled water (SDW). The seeds were then placed on a moist filter paper in a petri dish and vernalized at 4 °C for 1 day. Thereafter, the seeds were sown in Jiffy-7 pellet (Jiffy product international AS, Norway), covered with small amount of sterilized vermiculite, and grown in a controlled-environmental chamber (Biotron LH-220S, standard; Nippon Medical and Chemical Instruments, Osaka, Japan) at 23 °C under a 12-h light/12-h dark cycle (light intensity 30,000 Lx). Seven days after sowing, spinach seedlings were drenched with 3 mL of a bacterial cell suspension (OD_{600}=0.5). Control plants were treated with an equal volume of sterile 10mM $MgCl_2{\cdot}6H_2O$ without the bacteria. Both the control and bacteria-treated seedlings were maintained in the same controlled-environmental chamber. The plants were regularly irrigated throughout the growing period. After 20 days of drench treatment, spinach plants were harvested from the pots and their leaf area and shoot dry weight were measured. An image analysis software (LIA for Win32, https://www.agr.nagoya-u.ac.jp/~shinkan/LIA32) was used to measure the total leaf area of spinach plant from the scanned images of all the harvested leaves. To measure shoot dry weight spinach shoots (cut from the root and shoot junction) were stored in a ventilated constant temperature dryer (ADVANTEC® DRM620TB, Toyo Seisakusho Kaisha, Ltd., Japan) at

80°C for 2 days. In this experiment, five plants were used for each treatment and the experiment was repeated three times. Differences in the leaf area and shoot dry weight between the control and bacterial treatments were analyzed by the Dunnett′s test (P<0.05).

Secondary screening for PGP strains in spinach

GIC31, GIC41, and GIC51, three best performing strains in the above primary screening, were further evaluated in a pot experiment for their effect on spinach growth. Spinach seeds (cv. Banchu-summer-sky) were surface sterilized and vernalized as described above. Then, the seeds were sown in 150-mL plastic pots containing a double-autoclaved mixture of river sand and vermiculite at a ratio of 1:1 (v/v) and grown in a controlled environmental chamber at 23 °C under a 12-h light/12-h dark cycle. Seven days after sowing, the seedlings were drenched with 10 mL of a cell suspension of each strain (OD_{600}=0.5). Control plants were drenched with same amount of sterile 10mM $MgCl_2{\cdot}6H_2O$. The plants were regularly irrigated and fertilized once a week with 0.2% concentration of Hyponex solution (Type: 6−10−5, Hyponex Japan, Osaka, Japan) until excess solution leached out of a drainage hole in the bottom of pot. The first application was made at the day of drench treatment. After 20 days of drench treatment, the plants were harvested and their leaf area and shoot dry weight were measured as described above. Ten plants were used for each treatment and the experiment was repeated three times. Differences in the leaf area and shoot dry weight among treatments were analyzed by the Tukey′s test (P<0.05).

Evaluation of PGP effect of strain GIC41 under glasshouse conditions

As described later, strain GIC41 was chosen as a potential PGP candidate because it performed best in the secondary screening experiment. In this experiment, PGP effect of this strain

was evaluated under the glasshouse conditions. Surface-sterilized and vernalized spinach seeds (cv. Banchu-summer-sky) were sown in the plastic trays (Bee pot Y-49; Canelon Kaka Co. Ltd., Japan) containing a commercial potting soil mix ″Saika-ichiban″ (IBIKO Corporation, Ogaki, Japan) and grown in a controlled environmental chamber at 23 °C under a 12-h light/12-h dark cycle. After seven days of sowing, the seedlings were treated by drenching with the cell suspension of GIC41 (OD_{600}=0.5) at 10 mL per plant. Control seedlings were treated with sterile 10 mM $MgCl_2{\cdot}6H_2O$ instead of GIC41 cell suspension. These seedlings were then transplanted into the rectangular plastic containers (64 cm × 22 cm) containing commercial potting soil mix at five seedlings per container and grown on a bench in a glasshouse under natural sunlight for six weeks during which the plants were regularly irrigated without fertilization. Five containers were used as replicates for each treatment, and the experiment was repeated three times (designated as trial 1 to 3). Trial 1, 2, and 3 were conducted from March 26 to May 7, April 5 to May 17, and April 16 to May 28 in 2019, respectively.

At the end of the experiment in each trial, spinach plants were harvested and shoot dry weight (after drying for 2 days at 80 °C) was determined. Moreover, carbon and nitrogen concentrations of the shoot were measured by using the dry combustion method with an NC analyzer (SUMIGRAPH NC TR22, Sumika Chemical Analysis Service, Ltd., Osaka, Japan). To conduct this experiment, dried spinach shoots were grinded by using a blender and homogenized samples were prepared. Thereafter, N and C content of the powder was determined using the NC analyzer device. Since the growth of spinach plants in the control treatment markedly varied between trials, shoot dry weight data obtained from three trials were subjected to a random effects meta-analysis using the R package ″meta″ (ver. 2.6-1) (Schwarzer *et al.*, 2015). Differences in the carbon and nitrogen concentrations between the control and GIC41 treatment were analyzed by the Student′s *t*

-test (P<0.05).

Evaluation of PGP effect of GIC41 at different fertilization levels

In this experiment, PGP effect of GIC41 at different fertilization levels was examined. Experimental set up was identical to the second screening, except for growth chamber condition and fertilizer concentration. The experiment was conducted in a controlled-environmental chamber (MLR-350, Sanyo Electric Co. Ltd. Japan) at 23 °C under a 12-h light/12-h dark cycle (light intensity 20,000 Lx) and three different concentrations (0.05, 0.1 and 0.2%) of Hyponex solutions were given to the spinach plants. Twenty days after drenching with GIC41 cell suspension or sterile 10 mM $MgCl_2 \cdot 6H_2O$, spinach plants were uprooted from the pots and their shoot and root dry weight, and leaf area were measured. For each treatment five plants were used, and the experiment was repeated three times. Differences in the leaf area and shoot and root dry wight between the control and bacterial treatment were analyzed by the Tukey′s test (P<0.05).

Effect of dead bacterial cells on the PGP effect of GIC41

To evaluate the effect of dead bacterial cells of GIC41 on the plant growth promotion, this experiment was conducted. The experimental set up was identical to the first screening experiment except the treatments. Three treatments (control, application of dead bacterial cell suspension of GIC41, application of alive bacterial cells of GIC41) were performed. The application concentration and amount of dead and alive bacterial cells of GIC41 was same as the screening experiment. To prepare the dead bacterial cells, the cell suspension of GIC41 was prepared in the same manner of alive bacterial cells. After adjusting the OD, the cell suspension was autoclave at 121°C temperature for 20 min. Finally, after cooling down, dead bacterial cell suspension was applied to the spinach seedlings. Twenty days after drenching with GIC41 cell suspension or

sterile 10 mM $MgCl_2 \cdot 6H_2O$, spinach plants were cut from the root and shoot junction. Afterward, leaf area and shoot dry weight were measured. For each treatment five plants were used, and the experiment was repeated three times. Differences in the leaf area and shoot and root dry wight between the control, dead and alive bacterial treatment were analyzed by the Tukey′s test (P <0.05).

Statistical analysis

All statistical analyses were performed with EZR version 1.41 (Saitama Medical Center, at http://www.jichi.ac.jp/saitama-sct/ SaitamaHP.files/statmedEN.html), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 3.6.1).

Results

Molecular characterization of *Lysinibacillus* strains

Ten *Lysinibacillus* strains used in this study were identified by sequencing analysis of the nearly full-length of the 16S rRNA gene. The results showed that they possessed high similarity to species of *L. xylanilyticus*, *L. pakistanensis*, *L. capsici*, and *L. fusiformis* (Table 1). To clarify the phylogenetic position of these strains, phylogenetic tree was constructed based on the 16S rRNA gene sequences of these strains and recognized type strains of *Lysinibacillus* species (Fig. 1). As a result, strains GIC41, GIC119, and C75 formed a cluster with *L. xylanilyticus* KCTC 13423^T. Strains GIC31, GIC51, and GUCS34 were positioned in a cluster of *L. pakistanensis* NCCP-54^T. Strains T20 and W30 formed a single cluster along with the most closely related strain of *L. fusiformis* ATCC 7055^T. On the other hands, remaining two strains, GIC81 and T16, were placed in a cluster which includes *L. capsici* NRRL B-65515^T, *L. macroides* DSM 54^T, and *L. boronitolerans* DSM 17140^T.

In vitro plant growth promoting traits

Lysinibacillus strains were tested in vitro for their ability to produce IAA, siderophore, and ACC deaminase, as well as solubilize phosphate and fix nitrogen. All of the strains were capable of producing IAA but lacked the other traits (Table 2). Among the strains, IAA production was the highest in C75 with 2.0 µg mL^{-1} of IAA, followed by T20 and GUCS34 which produced 1.9 and 1.8 µg mL^{-1} of IAA, respectively.

Screening for effective plant growth promoting *Lysinibacillus* strains in spinach

Two step screening was conducted to identify the potential *Lysinibacillus* strain which can enhance spinach growth. In primary screening, spinach seedlings were grown in the pellets under controlled environmental condition. As a result of primary screening, majority of the *Lysinibacillus* strains increased spinach growth in compared to the plants with no bacterial inoculations after 20 days of drench-treatment (Fig. 2). Significant enhancement of leaf area was observed in the plants treated with GIC31, GIC41, and GIC51. Along with that, GIC31 and GIC41 significantly increased the dry weight of the spinach plants also.

Based on the results of primary screening, further secondary screening was conducted with the best performing three strains (GIC31, GIC41, GIC51) and their efficiency was evaluated on spinach plants grown in fertilized sand and vermiculite mixture in controlled environmental condition. According to the results, 20 days after drench treatment all the three *Lysinibacilllus* strains (GIC31, GIC41, and GIC51) enhanced spinach growth greater or lesser extent (Fig. 3). However, among the treatments, GIC41 treated plants could significantly enhance the leaf area and dry weight of spinach seedlings. Therefore, GIC41 was finally chosen for the greenhouse experiments.

Efficacy of GIC41 on spinach growth under glasshouse conditions

PGP effect of strain GIC41 was evaluated on spinach plants grown for six weeks in three independent trials that were performed under the glasshouse conditions. As a result, drench-treatment with GIC41 on the transplanting day consistently enhanced spinach growth across all trials, resulting in an approximately 12 to 49% increase in shoot dry weight compared to control treatment (Fig. 4A, B). A meta-analysis of data from three independent trials showed that the mean difference (MD) between GIC41 treatment and control treatment was 0.95 (95% confidence interval: 0.58-1.31) (Fig. 4B), indicating that GIC41 treatment displayed a significant promoting effect on the shoot growth of spinach plants under the glasshouse conditions.

In order to verify the influence of GIC41 treatment on the physiological status of spinach metabolism, the ratio between carbon and nitrogen in shoot tissues of spinach plants were determined. However, no significant difference was found in the carbon and nitrogen ratio between control and GIC41 treated plants (Table 3).

Evaluation of nutrient-dependent efficacy of GIC41

Following experiments were conducted to evaluate the efficacy of GIC41 under varied soil nutrient condition. To conduct this experiment, different concentrations (0.05, 0.1, and 0.2%) of fertilizer were applied to the sand and vermiculite mixtures for making difference in the nutrient condition of the plant growing media. Spinach plants were grown up to 20 days after drench-treatment with GIC41. Results showed that GIC41 treatment increased leaf area, shoot dry weight and root dry weight of the spinach plants in almost all the concentrations of fertilizer (Fig. 5). However, statistically significant difference was found in the leaf area and shoot dry weight of GIC41 treated spinach plants with 0.2% concentration of fertilizer which indicate that efficacy of GIC41 may differ with varying nutrient condition of the soil. Root dry weight was also enhanced

in the plants treated with GIC41 with the 0.2% concentration fertilizer which may be due to the enhancement of lateral roots (Fig. 7).

Evaluation of the dead cell effect of GIC41

To understand the nutrient effect of dead bacterial cells, the experiments were performed. To perform the experiment, spinach seedlings were treated with $MgCl_2$. $6H_2O$, dead and alive cell suspension of GIC41, separately. According to the results, drench treatment with alive cell suspension of GIC41 showed significant enhancement of leaf area and shoot dry weight in compared to the control and dead bacterial cell treatments (Fig. 8, 9). However, there was no significant difference between the leaf area and shoot dry weight of dead bacterial cell suspension and control treatments (Fig. 8).

Table 1. *Lysinibacillus* species identified by BLAST analysis of nearly full-length 16S rRNA sequence.

Strain	Origin	% similarity
GIC31	Paddy field soil	99.3% with *L. xylanilyticus* DSM 23493
GIC41	Paddy field soil	99.5% with *L. xylanilyticus* DSM 23493
GIC51	Paddy field soil	98.27% with *L. xylailyticus* DSM 23493
GIC119	Paddy field soil	99.09% with *L. xylanilyticus* DSM 23493
C75	Chinese chive rhizosphere soil	99.58% with *L. xylailyticus* DSM 23493
GUCS34	Tea garden soil	99.67% with *L. pakistanensis* JCM 18776
T16	Tomato rhizosphere soil	99.25% with *L. capsici* PB300
GIC81	Paddy field soil	100% with *L. capsici* PB300
W30	Welsh onion rhizosphere soil	99.92% with *L. fusiformis* NBRC 15717
T20	Tomato rhizosphere soil	99.67% with *L. fusiformis* NBRC 15717

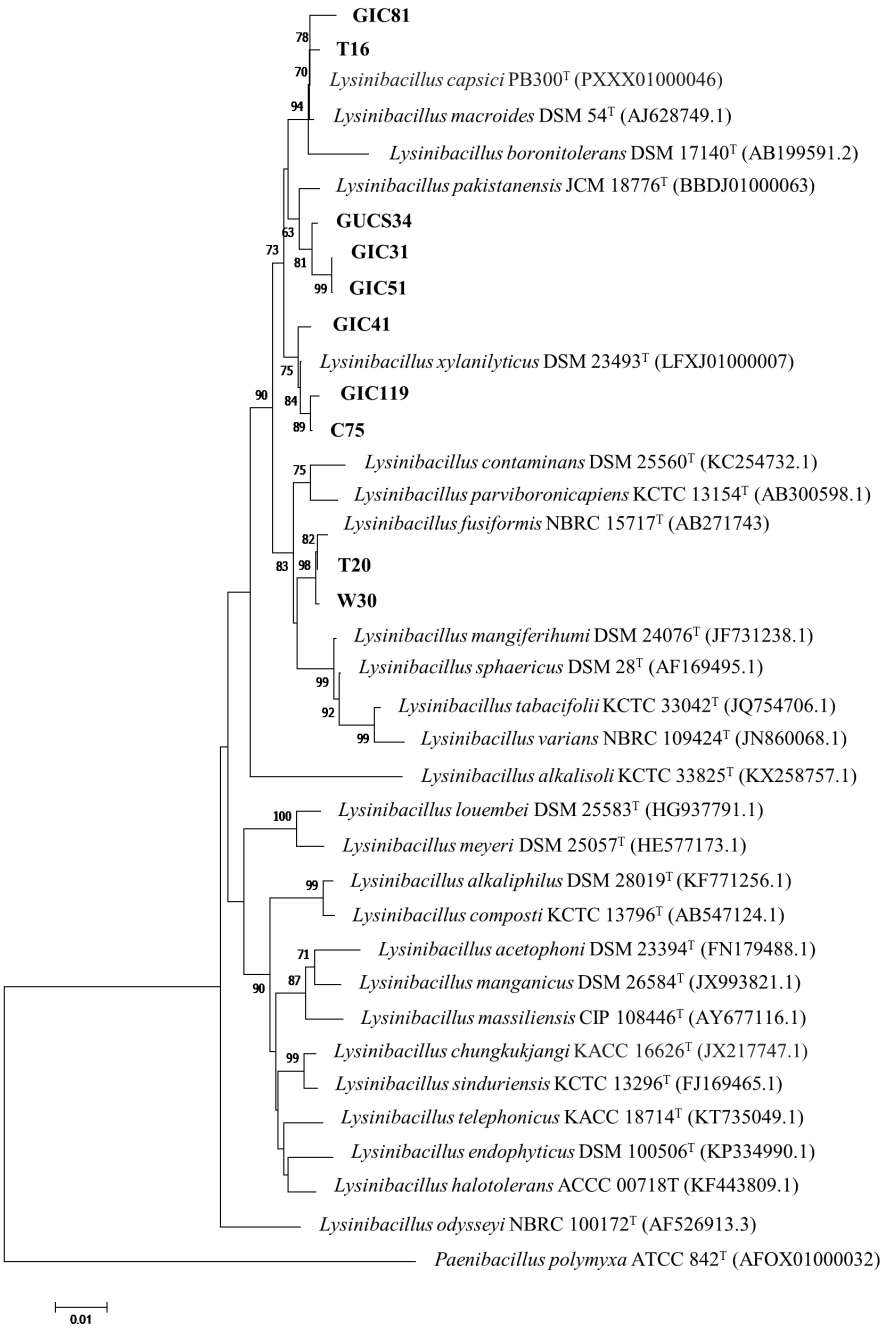

Fig. 1. Phylogenetic position of *Lysinibacillus* strains based on complete 16S rRNA gene sequence analysis. Bootstrap values of 1000 replicates are shown next to the branches based on a neighbor-joining analysis.

Strain name	IAA production (µg/mL)	Phosphate solubilization	Siderophore production	ACC deaminase production	Nitrogen fixation
GIC31	1.2	-	-	-	-
GIC41	1.5	-	-	-	-
GIC51	1	-	-	-	-
GIC119	1	-	-	-	-
C75	2	-	-	-	-
GUCS34	1.8	-	-	-	-
T16	1.4	-	-	-	-
GIC81	1.2	-	-	-	-
W30	1.1	-	-	-	-
T20	1.9	-	-	-	-

Table 2. In vitro plant growth promoting traits of the *Lysinibacillus* strains.

(-) indicate the lack of production ability of the strains.

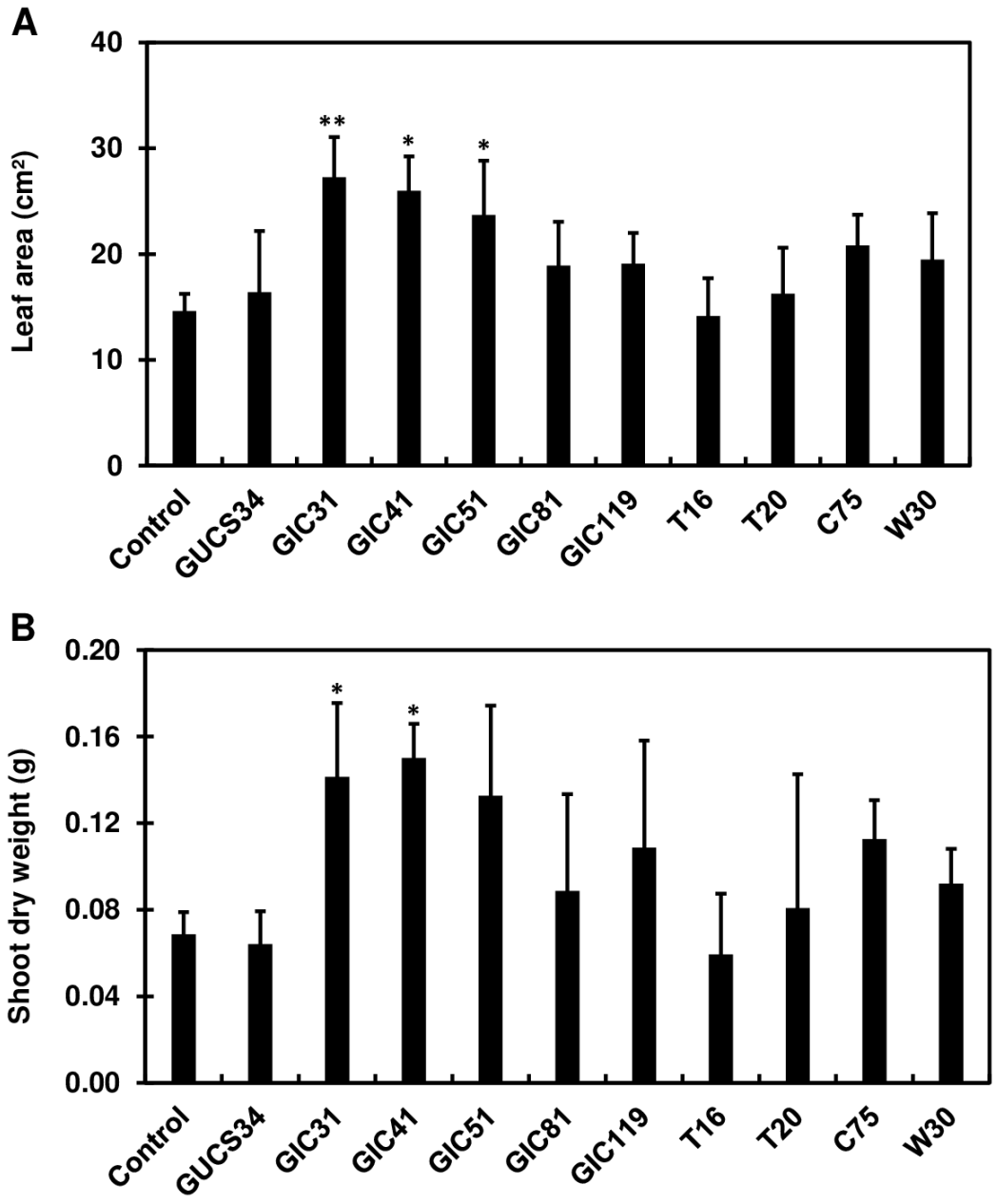

Fig. 2. Influence of *Lysinibacillus* inoculation on leaf area (A) and shoot dry weight (B) of spinach plants grown on Jiffy-7 pellets. Spinach plants were sampled at 20 days after treatment. Bars represent mean ± standard deviation of three independent experiments. Statistically significant differences between the control and the treatment groups are indicated by asterisks

(One-Way ANOVA, Dunnett′s test, *P<0.05, **P<0.01).

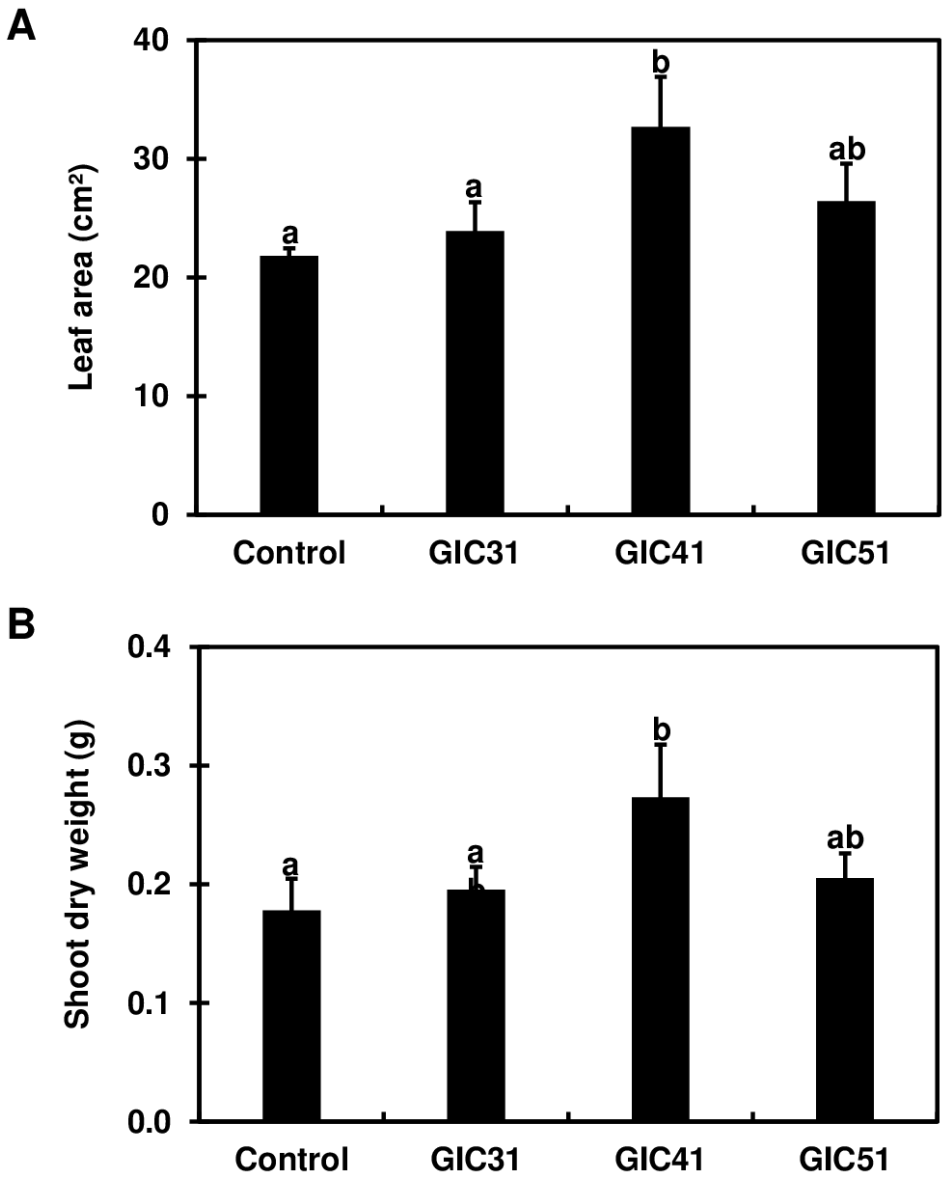

Fig. 3. Influence of *Lysinibacillus* inoculation on leaf area (A) and shoot dry weight (B) of spinach plants grown in sand and vermiculite mixture (1:1, v/v). Spinach plants were sampled at 20 days after treatment. Bars represent mean ± standard deviation of three independent

experiments. Statistically significant differences between the treatments are indicated by different letters (One-Way ANOVA, Tukey′s multiple comparison of means, $P < 0.05$).

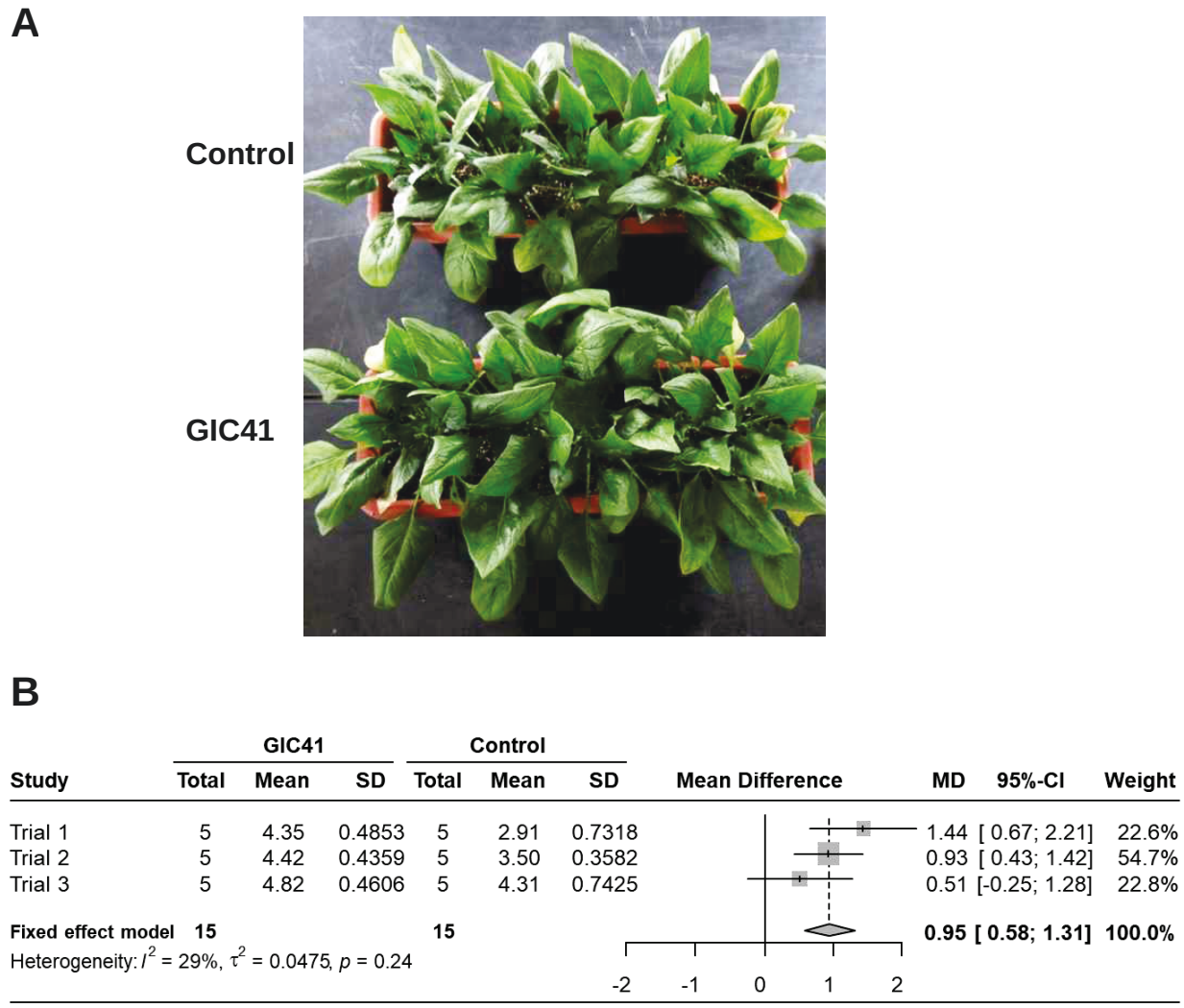

Fig. 4. Growth promoting effect of strain GIC41 on spinach plants grown under glasshouse conditions. A) Representative examples of control and GIC41-treated spinach plants grown for six weeks. B) Forest plot of a meta-analysis comparing GIC41 treatment and control treatment for the shoot dry weight (g) in the glasshouse experiment. Shoot dry weight data obtained from three independent trials were analyzed by a meta-analysis. Mean, SD, and MD represent mean shoot dry weight (g), standard deviation, and mead difference, respectively. The

gray boxes indicate the mean difference for each trial and horizontal bars indicate corresponding 95% confidence interval (95%-CI). Diamond indicates the pooled mean difference across trials. Fixed model was used.

Table 3. Effect of GIC41 treatment on C/N ratio in the shoots of spinach plants

Treatment	Carbon content (%)	Nitrogen content (%)	C/N ratio
Control	34.5 ± 1.6 a	4.3 ± 1.3 a	8.6 ± 2.8 a
GIC41	35.1 ± 1.6 a	3.8 ± 1.1 a	9.7 ± 2.8 a

Data represent the mean ± standard deviation of three replicates. Mean was analyzed for significant differences using Student′s t-test. Values in columns followed by same letters are not significantly different at $P<0.05$.

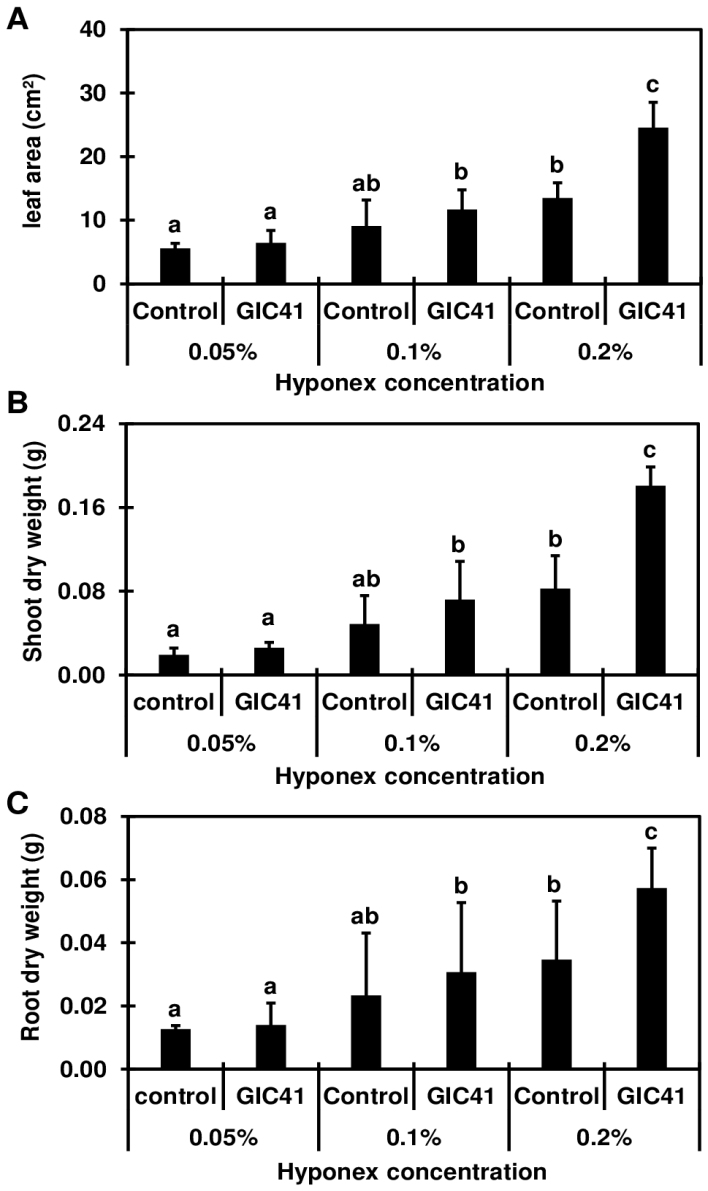
A
leaf area (cm²)
40
30
20
10
0
a
a
ab
b
b
c
Control
GIC41
Control
GIC41
Control
GIC41
0.05%
0.1%
0.2%
Hyponex concentration
B
Shoot dry weight (g)
0.24
0.16
0.08
0.00
a
a
ab
b
b
c
control
GIC41
Control
GIC41
Control
GIC41
0.05%
0.1%
0.2%
Hyponex concentration
C
Root dry weight (g)
0.08
0.06
0.04
0.02
0.00
a
a
ab
b
b
c
control
GIC41
Control
GIC41
Control
GIC41
0.05%
0.1%
0.2%
Hyponex concentration

Fig. 5. Effect of drench-treatment with GIC41 with three different concentrations (0.05, 0.1, and 0.2%) of fertilizer on the (A) Leaf area, (B) Shoot dry weight and (C) Root dry weight of spinach plants grown in sand and vermiculite mixture (1:1, v/v). Spinach plants were sampled at 20 days after treatment. Bars represent mean ± standard deviation of three independent experiments. Statistically significant differences between the treatments are indicated by different letters (One-Way ANOVA, Tukey′s multiple comparison of means, $P < 0.05$).

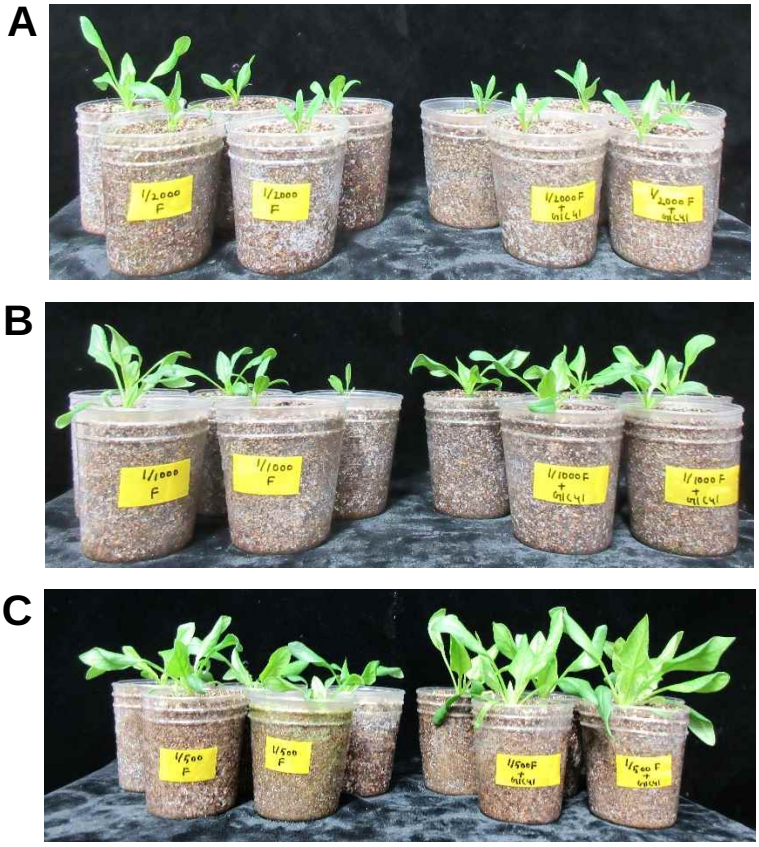

Fig. 6. Effect of GIC41 drench- treatment on the growth of spinach grown in different concentrations (0.05, 0.1, and 0.2%) of fertilizer. Left side pots are treated with different concentration of fertilizer and right-side pots are treated with different concentration of fertilizer along with GIC41 drench-treatment, (A) 0.05; (B) 0.1; (C) 0.2% concentration of fertilizer.

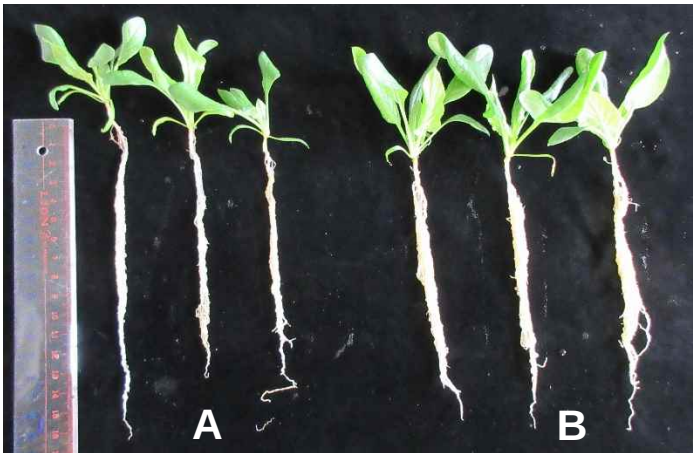

Fig. 7. Enhancement of lateral root growth in GIC41 drench- treated plants at the fertilizer concentration of 0.2%. Left side plants are treated with 0.2% concentration of fertilizer (A) and right-side pots are treated with 0.2% concentration of fertilizer along with GIC41 drench-treatment (B).

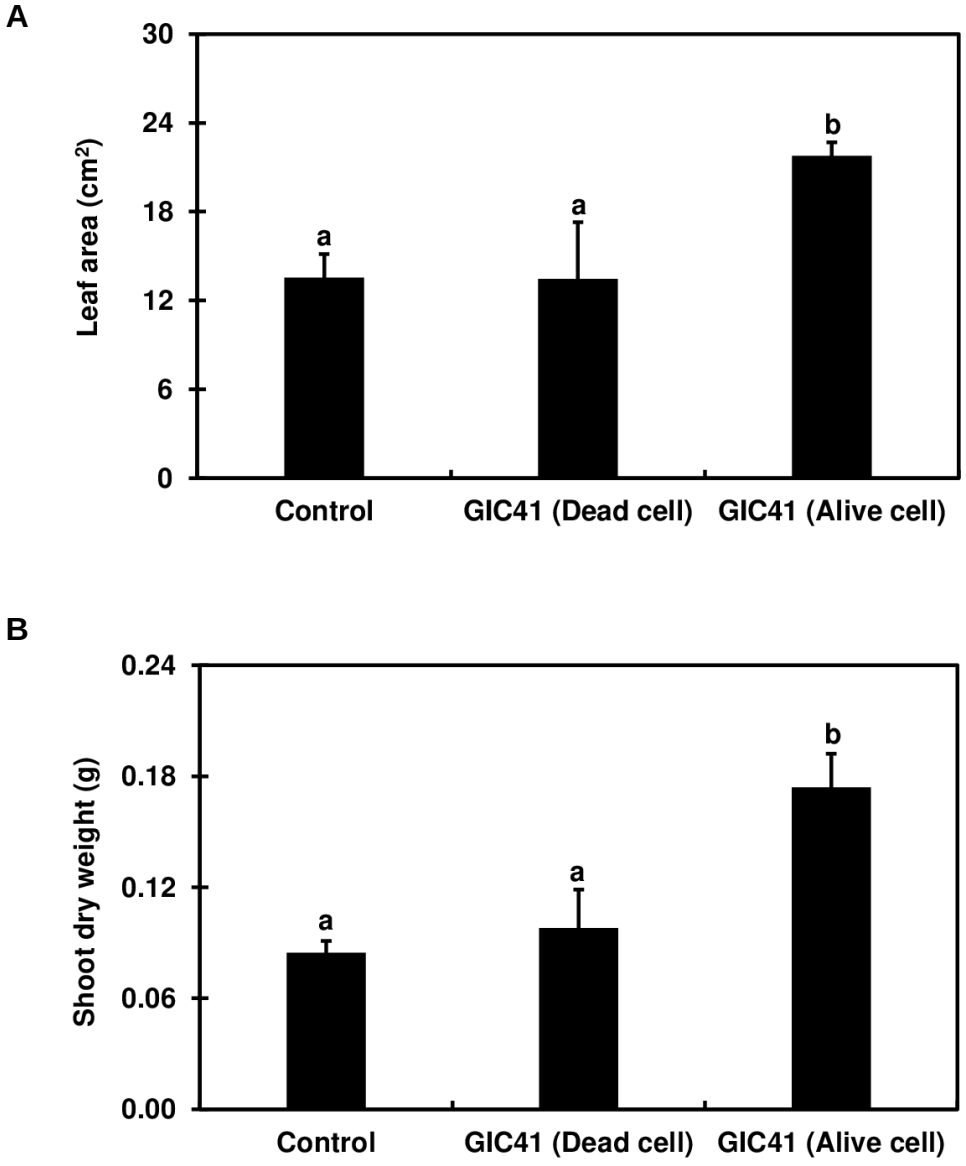

Fig. 8. **Effect of the dead and alive bacterial cell on the (A) leaf area and (B) shoot dry weight of spinach plants grown in the peat pellets.** Spinach plants were sampled at 20 days after treatment. Bars represent mean ± standard deviation of three independent experiments. Statistically significant differences between the treatments are indicated by different letters (One-

Way ANOVA, Tukey′s multiple comparison of means, $P < 0.05$).

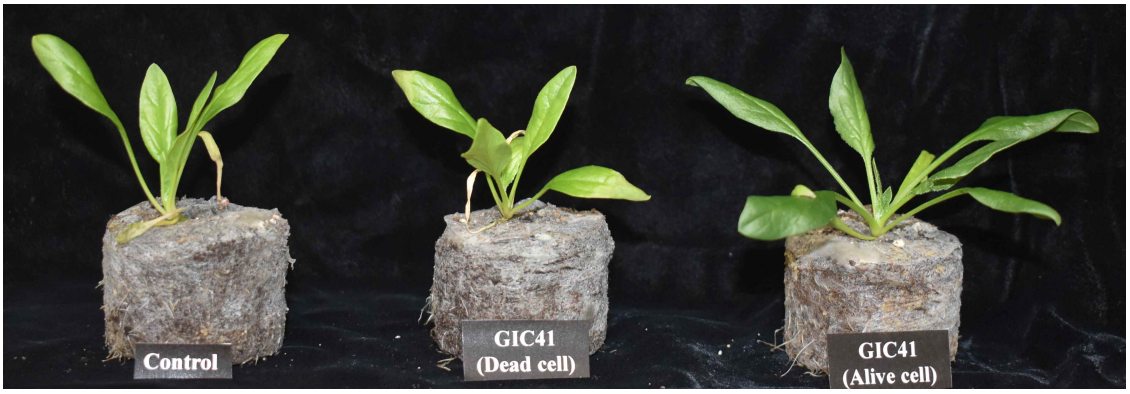

Fig. 9. Representation of the PGP activity of GIC41 with dead and alive cell suspension. The left-side plant is uninoculated control, the middle one is treated with the suspension of dead cells of GIC41 and the right-side plant is treated with the suspension of alive cells of GIC41.

Discussion

The present study aimed to identify a *Lysinibacillus* strain that offers the potential for use as a plant biostimulant. For this purpose, we screened 10 *Lysinibacillus* strains, previously isolated from soil and plant rhizosphere, for their growth-promoting effect on spinach plants. Accordingly, the strain GIC41, isolated from the paddy field soil, was selected as the best candidate strain because of its consistent performance across screening experiments using spinach seedlings grown in a controlled environmental chamber (Fig. 2). Moreover, drench treatment with strain GIC41 on the day of transplanting was found to boost spinach growth and significantly increase shoot biomass 6 weeks after transplantation under glasshouse conditions (Fig. 4), indicating the potential of this strain as a plant biostimulant.

Several past reports have demonstrated plant growth promotion by inoculation with *Lysinibacillus* species, such as *L. sphaericus*, *L. fusiformis*, and *L. chungkukjangi* (Sharma and Shaharan, 2015; Yu *et al*., 2016; Sahu *et al*., 2018; Borah *et al*., 2019; Ahsan and Shimizu, 2021). According to the phylogenetic analysis of the 16S rRNA gene sequences, the strain GIC41 was identified as *L. xylanilyticus* (Fig. 1). The species *L. xylanilyticus* was first discovered from forest humus as a xylan-degrading bacterium and validly reported by Lee *et al*. (2010). Since then, several *L. xylanilyticus* strains have been isolated from soil, rhizosphere, and plant tissues (Esmaeili *et al*., 2013; Verma *et al*., 2014; Tan *et al*., 2015). Moreover, *L. xylanilyticus* strains have been reported to exhibit certain PGP-associated traits, such as the IAA production and phosphate

solubilization (Verma *et al*., 2016; De Mandal *et al*., 2018). However, to the best of our knowledge, no study has yet confirmed its actual capacity to enhance plant growth. Therefore, this study may be the first one to describe the PGP capability of *L. xylanilyticus*.

Several previous studies have reported that nutrients released from the dead cells of introduced PGPB were taken up by plants and increased their biomass (Macedo-Raygoza et al., 2019; Seerat et al., 2019). However, inoculation with dead GIC41 cells had no influence on spinach growth (Fig. 8), demonstrating that PGP effect of this strain is due to particular activities of its living cells.

Many PGPB are known to improve plant growth by increasing the bioavailability of soil nutrients through solubilization of key mineral nutrients such as phosphorus and iron (Kenneth et al., 2019; Rawat et al., 2018). However, the strain GIC41 lacked the ability to solubilize phosphate as well as to produce siderophore, an iron solubilizing agent (Table 2). Furthermore, simple *in vitro* assay demonstrated that this strain did not have nitrogen-fixing ability, which is another important trait of many PGPB that stimulate promote plant growth (Table 2). It has been reported that the inoculation of nitrogen fixing PGPB increased the plant biomass while also enhancing the nitrogen content in the plants (Islam et al., 2013; Rokhzadi and Toashih, 2011). GIC41 inoculation, on the other hand, had no influemce on the nitrogen content of spinach plants (Table 3), suggesting that PGP-effect of this strain is not attributable to nitrogen fixtation.

When compared to the control treatment, the GIC41 treatment boosted lateral root development (Fig. 6), which resulted in an increase in the root biomass (Fig. 5C). The lateral roots contribute to nutrient and water absorption from the soil by increasing the overall surface area of the root system (Vessey, 2003). Therefore, enhanced shoot growth in GIC41-treated spinach

plants may be attributed to the improved uptake of nutrients through the lateral roots. Several PGPR have also been shown to increase the number and/or length of lateral roots, consequently enhancing the growth of the whole plant (Cao *et al*., 2020; Grover *et al*., 2021).

It is already well known that the lateral root formation is regulated by the phytohormone auxin and that the exogenous auxin promotes the production of lateral roots (Fukaki and Tasaka, 2009; Moriwaki *et al*., 2011; Waidmann *et al*., 2020). *In vitro* assays have revealed that the strain GIC41 possessed IAA-producing ability (Table 2), implying that IAA secreted by this strain plays a key role in the promotion of lateral root growth. The enhancement of the shoot and root growth by GIC41 treatment was more pronounced under the moderately fertilized condition (fertilized with 0.2% Hyponex solution, the supplier-recommended concentration) than under the less-fertilized conditions (fertilized with 0.1% and 0.05% Hyponex solution) (Fig. 5). A similar result was also reported by Lamont *et al*. (2014), who found that *Viminaria juncea* inoculated with an IAA-producing *Bacillus megaterium* strain generated a larger root system under moderate nitrogen conditions than under less nitrogen conditions. It has also been reported that IAA production by bacteria depends on the availability of nitrogen (Thuler *et al*., 2003;Tamaki and Mercier, 2007; Shokri and Emtiazi, 2010). Therefore, it is assumed that the soil nutrient status, particularly the nitrogen level, is a key factor for the strain GIC41 to exert its PGP effect.

Remarkably, although other *Lysinibacillus* strains used in this study were also capable of producing IAA, their PGP effect was found to be inferior to that of the strain GIC41, which may be attributed to several reasons. For instance, bacteria exhibit different responses to environmental conditions, and these variations affect the production of IAA (Sasirekha *et al*., 2012; Bharucha *et al*., 2013; Chandra *et al*., 2018). For example, Mohite (2013) reported that the quantity of IAA

produced by rhizospheric *Bacillus* and *Lactobacillus* strains varies significantly depending on the temperature, pH, and the types of carbon and nitrogen sources in the culture medium. Therefore, it is assumed that the environmental conditions, particularly the rhizospheric conditions of spinach plants, may be more favorable for the strain GIC41 to synthesize IAA than for the other strains. The second possible reason is the involvement of plant growth-stimulating substances other than IAA. All the *Lysinibacillus* strains used in this study demonstrated only IAA-producing ability out of the five major PGP-associated traits (i.e., IAA production, siderophore production, ACC deaminase production, phosphate solubilization, and nitrogen fixation) (Table 2). However, previous studies have reported that the production of plant hormones other than IAA, viz. cytokinins and gibberellins, also participate in the PGP effect of PGPB (Ortíz-Castro *et al*. 2008; Kang *et al*., 2014). Moreover, recent investigations have revealed that volatile organic compounds produced by PGPR can activate the phytohormone signaling pathways and enhance plant growth (Jishma *et al*., 2017 Tahir *et al*., 2017). Therefore, it is possible that, in addition to IAA, the strain GIC41 produces cytokinins, gibberellins, and/or VOCs, and that the coordinated action of these compounds may be responsible for the superior PGP effect of the strain GIC41. Another possible explanation for this result is the difference in the colonization capacity of the strains. Successful root colonization is an essential step for PGPR to exert their beneficial effects on plants (Ansari and Ahmad, 2018; Gamez *et al*., 2019). Although the root-colonization capacity of *Lysinibacillus* strains was not investigated in the present study, the strain GIC41 may have colonized the spinach roots more efficiently than the other strains and continuously secreted IAA in the rhizosphere; therefore, this strain exhibited a greater and more stable PGP effect than the other strains.

Further research is thus needed to uncover the detailed mechanisms underlying the PGP activity of the strain GIC41. In addition, we plan to examine the PGP effect of this strain on

various crops in the follow-up future studies.

In conclusion, the results of the present study demonstrated that the *L. xylanilyticus* strain GIC41 can effectively promote the growth of spinach plant, and thus, may contribute to the development of a new plant biostimulant.

Chapter 2 Evaluation of the potential *Lysinibacillus* strain with disease suppression ability against seedling damping off disease caused by soil borne pathogens

Introduction

Global food security is in an alarming state. The losses due to plant disease are worsening the situation. The estimated potential yield losses caused by plant pathogens is up to 16% globally (Ficke et al., 2018). Protection against losses caused by crop pests, plant diseases can play a critical role in improving food security worldwide. There are several plant protection strategies such as crop rotation, cultivar selection, use of pesticides, removal of diseased plants. Though these strategies are effective in controlling plant disease to some extent, however, they have some limitations also. For example, some pathogens have wide host range therefore crop rotation cannot be effective in controlling disease caused by such kind of pathogens. On the other hand, cultivar selection is expensive and time-consuming process. Among the strategies use of chemical pesticide is the most effective way to control plant disease. However, long term and indiscriminate use of the pesticides may cause harm to the environment, and people are preferring the foods grown in chemical freeways. Moreover, long term use of pesticides may generate pesticide resistant insects and pests. Therefore, plant probiotic bacteria, a newly emerging field is acquiring the attention of researchers and gaining popularity in recent years due to its safe and eco-friendly nature.

Bacillus, *Paraburkholderia*, *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, and *Serratia* are the major genera of plant probiotic bacteria (Menendez and Garcia-Fraile, 2017; Carro and Nouioui, 2017; Rahman et al., 2018). Among them, some probiotic bacteria show inconsistent results in the field condition which is mainly due to the low survival rate, bacterial rhizosphere competence and lack of adaptation ability in wide range of environmental condition (Dutta and Podile, 2010; Bach et al., 2016). Therefore, gram positive *Bacillus* strains have gained popularity among the growers for their comparatively consistent effectiveness in the field condition. Considering that, scientists are continuously searching for other endospore forming plant probiotic bacteria to increase the number of promising plant probiotic agents.

Lysinibacillus is a gram-positive bacterium with endospore forming ability which make the bacteria more suitable as a plant probiotic agent. In recent years, two species of *Lysinibacillus* (i.e. L. fusiformis and L. sphaericus) have been reported for their disease suppression ability (Singh et al., 2013; Naureen et al., 2017; Shabanamol et al., 2017; Passera et al., 2020). Therefore, along with the evaluation of plant growth promotion ability of the *Lysinibacillus* strains (Chapter 1), we also become interested to investigate their disease suppression ability. Soil borne pathogens cause significant yield loss to numerous agricultural crops throughout the world (Raaijmakers et al., 2009, Dixon and Tilston, 2010). Therefore, in this chapter, we evaluated the disease suppression ability of the previously identified (Chapter1) *Lysinibacillus* strains against two soil borne pathogens, *Sclerotinia sclerotiorum* and *Pythium aphanidermatum* which cause disease to a wide range of crops.

Materials and methods

Bacterial strains, culture condition, and inoculum preparation

Lysinibacillus strains (GIC31, GIC41, GIC51, GIC81, GIC119, C75, W30, T16, T20, and GUCS34), previously identified and investigated for plant growth promotion ability (Chapter 1) were evaluated for disease suppression ability against *Sclerotinia sclerotiorum* and *Pythium aphanidermatum*, in this study. Bacterial strains were cultured on nutrient broth (NB) (Nissui Pharmaceutical Co., Tokyo, Japan) at 30 °C for 2 days with shaking at 200 rpm. Cells were harvested by centrifugation at 9,900×*g* for 10 min and washed twice with 10mM $MgCl_2{\cdot}6H_2O$. Washed cells were resuspended in 10mM $MgCl_2{\cdot}6H_2O$ and adjusted to an optical density at 600 nm (OD_{600}) of 0.5 (ca. 10^7 CFU/mL).

Pathogenic strains, culture condition and inoculum preparation

Sclerotinia sclerotiorum (Lib.) de Bary used in this study was kindly provided by Dr. Hirofumi Suzuki (Mie Prefectural Agricultural Research Institute). The fungal strain was grown on Potato dextrose agar (PDA) plate at 25°C for 3 days. Growing hyphal disc was used for the

experiments.

Pythium aphanidermatum used in this study was kindly provided by Prof. Koji Kageyama (Gifu University). The strain was grown on handmade CMA (Corn Meal Agar) medium. Mycelial disc was placed on the CMA media and incubated at 25°C for 24h. Growing mycelial disc were obtained from the edge of the petridish. For induction of zoospores 10 mycelial discs were placed in the glass petridish (9 cm) containing 30ml of PWD (autoclaved pond water diluted 1:2 with sterile distilled water) and kept at 25°C. 24h later, zoospores were collected and adjusted at the concentration of 10^4 spores/mL.

Screening of *Lysinibacillus* strains for suppressive effect on damping-off disease of cabbage seedlings caused by *S. sclerotiorum*

Ten *Lysinibacillus* strains were investigated for the disease suppression ability against damping off disease of cabbage seedlings caused by *S. sclerotiorum*. To conduct this experiment, growing hyphal disc (3 mm diameter) was placed inside the small bottles (height 9cm x width 5 cm) containing 3 ml PDA media and incubated at 23°C for 3 days. Bottles contained only PDA media (without fungal hyphae of *S. sclerotiorum*) was used as uninoculated control. Three days later, the bottles were filled with 3 g of sterilized commercial soil (Saika ichiban, Ibigawa kougyou, Ogaki city, Gifu prefecture, Japan). Cabbage seeds (Cv. Okina) were surface sterilized with 70% (v/v) ethanol for 1 min, followed by 2% sodium hypochlorite for 5 min, then thoroughly rinsed with sterilized distilled water (SDW). After surface sterilization, five seeds were placed on the soil in the bottle. Then the seeds were covered with small amount of sterilized soil. 3ml cell suspensions (OD_{600}=0.5) of each bacterial strains were applied to the soil in the bottle. 10mM $MgCl_2$ was applied for control treatment. The bottles were covered with cap and grown in a

controlled-environmental chamber (Biotron, standard; Nippon Medical and Chemical Instruments, Osaka, Japan) at 23 °C under a 12-h light/12-h dark cycle. Disease severity was measured daily from the 4th day to 10th day of bacterial inoculation. Five plants were used for each treatment. The experiment was repeated three times and five replicates were used in each time. Differences in the disease severity between the treatmentments were analyzed by the Tukey′s test (P<0.05).

Development of disease symptoms

The damping off symptoms of cabbage seedlings were monitored daily based on a disease scale ranging from 0 to 4, as described by Shrestha et al. (2015) with slight modification, where 0 = No disease symptom (healthy), 1= Reduced growth than the healthy seedling, 2= Shrinked seedling, 3= Wilted hypocotyl showing reddish, 4= Completely damped of or ungerminated (Fig. 1). Disease severity was measured in daily basis up to seven days after seeding. Disease severity, the area under disease severity progress curve (AUDPC), and reduction of AUDPC (%) were calculated using the following formulas:

Disease severity = [(The number of diseased plants in each scale x disease scale)/ (Total number of plants investigated x the highest disease scale)] x 100.

AUDPC was calculated based on disease severity using the trapezoid integration of disease progress curve over time according to the following formula:

AUDPC= Σ [0.5 (X_i +$X_{i\text{-}1}$)] (t_i-$t_{i\text{-}1}$), where X_i and $X_{i\text{-}1}$ are disease severity at time t_i and $t_{i\text{-}1}$, respectively, and t_i and $t_{i\text{-}1}$ are consecutive evaluation dates with t_i and $t_{i\text{-}1}$ equal to 1.

Evaluation of GIC41 for disease suppressive effect on damping off of spinach seedlings caused by *Pythium aphanidermatum* in controlled environmental condition

Based on the results of the screening experiment of *Lysinibacillus* strains for the suppressive effect against *S. sclerotiorum*, the strain GIC41 was chosen for the further experiments. Afterward, GIC41 was tested for the suppression of spinach seedling dumping off disease caused by *P. aphanidermatum* to understand its efficacy towards different devastating diseases. In order to conduct this experiment, spinach seeds (*Spinacia oleracea* L. cv. Banchu-summer-sky) were surface sterilized with 70% (v/v) ethanol for 1 min, followed by 2% sodium hypochlorite for 5 min, then thoroughly rinsed with sterilized distilled water (SDW). The seeds were then placed on a moist filter paper in a petri dish and vernalized at 4 °C for 1 day. Followed by vernalization, seeds were sown in the plastic pots (8 cm x 7.5 cm) containing 150g of double sterilized commercial soil (Saika ichiban, Ibigawa kougyou, Ogaki city, Gifu prefecture, Japan) and grown in a controlled-environmental chamber (Biotron, standard; Nippon Medical and Chemical Instruments, Osaka, Japan) at 23 °C under a 12-h light/12-h dark cycle. Seven days after sowing, each pot was drenched with 5 mL of spore solution of *P. aphanidermatum* adjusted at the concentration of 10^4 spores/mL. For GIC41 treatment, along with pathogen inoculation 5 mL cell suspension of GIC41 (OD= 0.5) was applied to each pot. Control plants were treated with an equal volume of sterile 10mM $MgCl_2{\cdot}6H_2O$ without the pathogen and bacterial inoculation. Both the control and bacteria-treated seedlings were maintained in the same controlled-environmental chamber. The plants were regularly irrigated throughout the growing period. Three seedlings were maintained in each pot. Three pots were used as replicates for each treatment, and the experiment was repeated three times. Disease severity was monitored daily based up to 15 days after pathogen inoculation. Differences in the disease sverity between the control and GIC41 treated plants were analyzed by the Student′s *t*-test ($P<0.05$).

Development of disease symptoms

The damping off symptoms of spinach seedlings were monitored daily based on a disease scale ranging from 0 to 4, as described by Lookabaugh et al. (2015) with slight modification, where on a disease scale ranging from 0 to 4, where 0 = No disease symptom (healthy), 1= slightly stunted seedling, 2= moderate stunting or chlorosis, 3= severe stunting or wilting, 4= completely wilted or died (Fig. 2). The disease severity, and AUDPC was calculated by applying the same formula which were used for *S. sclerotiorum* affected plants.

Investigation of Antifungal ability of the GIC41 against *P. aphanidermatum*

In order to understand the mechanism related with the disease suppression ability of GIC41 the antifungal ability of the strain was evaluated against *P.* a*phanidermatum* according to the method of Sharma et al. (2016) with slight modifications. Briefly, *P.* a*phanidermatum* was cultured on CMA at 25 °C for 2 days. Thereafter, 8 mm diameter disc from actively growing colony of pathogen was cut with a sterile cork borer, and placed near the periphery (1 cm from the edge) of the plate (diameter of petridish 9 cm) containing three different medium (CMA, PDA, mixture of PDA and NA (1:1)). Similarly, *Lysinibacillus* strain GIC41 was grown on the nutrient agar (NA) media for two days. Afterward, a loop of bacterial cell was transferred on the other side (at the same distance from the edge) of the petridish at an 180° angle. Plates with no bacterial strains served as control for the pathogen. The plates were sealed with plastic wrap and incubated at 25 °C for five days. Mycelial growth inhibition percentage was calculated by using following formula: Inhibition Percentage (%) = $(A_1 - A_2 / A_1) \times 100$, where A1 is colony diameter in the control, A_2 is colony diameter in the dual culture.

Colonization by *Pythium* in GIC41 treated seedlings

Experimental set up was identical to the experiment conducted to evaluate the disease suppression ability of GIC41 against *Pythium aphanidermatum*. To estimate roots colonization percentage by *Pythium*, the roots of the plants were washed under tap water. Afterward, lateral roots were removed from the primary roots and finally the primary root was cut into several segments with 1cm length. Five segments (two pieces from proximal and distal portions and one piece from the middle portion of the primary root) were plated in a selective medium containing nystatin (10 mg L^{-1}) and miconazole (1 mgL^{-1}) in cornmeal agar (CMA) (Senda et al. 2009). *Pythium* colonies was estimated after incubation at 23°C for 24 h. *Pythium* colonization was estimated 3-7 days after pathogen inoculation. For each treatment five plants were used, and the experiment was repeated three times. Differences in the pathogen and bacterial treatment were analyzed by the Student′s *t*- test ($P<0.05$).

Statistical analysis

The data of disease suppression ability of *Lysinibacillus* strains against *S. sclerotinia* was compared by using Dunnett′s test ($P<0.05$). The data of disease suppression ability of GIC41 was compared by Student′s *t*-test ($P<0.05$). All statistical analyses were performed with EZR version 1.41 (Saitama Medical Center, at http://www.jichi.ac.jp/saitama-sct/ SaitamaHP.files/statmedEN.html), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 3.6.1).

Results

Bacterial strains

The *Lysinibacillus* strains (GIC31, GIC41, GIC51, GIC81, GIC119, C75, W30, T16, T20, and GUCS34) investigated for the plant growth promotion ability in chapter 1 were evaluated for their disease suppressive ability in this chapter.

Investigation of disease suppressive ability of *Lysinibacillus* strains against damping off disease caused by *S. sclerotiorum*

In the search of effective plant probiotic agent, we evaluated the disease suppression ability of ten *Lysinibacillus* strains against *S. sclerotiorum*. The suppressive effect of *Lysinibacillus* strains

against *S. sclerotiorum* was examined using the cabbage seedling bioassay. The disease severity was measured daily from the 4th to 10th day after seeding. All the *Lysinibacillus* strains showed disease suppressive ability in compared to the control treatment to a greater or lesser extent (Fig. 3). Among the strains GIC41 exhibited stronger suppressive effect against *S. sclerotiorum* initiated damping off by reduction of 49% disease severity in the cabbage seedlings. However, statistically no significant difference was found between the control and GIC41 treatment. Along with reduction of disease severity, the strain GIC41 reduced the AUDPC value 48% (Fig. 5). Therefore, the strain GIC41 was chosen for the further experiment.

Evaluation of the performance of GIC41 to suppress damping off disease of spinach seedlings caused by *P. aphanidermatum*

In order to evaluate the efficacy of GIC41 to suppress damping off disease caused by other pathogen, we examined the performance of GIC41 against *P. aphanidermatum*. Using spinach seedlings grown in the plastic pots (8 cm x 7.5 cm), the effect of GIC41 drenching on the damping off caused by P. *aphanidermatum* was assessed. The disease symptoms of spinach plants were measured 7 to 15 days after pathogen inoculation. In the control treatment, disease severity gradually increased from 34% to 73% by 15 days after pathogen inoculation (Fig. 6). In contrast, GIC41 treatment consistently suppressed the disease compared with the control and reduced the disease severity around 51% up to 15 days after pathogen inoculation which is significantly different from the control treatment. The area under disease progress curve (AUDPC) also reduced about 74% in the GIC41 treated plants (Table 1).

Antifungal ability of GIC41

To investigate the mechanisms related with the disease suppression ability of the strain GIC41, the in-vitro antifungal ability was examined in different media condition (CMA, PDA and the mixture of PDA and NA). However, during the in vitro test the stain GIC41 could not suppress the mycelial growth of *P. aphanidermatum* on CMA and PDA media (Fig. 8). However, the strain showed a week antifungal ability (a noticeable reduction of mycelial density around the colony of GIC41) on the PDA and NA mixture medium.

Evaluation of colonization by *Pythium* in GIC41 treated seedlings

Colonization ability of *Pythium* was investigated to understand the disease suppression mechanism of the strain GIC41. Spinach root segments of *Pythium* and GIC41 inoculated plants were investigated to evaluate the difference in the colonization ability of *Pythium* due to the application of GIC41. The results showed that plants treated with GIC41 significantly reduced the *Pythium* colonization of the spinach root segments up to 4^{th} day of the application of GIC41 (Fig. 9). After that, though the colonization rate of *Pythium* was lower, but there was no significant difference between the plants inoculated with pathogen and treated with GIC41 (Fig. 9).

Fig. 1. The disease severity scale implemented for disease rating on cabbage seedlings. Disease severity scores of the seedlings were based on the degree of stunting and wilting. 0 = No disease symptom (healthy), 1= Reduced growth than the healthy seedling, 2= Shrinked seedling, 3= Wilted hypocotyl showing reddish. Disease scale 4 indicate completely damped of or ungerminated seedlings therefore, no picture was available.

Fig. 2. The disease severity scale implemented in order to measure disease severity in spinach seedlins caused by *P. aphanidermatum*. Disease severity scores of the seedlings were based on the degree of stunting and wilting ranging from 0 to 4, where 0 = No disease symptom (healthy), 1= slightly stunted seedling, 2= moderate stunting or chlorosis, 3= severe stunting or wilting, 4= completely wilted or died.

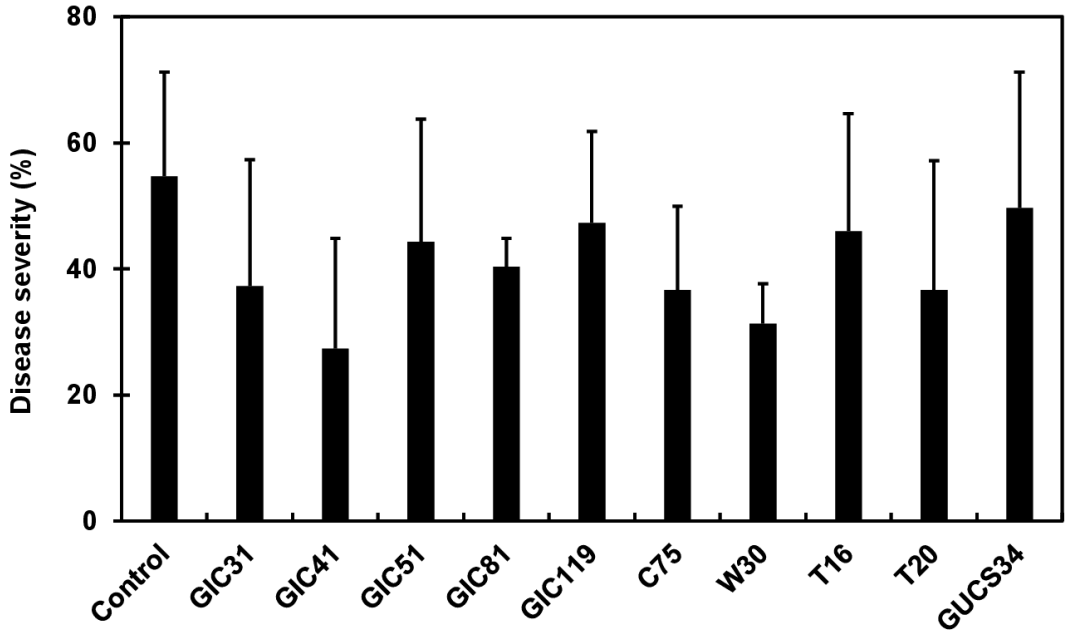

Fig. 3. Suppressive effect of *Lysinibacillus* strains against damping off of cabbage seedlings caused by *S. sclerotiorum*. Disease severity = [(The number of diseased plants in each scale x disease scale)/ (Total number of plants investigated x the highest disease scale)] x 100. Bars represent the mean ± standard deviation of three independent experiments.

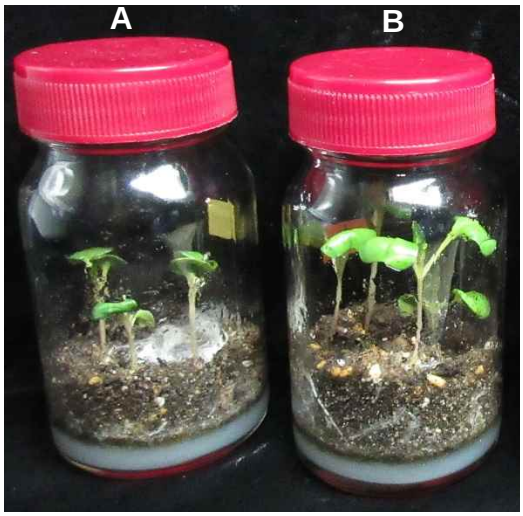

Fig. 4. Effect of GIC41 application on the suppression of damping off disease caused by *S. sclerotiorum*. (A) Control, (B) GIC41 treatment. Photo was taken at 10 days after seeding.

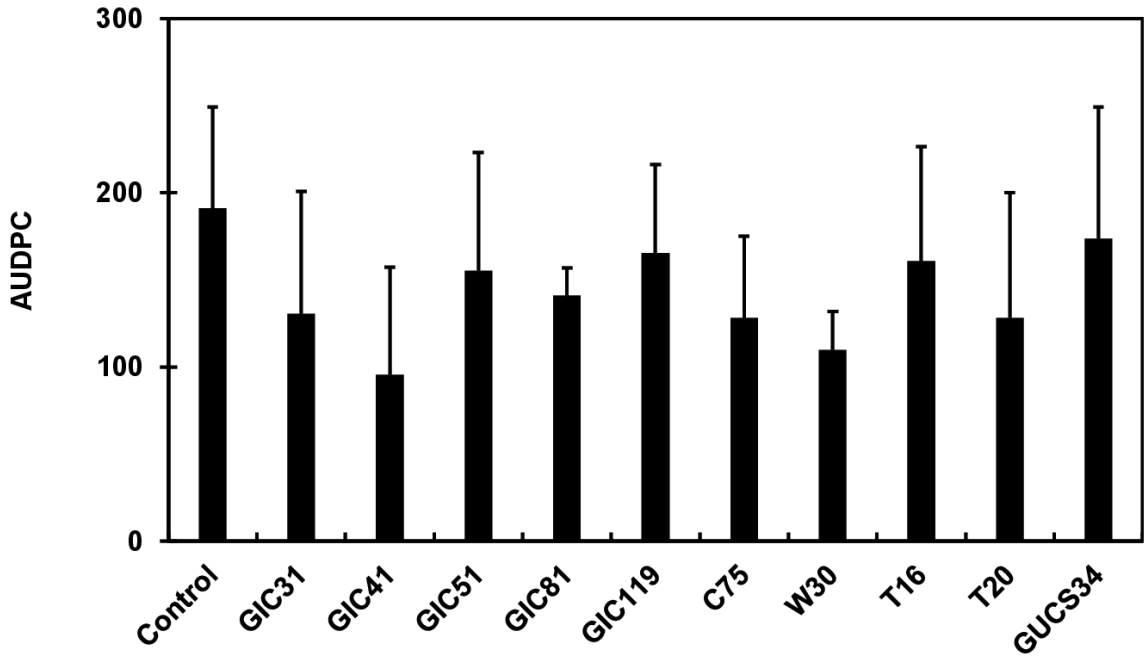

Fig. 5. The area under the disease progress curve (AUDPC) of damping off (*S. sclerotiorum*) in cabbage seedlings under the application of *Lysinibacillus* strains as plant probiotic agents.

Disease severity = [(The number of diseased plants in each scale x disease scale)/ (Total number of plants investigated x the highest disease scale)] x 100.

AUDPC was calculated based on disease severity using the trapezoid integration of disease progress curve over time according to the following formula:

AUDPC= Σ [0.5 (X_i +$X_{i\text{-}1}$)] (t_i-$t_{i\text{-}1}$), where X_i and $X_{i\text{-}1}$ are disease severity at time t_i and $t_{i\text{-}1}$, respectively, and t_i and $t_{i\text{-}1}$ are consecutive evaluation dates with t_i and $t_{i\text{-}1}$ equal to 1.

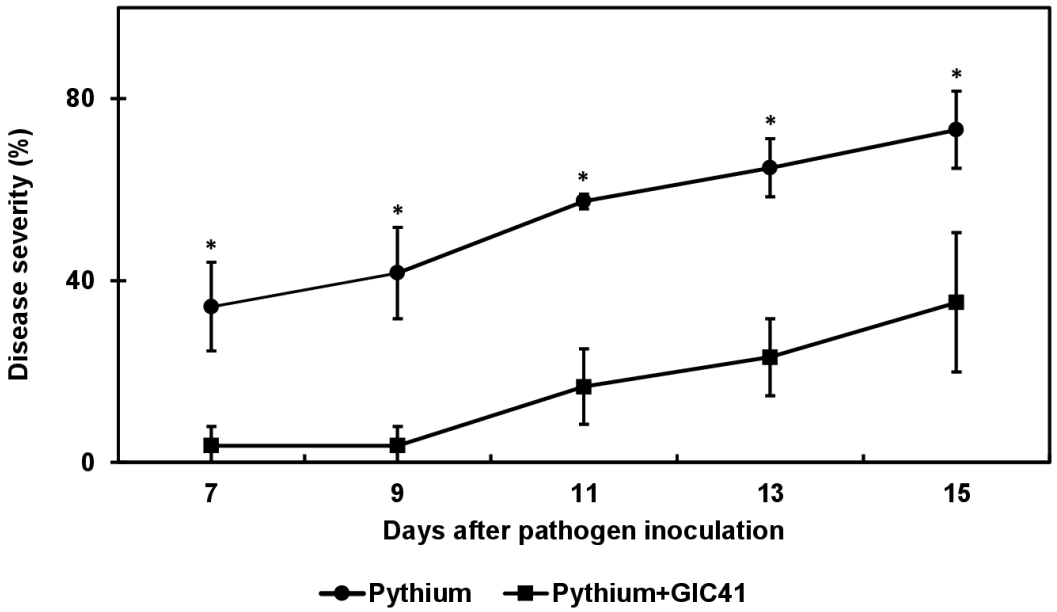

Fig. 6. Effect of GIC41 inoculation on the disease severity of spinach seedlings against damping off disease caused by *P. aphanidermatum* from 7 to 15 days after pathogen inoculation grown under control environmental condition. Disease severity = [(The number of diseased plants in each scale x disease scale)/ (Total number of plants investigated x the highest disease scale)] x 100. Bars represent the mean ± standard deviation of three replicates per treatment with three times repetitions. An asterisk indicates significant difference between treatments according to Student′s *t*-test at $P < 0.05$

Table 1. Suppressive effect of GIC41 drench treatment on spinach seedlings against damping off disease caused by *P. aphanidermatum* at 15 days after pathogen inoculation

Treatment	Disease severity (%)	AUDPC
Control	73.2 ± 8.5a	555.1 ± 82.7a
GIC41	35.2 ± 15.3b	138.9 ± 47.3b

Disease severity = [(The number of diseased plants in each scale x disease scale)/ (Total number of plants investigated x the highest disease scale)] x 100.

AUDPC was calculated based on disease severity using the trapezoid integration of disease progress curve over time according to the following formula:

AUDPC= Σ [0.5 (X_i +X_{i-1})] (t_i-t_{i-1}), where X_i and X_{i-1} are disease severity at time t_i and t_{i-1}, respectively, and t_i and t_{i-1} are consecutive evaluation dates with t_i and t_{i-1} equal to 1.

Each value represents a mean ± standard error. Values with the same lower-case letters in a row within the column are not significantly different at $P< 0.05$ (Student′s *t*-test).

Fig. 7. Examples of suppressive effects provided by soil drenching treatment with GIC41 against seedling damping off disease caused by *P. aphanidermatum*. (A) uninoculated seedlings, (B) seedlings inoculated with pathogen *P. aphanidermatum*, (C) seedlings inoculated with pathogen *P. aphanidermatum* and *Lysinibacillus* strain GIC41. The photo was taken 15 days after pathogen inoculation.

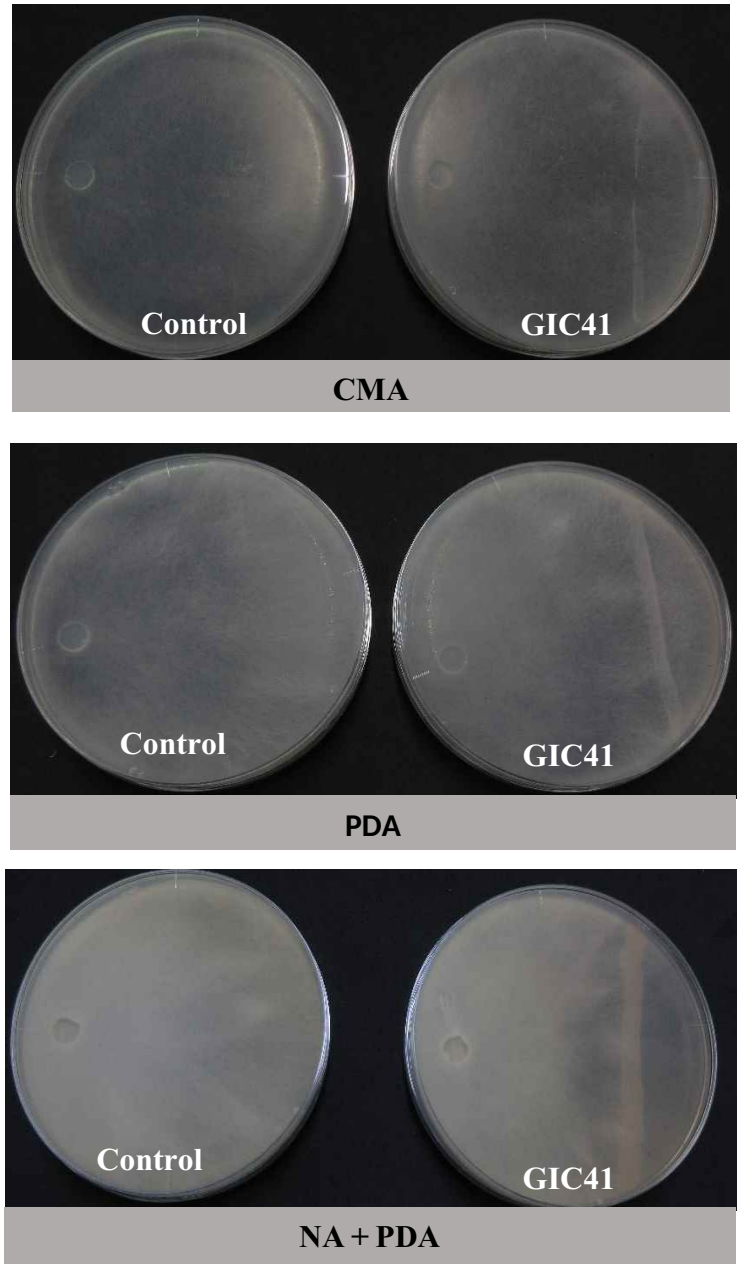

Fig. 8. Dual culture assay showing difference in the antifungal ability of the strain GIC41 against *P. aphanidermatum* grown in different media. The Photo was taken after the incubation for five days at 25°C.

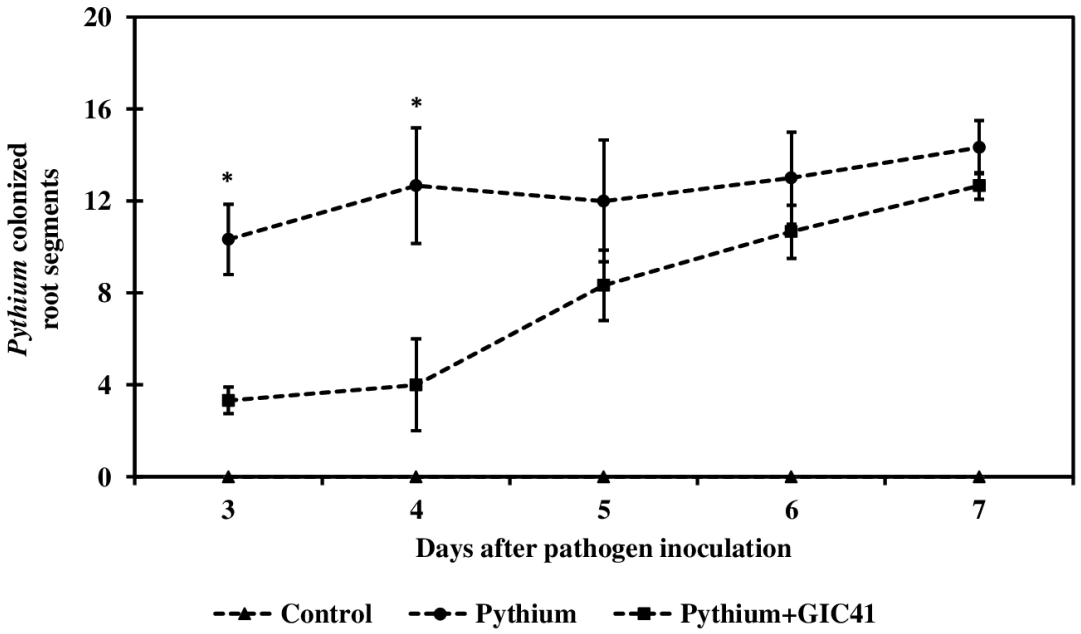

Fig. 9. Effect of GIC41 application on the colonization ability of *P. aphanidermatum* based on the root segments in which the pathogen was recovered with in 24h. Bars represent the mean ± standard deviation of three replicates per treatment with three times repetitions. An asterisk indicates significant difference between treatments according to Student′s t-test at $P < 0.05$

Discussion

The aim of the present study was to evaluate the disease suppression potential of the *Lysinibacillus* strains against two soilborne pathogen *Sclerotinia sclerotiorum* and *Pythium aphanidermatum*. Ten *Lysinibacillus* strains were tested in this chapter for their disease suppression ability which were previously identified in our laboratory and evaluated for their growth promotion ability in chapter1. Among the strains, GIC41 showed strong disease suppression ability against *S. sclerotiorum* and reduced the disease severity by 49% compared to the control treatment. Moreover, the disease suppression ability of GIC41 was tested against another pathogen *P. aphanidermatum* causing damping off spinach seedlings grown in the small pots. According to the results, GIC41 can reduce disease severity by 51% (Fig. 6) and AUDPC was reduced by 74% (Table 1) indicating that GIC41 could be a promising agent in the suppression of damping off disease caused by *Sclerotinia sclerotiorum* and *Phythium aphanidermatum*.

As described in the chapter 1, GIC41 was found to be *L. xylanilyticus*. In recent years, *Lysinibacillus* species have been reported for the biocontrol potential to a wide range of pathogens (Abiala et al., 2015; Shabanamol et al., 2018; Naureen et al., 2018). Passera et al. (2020) reported that *L. fusiformis* can reduce the disease severity symptoms caused by *Botrytis cinerea* in detached tomatos and grapevine leaves. Another species, *Lysinibacillus sphaericus* was reported for the suppression of the pathogenic fungi *Rhizoctonia solani* which cause sheath blight disease in rice (Shabanamol et al. 2017). Till now, around 28 *Lysinibacillus* species have been identified. Among them, only two species, *L. sphaericus* and *L. fusiformis*, were reported for the potential to control

plant diseases (Ahsan and Shimizu, 2021). To the best of our knowledge, there is no study about the biocontrol ability of *L. xylanilyticus* against *S. sclerotiorum* and *P. aphanidermatum*. Therefore, this might be the first study to describe the potential of *L. xylanilyticus* in disease suppression against soil borne pathogens.

During the screening experiment, most of the *Lysinibacillus* strains showed disease suppressive ability against *S. sclerotiorum*. However, the maximum reduction (48%) of disease progress was obtained with the GIC41 treatment. Previously, *Lysinibacillus sphaericus* has been reported for the antagonistic behavior against *S. sclerotiorum* (Naureen et al., 2017). However, no other strain of *Lysinibacillus* has yet been reported for the disease suppression ability against *S. sclerotiorum*. To the best of our knowledge, it is the first report about the disease suppression ability of *L. xylanilyticus* against *S. sclerotiorum*.

The strain GIC41 also could successfully suppress the disease caused by *P. aphanidermatum* in control environmental condition (Fig 7). Several reports explained that the biocontrol ability of *Lysinibacillus* spp. is associated with their antimicrobial compound production ability (Passera et al., 2020; Shabanamol et al., 2017; Naureen et al., 2017). In our study, GIC41 could not show any antifungal ability against *P. aphanidermatum* when grown in CMA and PDA media. Contrarily, when GIC41 was tested for the antagonistic ability on the PDA and NA mixed (1:1) media, the strain showed a weak antifungal ability (Fig. 8). This result indicate that the strain may have some antifungal ability against *P. aphanidermatum* which vary greatly with the growing condition of the strains. This finding is identical with the result of Darmawan et al., (2021) where they found that the extracellular antifungal production by *Serratia plymuthica* strain UBCF_13 is influenced by the culture media where the bacteria was grown. Along with the possibilities of the involvement of

antagonistic ability of GIC41 for the disease suppression, some other mechanisms may also involve with the disease suppression ability of GIC41. We know that colonization is very important to exert disease suppressive effect against soil borne pathogens (Raaijmakers et al., 2002). In this chapter, we examined the colonization ability of *P. aphanidermatum* on the plants treated with GIC41. According to the results, GIC41 treated plants reduced *Pythium* colonization significantly up to 4th day of treatment, which indicate that reduction of the colonization capacity of *Pythium* may be involved with the disease suppression ability of GIC41. Nutrient competition between pathogen and GIC41 may be related with the reduction of colonization ability of *Pythium* in GIC41 treated plants.

Another possible reason which may involve with the disease suppression ability of GIC41 is induction of systemic resistance. Drenching application of GIC41 may induced the systemic resistance of the plant. As a result, the plants may accumulate higher level of defense enzymes in the roots. The induction of systemic resistance by the application of GIC41 may be associated with the biosynthesis of lignin, reactive oxygen, and antimicrobial compounds that have been considered as resistant mechanisms against plant diseases. In several reports, enhancement of defense enzymes has contributed to the disease suppression ability of beneficial bacteria. For example, Saravanakumar et al. (2007) have been reported that application of *Pseudomonas fluorescens* induced defense enzymes such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, chitinase, and β-1,3-glucanase which may be related to the reduction of the incidence of blister blight in tea plant. Similarly, Ganeshamoorthi et al. (2008) explained that induction of defense enzymes might have the contribution in restricting the invasion of *Macrophomina phaseolina* in mulberry roots. However, to clarify the mechanisms related with the disease suppressive ability of GIC41, further experiment is needed such as induction of defense

enzyme activity in the root of spinach seedlings and defense related gene expression analysis.

In conclusion, the findings from the present study evident that soil drenching with GC41 is effective in suppressing different soil borne diseases. Therefore, GIC41 could be a promising candidate to formulate a novel biocontrol agent in future.

General discussion

Results from the present study clearly demonstrated that *Lysinibacillus xylanilyticus* GIC41 is a potential plant probiotic agent. The strain could enhance the growth of spinach plant (Chapter 1) and also successfully suppress the seedling damping off disease caused by two soil borne pathogens, *S. sclerotiorum* and *P. aphanidermatum* (Chapter 2). Among the tested 10 strains GIC41 was the best performed strain which showed constant efficacy during both plant growth promotion and disease suppression experiments. The same growth enhancement trend was observed in spinach plants when treated with GIC41 in control environmental condition and glass house condition (Chapter 1). During the disease suppression investigation, the strain GIC41 showed highest (49%) suppressive effect against seedling damping off of cabbage caused by *S. sclerotiorum* in compared to other *Lysinibacillus* strains. Along with that the strain GIC41 strongly (51%) suppressed the spinach seedling damping off disease caused by *P. aphanidermatum* in controlled environmental condition (Chapter 2). Here, *Lysinibacillus xylanilyticus* GIC41 have been reported for the first time as plant probiotic agent which has capacity to enhance plant growth and suppress disease against two devastating soil borne diseases.

The growth promotion ability of the strain (Chapter 1) may be related with the enhancement of nutrient and water uptake ability of the plants. Due to the soil drenching with GIC41, the number of lateral roots enhanced which subsequently enhanced the nutrient and water uptake of the plants resulting the enhancement of the growth. As we know, Lateral roots contribute to nutrient and water absorption from soil by increasing the overall surface area of the root system

(Vessey, 2003). Enhancement of nutrient uptake capacity of GIC41 treated plants can be explained with the results of nitrogen percentage of the plants. Though the shoot dry weight increased significantly in GIC41 treated plants, however, there was no significant difference in the nitrogen content of GIC41 treated plants with control treatment. This result indicated that the total amount of nitrogen uptake from soil was increased in the GIC41-treated plants.

Production of phytohormone (IAA) may also play an important role in the growth promotion ability of the strain GIC41 (Chapter 1). It is well known that the lateral root formation is regulated by the phytohormone auxin and exogenous auxin promotes the production of lateral roots (Moriwaki et al., 2011; Fukaki and Tasaka, 2009; Waidmann et al., 2020). Since strain GIC41 exhibited IAA-producing capacity, it was assumed that IAA synthesized by strain GIC41 play an important role in the enhancement of lateral root development. The increase in root biomass after GIC41 treatment was more pronounced under the moderately fertilized conditions (fertilized with 0.2% Hyponex solution, the concentration recommended by the supplier) than under the less fertilized conditions (fertilized with 0.1% and 0.05% Hyponex solution). As we know that IAA production by bacteria depends on the availability of nitrogen (Thuler et al., 2003; Tamaki and Mercier, 2007; Shokri and Emtiazi, 2010). Therefore, it is assumed that soil nutrient status, particularly nitrogen level, would be a key factor for strain GIC41 to exert its PGP effect. Moreover, the concentration of IAA produced by the bacteria may also differ from the in vitro experiment to spinach rhizosphere condition which contribute to the enhanced root growth in compared to other strains. Because the concentration of IAA differs with the difference in growing condition and substrate availability (Sasirekha et al., 2012; Chandra et al., 2018). The colonization ability of the strain GIC41 in spinach rhizosphere may also contribute to the more stable PGP effect in compared to other strains. Although the root colonization capacity of *Lysinibacillus*

strains was not investigated in this study. Another possible explanation is that the involvement of the mechanisms other than IAA production. Previous studies have shown that production of plant hormones other than IAA, viz. cytokinins and gibberellins, also participate in the PGP effect of PGPR (Ortíz-Castro et al. 2008; Kang et al., 2014). Moreover, recent investigations revealed that volatile organic compounds produced by PGPR can activate phytohormone signaling pathways and enhance plant growth (Jishma eta al., 2017 Tahir et al., 2017. Therefore, it is possible that, in addition to IAA, strain GIC41 produces cytokinins, gibberellins, and/or VOCs, and that the coordinated action of these compounds and IAA may be responsible for the strain GIC41′s superior PGP effect.

Very few studies have been conducted on the disease suppression ability of *Lysinibacillus* strains of plants (Trivedi et al., 2011; Singh et al., 2013; Abiala et al., 2015). Mostly, two species, *L . sphaericus* and *L. fusiformis* have been studied for their disease suppression ability against various plant pathogens (Ahsan and Shimizu, 2021). Several reports explained that the biocontrol ability of *Lysinibacillus* spp. is associated with their antimicrobial compound production ability (Passera et al., 2020; Shabanamol et al., 2017; Naureen et al., 2017). However, in our study (Chapter 2), GIC41 could not show any antifungal ability against *P. aphanidermatum* indicating that production of antimicrobial compound by GIC41 may not be involved with the disease suppression ability of the strain. Except antimicrobial compound production, the plant probiotic bacteria can suppress disease through other mechanisms such as production of lytic enzymes, competition, production of siderophore, ACC deaminase production and induced systemic resistance (Glick, 2012). As we conducted the in vitro experiment in chapter 1 to know the potential ability of the strain GIC41, from that we can know that the strain is also lack of siderophore and ACC deaminase production ability. Therefore, we can assume that the disease

suppression ability of GIC41 may be related with induction of systemic resistance of the plant. As a result of induction of systemic resistance, the plants may accumulate higher level of defense enzymes in the roots which subsequently, suppressed the damping off disease caused by *P. aphanidermatum*. Moreover, colonization ability of GIC41 may also involve with their disease suppression ability because colonization is very important to exert disease suppressive effect against soil borne pathogens (Raaijmakers et al., 2002). As GIC41 could suppress the damping off disease caused by *P. aphanidermatum* around 51% indicate that may be the bacteria can colonize in the rhizosphere of spinach plant which might contribute to the suppression of pathogen multiplication.

Conclusion

In this study, we successfully obtained a *Lysinibacillus* strain, GIC41 as a novel plant probiotic agent which is capable to enhance plant growth as well as can suppress devastating soil borne diseases. In future, the strain could be a promising candidate to formulate a novel biostimulant or biocontrol agent.

The strain exhibited constant plant growth promotion ability in controlled environmental condition and glass house condition (Chapter 1), however, field experiments are needed to confirm its efficacy in wide scale cultivation. Besides, we speculated that the colonization ability of the strain and the production of phytohormone may be involved with the root growth of the plant, resulting the higher growth enhancement of the plant. However, for better understanding, more detail study about the mechanisms are needed such as involvement of other hormones with the plant growth promotion ability of the strain, analysis of growth promotion related gene expression, investigation of the the colonization and IAA production ability of the strain in spinach rhizosphere.

In this study (Chapter 2), we successfully screened the strain GIC41 for disease suppressive ability against two soil borne pathogens *S. sclerotiorum* and *P. aphanidermatum* in controlled environmental condition. However, to confirm the disease suppression efficacy of the strain greenhouse and field experiments are needed to conduct in future. Moreover, during the in vitro test as the strain did not show any antibacterial ability therefore, it is needed to investigate the

related mechanisms such as expression of defense related genes or accumulation of defense enzymes which are really involved with the disease suppression ability of the strain GIC41.

Summary

In recent years, plant probiotic bacteria have drawn special attention of the scientific communities due to their potential to enhance crop productivity in an environment-friendly manner. To date, a wide range of plant probiotic bacteria have been discovered and some of them have been commercialized also. However, sometimes their performance in the field condition is inconsistent most likely due to the influence of biotic and abiotic factors. Therefore, there is a continuous search for in the search of effective plant probiotic agents. In this study, we evaluated the plant growth promoting and disease suppression potential of *Lysinibacillus* strains. As the *Lysinibacillus* can produce endospores, and adapt to a wide range of environment, thus we chose the bacteria of genus *Lysinibacillus* for our study in the search of a novel plant probiotic agent.

Chapter 1. Evaluation of the plant growth promoting potential of *Lysinibacillus* spp.

In this chapter, we evaluated the plant growth-promoting (PGP) ability of 10 *Lysinibacillus* strains which were chosen from our laboratory′s bacterial collection based on their partial 16S rRNA gene sequences. In the primary screening, the PGP effect of the strains were evaluated on spinach plants grown on the Jiffy-7 pellets in a controlled environmental chamber. As a result, three strains of *L. xylanilyticus* (GIC31, GIC41, and GIC51) significantly increased the shoot biomass. Subsequently, a pot experiment was conducted in controlled environmental condition.

Spinach seeds were sown in 150 mL-pots containing sand and vermiculite mixture. Seven days after sowing, spinach seedlings were drench treated with a cell suspension of each selected strains and grown for 20 days with regular watering and fertilizing weekly up to the saturation level. As a result, the strain GIC41 showed the highest growth-promoting effect, so GIC41 was selected as the best candidate. In this study a glass house experiment was also conducted to evaluate the plant growth promoting effect of the strain GIC41. Seven days old spinach seedlings were drench treated with GIC41 and transplanted to the rectangular pot (64 cm x 22 cm) filled with commercial soil (5 plants/ pot) and grown in glasshouse condition for six weeks. Six weeks later, shoot biomass, carbon and nitrogen contents (%) were measured. According to the result, GIC41 significantly increased the shoot biomass suggesting the potentiality of the strain in enhancing plant growth promotion. However, the carbon and nitrogen content were not differ from that of the control treated plants. Thereafter, the effect of fertilizer levels on the PGP effect of GIC41 was evaluated. A pot experiment was conducted in controlled environmental chamber where different concentrations (0.05, 0.1, 0.2% Hyponex) fertilizer was applied to the GIC41 treated spinach seedlings. As a result, a significant growth promoting effect was found with the recommended concentration (0.2%) of fertilizer. Along with enhancement of shoot biomass and leaf area, root dry weight of the GIC41 treated plants were also increased. However, with the lower concentrations (0.05, and 0.1%) of fertilizer, the strain could not show such significant enhancement, indicating that certain nutrient condition of soil is needed to maintain for the PGP effect of GIC41. The enhancement of root dry weight of the GIC41 treated plants which is due to the enhancement of lateral roots suggesting the plant hormone indole acetic acid may be involved in the growth promoting mechanism. The results of in vitro test showed that, GIC41 produces IAA but lack of the other PGP associated traits including phosphate solubilization, siderophore

production ACC deaminase production, and nitrogen fixation. Taken together all the result it can be speculated that production of IAA may be related with the PGP effect of GIC41. IAA produced by GIC41 may increase the lateral roots in GIC41 treated plants which facilitate the plants to uptake more water and nutrient subsequently enhanced growth. However, further analysis is needed to elucidate the PGP effect of GIC41.

Moreover, the results clearly indicated that the *L. xylanilyticus* strain GIC41 has a potential for plant growth promotion, therefore, it could be a promising biostimulant in future.

Chapter 2 Evaluation of the potential *Lysinibacillus* strains with disease suppression ability against seedling damping off disease caused by soil borne pathogens

In this chapter, the disease suppressive ability of the *Lysinibacillus* strains was evaluated against the seedling damping off disease caused by different soil borne pathogens.

Firstly, the suppressive effect of the strains against *Sclerotinia sclerotiorum* was investigated. Growing hyphae of *Sclerotinia sclerotiorum* was transferred to the glass bottles (height 9cm x diameter 5 cm) containing 3 mL of potato dextrose agar (PDA) and grown for three days at 23°C. Three days later, the bottles were filled with sterilized commercial soil, and then sterilized cabbage seeds were sown. Thereafter, cell suspensions of each bacterial strains were applied to the soil in the bottle. The cabbage seedlings were grown in a controlled environmental chamber for 10 days. As a result, all the strains suppressed the seedling damping off to a greater or lesser extent. However, the highest suppressive effect was obtained by the treatment with the strain GIC41 which reduced the disease severity by 49%. The strain GIC41 was then evaluated for its suppressive effect against spinach damping off caused by *Pythium aphanidermatum*. The spinach

seeds were sown in the 9 cm pots containing commercial soil and grown in a controlled environmental chamber. One week later, seedlings were inoculated with zoospores of pathogen and GIC41. After 15 days of inoculation, the GIC41 treated plants showed lower disease symptoms and reduced the disease severity by 52%. Antifungal activity of the strain GIC41 was tested by dual culture method. However, the strain did not inhibit hyphal growth of the pathogen suggesting that the antibacterial effect of the secondary metabolites was not involved in the disease suppressing effect of the strain. However, further analysis is required to elucidate the disease control mechanisms in detail.

Finally, the findings from the present study evident that GC41 is effective in suppressing devasting soil borne pathogens suggesting the promising potential of the strain GIC41 as a biocontrol agent.

Conclusion

In this study, we have successfully found out a potential plant probiotic *Lysinibacillus* strain which has capacity to enhance plant growth and suppress seedling damping off disease caused by devastating soil borne pathogens. This is the first study to describe the PGP and biocontrol ability of *L. xylanilyticus*.

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