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Effects of inoculation with a commercial microbial inoculant *Bacillus subtilis* C-3102 mixture on rice and barley growth and its possible mechanism in the plant growth stimulatory effect

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Abstract

The effects of a microbial inoculant (Thervelics®: a mixture of cells of *Bacillus subtilis* C-3102 and carrier materials) on rice (*Oryza sativa* cv. Milkyprincess) and barley (*Hordeum vulgare* cv. Sachiho Golden) were evaluated in four pot experiments. In the first and second experiments, the dry matter production of rice and barley increased significantly by 10–20% with the inoculation of the mixture at a rate of 10^7 cfu $\cdot$ g^{-1} soil compared with the non-inoculated control. In the third experiment, the growth promoting effects of the mixture, the autoclaved mixture and the carrier materials were compared. The dry mater production of rice grains was the highest in the mixture, and it was significantly higher in the three treatments than in the control, suggesting that the carrier materials may also have a plant growth promoting effect and the living cells might have an additional stimulatory effect. To confirm the efficacy of the living cells in the mixture, only *B. subtilis* C-3102 cells were used in the fourth experiment. In addition, to estimate the mechanisms in growth promotion by *B. subtilis* C-3102, three *B. subtilis* strains with similar or different properties in the production of indole-3-acetic acid (IAA), protease and siderophore and phosphate-solubilizing ability were used as reference strains. Only *B. subtilis* C-3102 significantly increased the dry matter production of rice grains and the soil protease activity was consistently higher in the soil inoculated with *B. subtilis* C-3102 throughout the growing period. These results indicate that the microbial inoculant including live *B. subtilis* C-3102 may have growth promoting effects on rice and barley.

Keywords: *Bacillus subtilis* C-3102, indole-3-acetic acid (IAA), phosphate solubilization, plant growth promoting rhizobacteria (PGPR), protease

Introduction

Probiotics are considered to be beneficial to the host animal (Khochamit *et al.* 2015; Uyeno *et al.* 2015). *Bacillus subtilis* C-3102 has been used as a direct-fed probiotic for improved gut health in different livestock, such as broilers (Meure *et al.* 2010; Jeong and Kim 2014), matrinxa (Dias *et al.* 2012) and tilapia (He *et al.* 2013; Garcia-Marengoni and Mendez-Albuquerque 2015). Moreover, C-3102 is known to improve the composition and metabolic activity of the human intestinal microflora (Hatanaka *et al.* 2012) and has attracted attention.

Many plant-associated bacteria are well known for their ability to promote plant growth (Compant *et al.* 2010; Rainer 2011; Chauhan *et al.* 2015; Cherif-Silini *et al.* 2016; Islam *et al.* 2016). Among the commonly reported plant-growth promoting rhizobacteria, strains

belonging to the genus *Bacillus* are prevalent (Qiao *et al.* 2014). Root-colonizing *Bacillus* spp. are well documented to enhance growth of many crops by producing growth promotion-related chemical compounds, e.g. indole acetic acid (IAA) in *Bacillus subtilis* B579, *Bacillus subtilis* B4, *Bacillus subtilis* KPS-11, *Bacillus subtilis* AK 38 and *Bacillus velezensis* BAC03 (Chen *et al.* 2010; Park *et al.* 2013; Hanif *et al.* 2015; Meng *et al.* 2016; Karnwal 2017), protease in *B. subtilis* 38, *B. subtilis* V26 and *B. subtilis* BUU1 (Khedher *et al.* 2015; Uttatree and Charoenpanich 2016), siderophore in *B. subtilis* B579 and *B. subtilis* CAS15 (Chen *et al.* 2010; Yu *et al.* 2011), cytokine in *B. subtilis* IB-22 (Arkhipova *et al.* 2007), jasmonic acid, ethylene, abscisic acid and auxin in *B. subtilis* BBG111 (Chandler *et al.* 2015). In addition, some *Bacillus* strains suppress crop pests and thereby indirectly promote plant growth (Kumar *et al.* 2012a; Kumar *et al.* 2012b; Lin *et al.* 2014; Lee and Kim 2015; El-Bendary *et al.* 2016; Ge *et al.* 2016; Islam *et al.* 2016; Meng *et al.* 2016). Thus, there is growing interest in *Bacillus* related species as a biopesticide or biofertilizer (Qiao *et al.* 2014; Toyota 2015). As examples, enhanced plant growth has been documented in rice inoculated with *B. subtilis* MBI-600 (Kumar *et al.* 2012a), *B. megaterium* (Al-Taweil *et al.* 2009), *B. lichenifromis* (Wang *et al.* 2009), *B. altitudinis*, *B. pumilus* (Habibi *et al.* 2014), *Bacillus* sp. and *Bacillus cereus* (Shakeel *et al.* 2015).

These previous studies indicate that *B. subtilis* C-3102 might have a plant growth promoting effect. This strain was originally formulated as a probiotic product and its application has been expanded to agriculture because of its stimulatory effect on composting. *Bacillus subtilis* C-3102 is now formulated as a compost accelerating agent with the trade name of Thervelics. The objectives of this study were to evaluate a plant-growth-promoting effect of the live *Bacillus subtilis* C-3102 mixture on the growth of rice and barley, one of the classical cropping patterns in Japan (Sotome *et al.* 2010) and to estimate the possible mechanism in growth promotion by the mixture.

Materials and Methods

Commercial microbial inoculant *Bacillus subtilis* C-3102 mixture

In this study, we evaluated the effects of a commercially available microbial inoculant of *B. subtilis* C-3102 on rice and barley growth. This inoculant is a mixture of *B. subtilis* C-3102 cells, culture medium (soybean extract, 27% of the total weight), and calcium carbonate (73% of the total weight) (Thervelics®: containing 10^{10} cfu of *B. subtilis* C-3102 in 1 g powder, produced by Asahi Calpis Wellness Co., Ltd.).

Soil

Soil (gray lowland soil; soil texture, SiL) was collected from the surface layer of an experimental paddy field in the B1 plot at FM Hommachi, FS Center, Tokyo University of Agriculture and Technology (35°39'N, 139°28'E at an altitude of 48 m) at Fuchu, Tokyo, Japan. The collected soil sample was sieved through an 8-mm screen, mixed well and used for the following experiments.

Experiment 1: Effects of the *Bacillus subtilis* C-3102 mixture (BS mixture) on rice growth

Rice (*Oryza sativa* cv. Milkyprincess) seedlings were prepared by planting three 2-day pre-germinated seeds on May 11, 2014, into a small pot containing 50 g of the soil described above, with the addition of 0, 0.5 mg and 50 mg of the mixture powder in 20 replicates each, which corresponded to final densities of 0, 10^5 and 10^7 cfu · g^{-1} soil, respectively. In the treatment of 0.5 mg of the mixture to 50 g soil, 50 mg of the mixture was added with 50 g of soil and 0.5 g of the mixed soil wad was further added with 49.5 g of non-mixed soil. Then, the treated soils were fertilized with a compound chemical fertilizer (N : P_2O_5 : K_2O = 14 : 14 : 14) at a rate of 30 mg · kg^{-1} N soil. After inoculation and fertilization, soil in the pots was mixed well. The pots were kept flooded during the growing period. A total of 60 pots were grown in a glasshouse, Koganei campus, Tokyo University of Agriculture and Technology, until June 7, and then ten pots that showed average growth of seedlings were selected from each treatment for transplanting into larger pots (a surface area of 0.05 m^2), with 10 kg of the soil. In the larger pots, soil was added with the compound fertilizer at a rate of 30 kg · ha^{-1} N and with the mixture powder at the densities as described above on May 30 in the B-1 plot at FM Hommachi. A total of 30 larger pots were prepared for three treatments with ten replicates. On June 7, three seedlings of rice, prepared as above, were transplanted into each of the pots. Topdressing was done two times at rates of 20 kg N and 10 kg · ha^{-1} N on July 18 and August 5, respectively. During cultivation, the pots were placed in an open place at FM Hommachi and always kept flooded with tap water at 1 to 2 day intervals.

The growth parameters (plant height, tiller number and SPAD value) were monitored at 1 to 2 week intervals during the growing period. In the SPAD analysis, ten young leaves were measured per pot. Watering was stopped at the end of August and the aboveground tissues were harvested at a height of 2 cm from soil surface on September 12 and separated into the straw (stem and leaf) and grain parts. On

September 16, the root systems were collected from six pots with average aboveground growth, out of ten pots, and washed with running tap water to remove the adhering soil. The dry matter weights of the straw, grain and root parts were measured after oven-drying at 70°C for 4 days.

Soil samples were collected from a 0–15 cm depth using a root auger with a 2-cm diameter from three pots randomly selected at transplanting and from three pots with average plant growth at 31 days after transplanting and at harvesting. Soil samples were used for the measurements of available phosphate (P) content and the number of C-3102. Available P was measured with the Truog method using oven-dried soil samples prepared as described below. To determine the density of *B. subtilis* C-3102, a part (100 g) of the soil collected as above was dried for 2 days at 60°C. One g soil or root was added to 9 ml of sterilized distilled water and the suspensions were shaken vigorously on a vortex, treated at 65°C for 30 min to kill indigenous non-spore formers and then serially diluted. An aliquot (100 μl) of the suspensions was spread in triplicate on the tryptic soy medium and incubated at 37°C for 18 h. Colonies with special characters (large, mucoid, wrinkled and cone-shaped) were considered to be *B. subtilis* C-3102.

To confirm whether such colonies derived from *B. subtilis* C-3102, colony PCR was done using two species specific primer sets targeting the amylases of *B. subtilis* (primers 1 and 2, this study) and *B. amyloliquefaciens* (primers 3 and 4; Marubashi *et al.* 2009), since all the *B. subtilis* strains in Table 1, as described below (Experiment 4), reacted to primers 1 and 2 while only *B. subtilis* C-3102 reacted to primers 3 and 4. A loopful of colonies with the special characters mentioned above were treated using a PrepMan Ultra Sample Preparation Reagent (Thermo Fisher Scientific K.K.) as instructed by the manufacturer's manual and treated DNA solution was used as a template after ten times dilution for PCR using either primers 1 and 2 or primers 3 and 4. The PCR program was 1 min at 94°C, followed by 25 cycles of amplification, each consisting of 20 s at 98°C, 15 s at 60°C and 1 min at 72°C, with a final extension for 10 min at 72°C. The primer sequences were primer 1: 5'-CCTCTTTACTGC CGTTATTCGC-3', primer 2: 5'-CTGCATTTGCCAC GACAACGC-3', primer 3: 5'-GCACATACGAAAA GACTGGCTGAAA-3', and primer 4: 5'-CCCACGTT GTGATTAAAAGCAGCGAT-3'. Aliquots of PCR products were analyzed by electrophoresis on a 0.7% (w/v) agarose gel stained with ethidium bromide. All the *B. subtilis* strains in Table 1 showed a 1.2 kbp band in primers 1 and 2, while only *B. subtilis* C-3102 showed a 700 bp band in primers 3 and 4 in addition to the 1.2 kbp product (Table 1).

Experiment 2: Effects of *Bacillus subtilis* C-3102 mixture on barley growth

After the rice experiment the same pots were used for the barley experiment by re-inoculating with the *B. subtilis* C-3102 mixture powder at final densities of 0, 10^5 and 10^7 cfu · g^{-1} soil and fertilized with the compound chemical fertilizer as mentioned above on November 7, 2014. After inoculation and fertilization, soil in the pots was mixed well and then ten seeds of barley (*Hordeum vulgare* cv. Sachiho Golden) were directly planted in a pot. For 1 month after seeding, the pots were watered with tap water and not watered after 1 month except for precipitation. Aboveground tissues were harvested on May 6, 2015 and the weights of their straw and grain portions were separately measured after oven-drying at 70℃ for 4 days. At harvesting, soil samples were collected from a 0–15 cm depth using a root auger with a 2-cm diameter from three pots with average plant growth. Soil and root samples were used to measure the density of *B. subtilis* C-3102, as described above.

Experiment 3: Effects of autoclaved and non-autoclaved *Bacillus subtilis* C-3102 mixture on rice growth

Ten kg of the soil (moisture content = 28%) newly collected was put into a plastic pot with a surface area of 0.05 m^2 and mixed with the compound chemical fertilizers at a rate of 30 kg · ha^{-1} N. A total of 40 pots were prepared for four treatments in ten replicates. On June 17, 2016, three seedlings of rice prepared as described below, were transplanted. The treatments were: 1 – inoculation of the mixture powder (10 g) at a final density of 10^7 cfu · g^{-1} soil, 2 – inoculation of the autoclaved (121°C, 15 min) mixture power (10 g), 3 – application of the carrier materials of the mixture powder (CaCO$_3$ + soybean extract) at rates of 7.3 and 2.7 g · pot^{-1} soil, respectively, which is equivalent to the same amount of the mixture without the C-3102 cells, and 4 – no inoculation (control). Topdressing was done two times at rates of 20 kg N and 15 kg · ha^{-1} N on July 28 and August 18, respectively. The pots were placed in an open place at FM Hommachi during the cultivation period, and always flooded at 1 to 2 day intervals with tap water.

The seedlings were prepared in a seedling-tray by planting three seeds of rice on May 26 in each well containing 12 g of the soil in 20 replicates and fertilized as described in the previous experiment. The pots were kept flooded. A total of 80 wells were grown in the glasshouse for 3 weeks, and then the seedlings in ten wells with average growth were selected in each treatment and transplanted into the larger pots, as described above.

Table 1. Plant growth promoting traits of various *Bacillus subtilis* strains and their reaction of the specific species primer sets to *B. subtilis* (Bs) and *B. amyloliquefaciens* (Ba)

Bacterial strains	Protease activity*	IAA**	Phosphate solubilization	Siderophore production	Reaction to	
					Bs primers	Ba primers
C-3102	2.39	2.84	–	+	++	++
NBRC109335	1.31	2.41	–	+	++	–
NBRC104468	1.28	2.39	–	–	++	–
NBRC104443	0.70	1.58	++	++	++	–
NBRC101592	0.64	2.24	+	–	++	–
NBRC101584	0.57	1.02	++	++	++	–
NBRC101581	0.37	0.00	+	+	++	–
NBRC101240	0.36	0.00	++	–	++	–
NBRC104440	0.29	1.53	–	+	++	–
NBRC104463	0.28	1.36	+	++	++	–
NBRC101588	0.21	1.47	+	++	++	–
NBRC101246	0.19	0.00	–	+	++	–
NBRC101582	0.18	1.94	+	++	++	–
NBRC101590	0.18	1.93	+	+	++	–
NBRC104449	0.17	2.71	+	++	++	–
NBRC101239	0.15	1.36	++	+	++	–
NBRC104461	0.13	0.96	++	+	++	–
NBRC101243	0.12	1.08	++	+	++	–

*mg · ml⁻¹ tyrosine of bacterial culture, **μg · ml⁻¹ of bacterial culture; mean values (n = 2); strains shown in bold were used in the fourth pot experiment; „–" – no, „+" – weak abilities, „++" – strong abilities

Plant height, tiller number and SPAD values were monitored at 1 to 2 week intervals during the growing period. Watering was stopped in the middle of September and the aboveground tissues were harvested on September 25, 2016. The dry matter weights of the straw, grain and root parts were measured as described in the previous experiment. The root systems were collected from three pots with average aboveground growth, washed with tap water to remove the adhering soil and used to measure the density of *B. subtilis* C-3102.

Soil samples were collected from three pots randomly selected at transplanting, 1 and 4 weeks after transplanting, and from three pots with average plant growth at harvesting and used for the measurements of protease activity and the density of *B. subtilis* C-3102. The soil protease activity was measured using casein as a substrate according to the method described by Ladd and Butler (1972).

Experiment 4: Efficacy of *Bacillus subtilis* C-3102 and other *B. subtilis* strains on rice growth

To confirm the efficacy of the living cells in the mixture, only *B. subtilis* C-3102 cells were used. In addition,

to speculate the mechanism of growth promotion by *B. subtilis* C-3102, its efficacy was compared with other *B. subtilis* strains. Seventeen *B. subtilis* strains were purchased from NITE Biological Resource Center (NBRC) culture collections, because all the strains were isolated from soil and considered to be adapted to soil environment. Root-colonizing *Bacillus* spp. are well documented to enhance growth of many crops by producing growth promotion-related chemical compounds, e.g. indole acetic acid (IAA) and phosphate-solubilization (Karnwal 2017), protease (Uttatree and Charoenpanich 2016), siderophore (Yu *et al.* 2011). Thus, these activities were measured (Table 1). IAA producing ability of the strains was evaluated with the method reported by Gardan *et al.* (1992). Protease activity was determined with the method of Kembhavi *et al.* (1993). Siderophore production was estimated with the method of Perez-Mirand *et al.* (2007). Phosphate solubilizing ability was tested on Pikovskay's agar plate containing 5 g · l⁻¹ tricalcium phosphate as an insoluble phosphate source (Gupta *et al.* 2012).

Among 17 strains, three strains of *B. subtilis* NBRC109335, NBRC104449 and NBRC101240 (Table 1) were selected based on the following properties: *B. subtilis* C-3102 showed the highest protease activity and IAA production, NBRC109335 showed similar

properties to *B. subtilis* C-3102, although its protease activity and IAA production were lower, NBRC104449 showed high IAA production, but low protease activity, NBRC101240 showed low protease activity, no IAA production and high phosphate solubilizing ability.

Two and half kg of the soil (moisture content = 28%) newly collected was put into a plastic pot with a surface area of 0.02 m^2 and mixed with the compound fertilizers at a rate of 30 mg · kg^{-1} N soil. A total of 25 pots were prepared in five replicates for five treatments (*B. subtilis* C-3102, the three NBRC strains and no inoculation). The bacterial strains prepared as described below were separately inoculated into pots at a final density of 10^7 cfu · g^{-1} soil and mixed well. On January 6, 2017, three seedlings of rice prepared as described below, were transplanted into each pot. The pots were placed on a hot-mat (Seiwa Co. S & H), covered with plastics to increase the air temperature, and always kept flooded at 3 to 4 day intervals of watering with tap water. Topdressing was done two times at rates of 20 kg N and 15 kg · g^{-1} N soil on March 30 and April 30, respectively. The average soil temperatures during the growing periods were 19.5℃, 23.7℃, 23.7℃, 27.8℃, and 28.9℃ in January, February, March, April and May, respectively.

Each of the bacterial strains was cultured in a 300 ml glass flask, containing 100 ml of 0.09 g nutrient broth (NB; Eiken Chemical Co., Ltd.) for 24 h on a rotary shaker (Taitec Co., Ltd.) at 145 rpm at room temperature. The cells were then collected by centrifugation at 6,000 rpm for 10 min and re-suspended in sterile saline solution (0.85% NaCl). The cell concentrations were determined with the dilution plate method on 10^{-1} nutrient agar (NA; Eiken Chemical, Co., Ltd) medium.

Seedlings were prepared in a seedling-tray by planting three seeds on December 15, 2016, into each well containing 12 g of the soil inoculated with each of the bacterial strains separately at a final density of 10^7 cfu · g^{-1} soil in ten replicates and fertilized as described in the previous experiments. The trays were kept flooded with tap water during the growing periods. A total of 50 wells were grown in a climatron (Biotron LPH200, Nippon Medical and Chemical Instruments Co., Ltd.) at 28℃ (16 h light, 8 h dark) for 4 weeks, and then the seedlings in five wells with average growth were selected in each treatment and transplanted into the larger pots as described above.

The growth parameters, plant height, tiller number and SPAD values were monitored at 2 week intervals during the growing period. Watering was stopped in the middle of May and the aboveground tissues were harvested at a height of 1 cm from the soil surface on June 4, 2017. The dry matter weights of the straw, grain and root parts were measured after oven-drying as described in the previous experiments.

Soil samples were collected from three pots randomly selected at transplanting, 2, 4 and 6 weeks after transplanting, and from three pots with average plant growth at harvest and used for the measurement of protease activity. The density of *B. subtilis* C-3102 in soil was measured at transplanting and harvest. Whole root systems were collected from all pots at harvest and the roots from three pots with average plant growth were used to measure the density of the strain.

Statistical analysis

Results are expressed as means and standard deviations on an oven-dry (60°C for soil or 70°C for root) basis for *B. subtilis* C-3102 population, enzyme assay and plant biomass, and were statistically analyzed by ANOVA using the software Excel Statistics version 1.09 (SPSS Japan).

No remarkable disease or pest was observed during the growing periods in all the experiments and thus differences in the measured parameters were considered to be due to inoculation.

Results

Effects of the *Bacillus subtilis* C-3102 mixture on rice growth (Experiment 1)

In rice, both BS mixture-treated, and non-treated rice plants showed similar patterns in the periodical change of height (Fig. 1A). However, the number of tillers was consistently highest ($p < 0.05$) in the treatment with the mixture of 10^7 (Fig. 1B). SPAD values at the early stage were also markedly higher ($p < 0.05$) in the treatment with the mixture of 10^7 (Fig. 1C). The aboveground biomass of straw and grain, and root biomass were significantly ($p < 0.05$) higher by 27.0%, 25.0% and 20.5%, respectively, in the treatment with the mixture of 10^7 than in the control (Table 2). The number of panicles was also significantly ($p < 0.05$) higher by 13.0% in the treatment with the mixture of 10^7 than in the control, while there was no significant difference in the weight of 1,000 filled grains between the treatments (data not shown). There was also no difference in the soil available P among the treatments at transplanting and 1 month after transplanting (data not shown). The density of *B. subtilis* C-3102 in soil at transplanting was 14 × 10^4 and 500 × 10^4 cfu · g^{-1} soil in the treatments with the *B. subtilis* C-3102 mixture at 10^5 and 10^7, respectively, while its density in the control was 4.0 × 10^4. At harvest, *B. subtilis* C-3102 was detected at 8 × 10^4 and 250 × 10^4 cfu · g^{-1} soil in the inoculated treatments, respectively, while it was detected only at
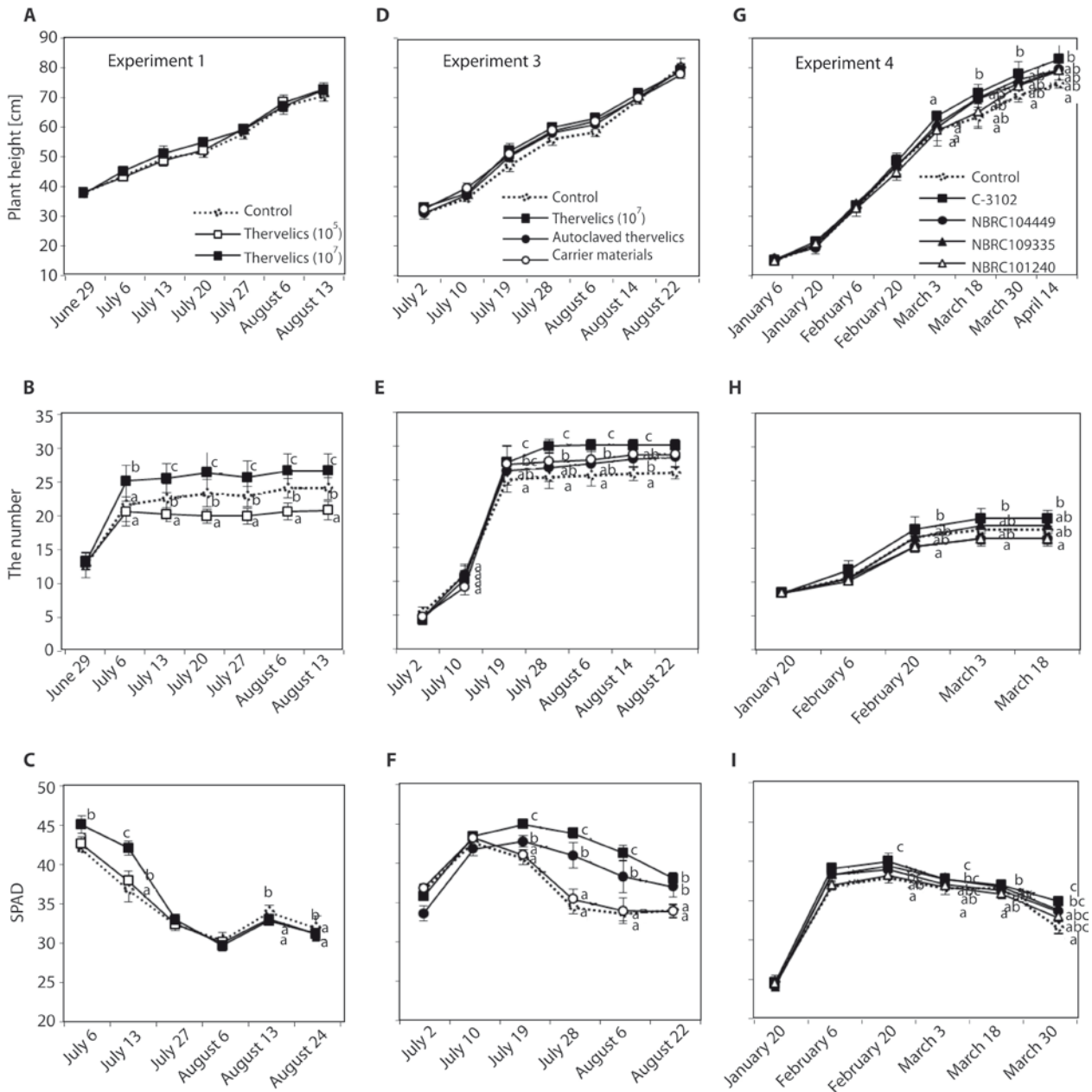
Fig. 1. Plant heights (A, D, G), tiller numbers (B, E, H) and SPAD values (C, F, I) for rice plants throughout the growing season in Experiments 1, 3 and 4; means ± SD; different letters in each sampling date indicate significant differences ($p < 0.05$); no letters indicate no significant differences among treatments

2×10^4 in the control (Fig. 2A). In root at harvest, *B. subtilis* C-3102 was detected at 3.0×10^3 cfu · g⁻¹ root only in the treatment with the mixture of 10^7, and its density in the other treatments was less than the detection limit (1.0×10^3 cfu · g⁻¹ root).

Effects of the *Bacillus subtilis* C-3102 mixture on barley growth (Experiment 2)

In barley, the dry matter of both straw and grain was higher ($p < 0.05$) by 10.3% and 17.7%, respectively, in the treatment with the mixture of 10^7 than in the other treatments (Table 3). *Bacillus subtilis* C-3102 was

detected in soil at 83×10^4 and $1,000 \times 10^4$ cfu · g⁻¹ soil at harvest in the treatments with the mixture of 10^5 and 10^7 and was detected in root at 26×10^4 and 320×10^4 cfu · g⁻¹ root in the inoculated treatments, respectively (Fig. 2B).

Effects of autoclaved and non-autoclaved *Bacillus subtilis* C-3102 mixture on rice growth (Experiment 3)

Plant heights exhibited similar patterns in all the treatments through the growing period (Fig. 1D). The numbers of tillers were the highest ($p < 0.05$)

Table 2. Dry matter production of rice plant tissues (*Oryza sativa* cv. Milkyprincess) and the number of panicle per pot among treatments

Experiment	Treatment	Straw [g · pot⁻¹]	Grain [g · pot⁻¹]	Root [g · pot⁻¹]	No. of panicle [g · pot⁻¹]
1	Control	45.5 ± 3.7 a	26.9 ± 2.5 a	7.3 ± 0.7 a	24.4 ± 1.4 a
	BS mixture (10^5)	47.1 ± 3.0 a	27.7 ± 2.6 a	6.5 ± 0.7 a	21.2 ± 1.5 a
	BS mixture (10^7)	57.9 ± 2.8 b	33.6 ± 3.1 b	8.8 ± 0.9 b	27.5 ± 2.5 b
3	Control	46.3 ± 2.8 a	40.4 ± 4.2 a	9.1 ± 1.5 a	26.9 ± 3.6 a
	BS mixture (10^7)	54.1 ± 1.0 b	56.4 ± 2.5 c	8.9 ± 1.9 a	30.6 ± 1.7 b
	Autoclaved BS mixture	51.7 ± 2.6 b	53.3 ± 2.1 bc	10.6 ± 3.7 a	30.4 ± 2.2 b
	Carrier materials	53.1 ± 3.1 b	52.7 ± 2.3 b	9.9 ± 1.0 a	30.6 ± 1.7 b
4	Control	16.4 ± 3.5 a	5.2 ± 0.1 b	2.2 ± 0.4 a	11.4 ± 1.3 bc
	C-3102	20.3 ± 1.0 a	7.0 ± 0.5 c	3.0 ± 0.4 a	13.2 ± 1.3 c
	NBRC104449	16.1 ± 1.8 a	5.0 ± 0.3 ab	2.6 ± 0.4 a	9.0 ± 1.2 a
	NBRC109335	17.0 ± 1.7 a	4.5 ± 0.2 a	2.6 ± 0.3 a	10.4 ± 0.5 ab
	NBRC101240	17.3 ± 2.8 a	4.5 ± 0.3 a	3.0 ± 0.7 a	11.0 ± 1.0 ab

BS – *Bacillus subtilis*; experiments 1 and 3 with 0.05 m² pots: $n = 10$, except for root ($n = 6$ in Experiment 1, $n = 3$ in Experiment 3); Experiment 4 with 0.02 m² pots: $n = 5$; (10^5) and (10^7) indicate the initial inoculation levels (cfu · g⁻¹ soil) of BS mixture; means ± SD; different letters each column indicate significant differences ($p < 0.05$)

Table 3. Dry matter production of barley tissues (*Hordeum vulgare* cv. Sachiho Golden) per 0.05 m² pot among treatments

Treatment	Straw [g · pot⁻¹]	Grain [g · pot⁻¹]
Control	8.7 ± 1.0 a	13.0 ± 2.1 a
BS mixture (10^5)	8.3 ± 1.3 a	12.5 ± 2.0 a
BS mixture (10^7)	9.6 ± 1.1 b	15.3 ± 2.4 b

BS – *Bacillus subtilis*; $n = 10$; (10^5) and (10^7) indicate the initial inoculation levels (cfu · g⁻¹ soil) of BS mixture; means ± SD; different letters indicate significant differences ($p < 0.05$)

in the mixture treated group in most of the growing period (Fig. 1E). The numbers were significantly ($p < 0.05$) higher in the carrier materials than in the control on July 19, July 28 and August 6, and there was no significant difference between the autoclaved mixture and the carrier materials. One month after transplanting, the SPAD values were the highest ($p < 0.05$) in the mixture (Fig. 1F). The treatment with the autoclaved mixture also showed significantly ($p < 0.05$) higher SPAD values than those in the control and in the carrier materials. The aboveground biomasses were significantly ($p < 0.05$) higher by 16.8%, 11.7% and 14.7% in straws, and 39.6%, 31.9% and 30.4% in grains, respectively, for the treatments treated with the mixture, autoclaved mixture and carrier materials than in the control (Table 2). The treatment with the BS mixture yielded a significantly ($p < 0.05$) higher amount of grain (7.0%) than its carrier materials, but it was not significantly different from the autoclaved mixture. The numbers

of panicles were significantly ($p < 0.05$) higher for the treatments treated with the mixture, autoclaved mixture and carrier materials by 13.8%, 13.0% and 13.8%, respectively. There were no significant differences in root biomass between the treatments.

Bacillus subtilis C-3102 was detected both in soil and root at 170×10^4 and 93×10^4 cfu · g⁻¹, respectively, at harvest in the treatment inoculated with the mixture (Fig. 2C) and its density in soil for the other treatments was detected at 0.3×10^4, 0.5×10^4 and 0.6×10^4 cfu · g⁻¹ soil in the autoclaved mixture, carrier materials and control, respectively. *Bacillus subtilis* C-3102 was detected in root at 1.7×10^4, 2.1×10^4 and 2.0×10^4 cfu · g⁻¹ root in the autoclaved mixture, carrier materials and control, respectively.

The soil protease activity was consistently higher in the non-autoclaved mixture during the 4 weeks after transplanting than in all the other treatments (Fig. 3A). The treatments with the autoclaved mixture and carrier materials also showed higher ($p < 0.05$) protease activity than in the control, except 1 week after transplanting.

Efficacy of *Bacillus subtilis* C-3102 and other *B. subtilis* strains on rice growth (Experiment 4)

The heights of rice plants were higher ($p < 0.05$) in the treatments with *B. subtilis* C-3102 than in the control, while no significant differences were observed between *B. subtilis* C-3102 and the NBRC treatments (Fig. 1G). The numbers of tillers in *B. subtilis* C-3102 and NBRC treatments were not different from those in
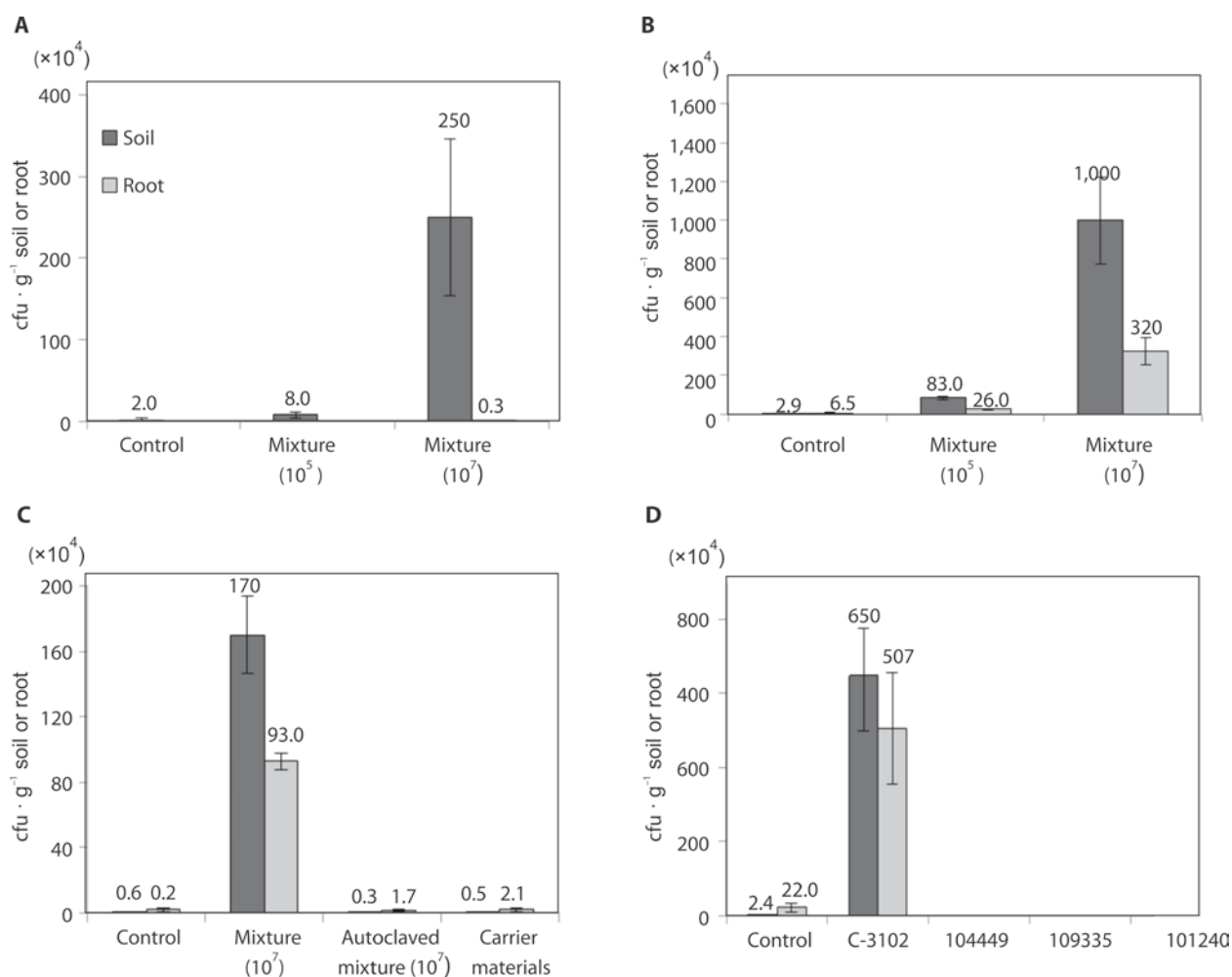
Fig 2. Density of *Bacillus subtilis* C-3102 in soil and rice root at harvest in Experiments 1 (A) to 4 (D); *n* = 3; the numbers above each bar indicate densities, expressed based on unit gram of oven-dried soil (60°C for 2 days) and root at (70°C for 4 days); 104449, 109335, 101240: NBRC104449, NBRC109335, NBRC101240

the control (Fig. 1H). The numbers of tillers were significantly (*p* < 0.05) higher in *B. subtilis* C-3102 than in NBRC104449 and NBRC101240, and the number of tillers were not significantly different between *B. subtilis* C-3102 and NBRC109335. One month after transplanting, the SPAD values were significantly higher (*p* < 0.05) in *B. subtilis* C-3102 than those in the control and NBRC101240, while no significant differences were observed between *B. subtilis* C-3102 and the other two NBRC treatments (Fig. 1I). NBRC109335 and NBRC104449 also showed higher (*p* < 0.05) SPAD values than in the control. The dry matter of straw was higher by 23.8% in *B. subtilis* C-3102 treatment than in the control, although there was no significant difference. The treatment with *B. subtilis* C-3102 yielded the highest amount of grains (Table 2) with statistical significance (*p* < 0.05). The dry matter of straw and grain was not markedly different between the other three *B. subtilis* strains and the control. Root biomass was higher by 36.4% in *B. subtilis* C-3102 than in the control, although there was no significant difference. The number of panicles was 15% higher in the treatment

with *B. subtilis* C-3102 than in the control, although there was no significant difference. The density of *B. subtilis* C-3102 in soil at transplanting was 14×10^4 although its inoculation density was $1,000 \times 10^4$, indicating that a majority of *B. subtilis* C-3102 cells were in a vegetative stage and were killed by the heat treatment. Its density in the control was 2.4×10^4. *Bacillus subtilis* C-3102 was detected both in soil and root at 650×10^4 and 507×10^4 cfu · g⁻¹, respectively. at harvest in the inoculated treatment (Fig. 2D), while its density in the control was as low as 2.4×10^4 and 22×10^4, respectively. Two weeks after transplanting, the soil protease activities were significantly (*p* < 0.05) higher in the inoculated treatments than in the control (Fig. 3B), while it was consistently increased in both *B. subtilis* C-3102 and NBRC109335 treatments during the first month after transplanting but after 6 weeks its activity was significantly (*p* < 0.05) higher in *B. subtilis* C-3102 than in NBRC109335, though both of the strains produced significantly higher protease activity than those of the other NBRC strains based on the *in vitro* tests.
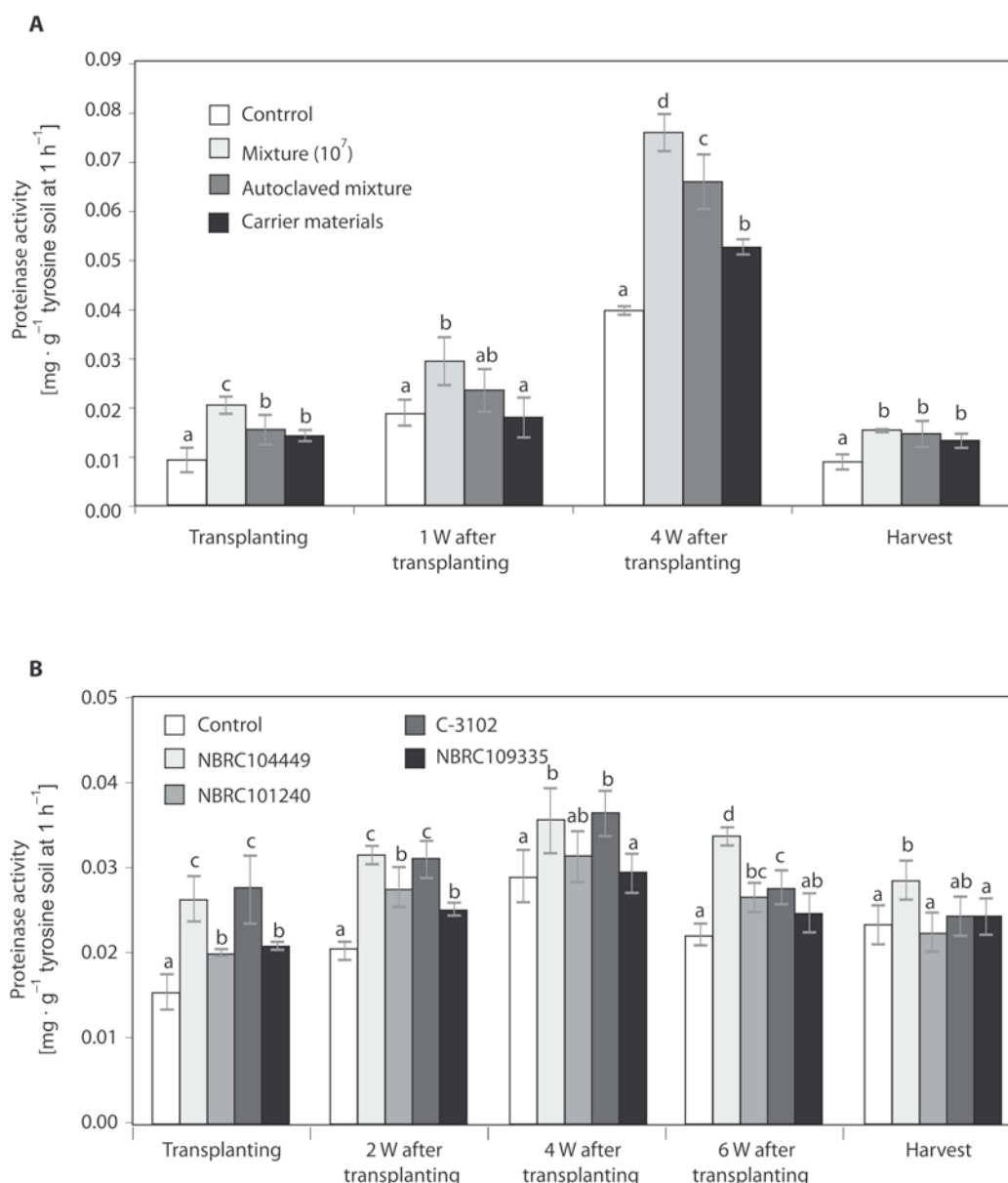
A

B

Fig. 3. Periodical change in soil protease activity in Experiments 3 (A) and 4 (B); W = weeks; different letters in each period indicate significant difference ($p < 0.05$); each bar indicates means ± SD ($n = 3$) of soil protease activity, expressed based on unit gram of oven-dried soil at 60°C for 2 days

Discussion

The present study demonstrated a 25% (Experiment 1) increase in the grain production of rice by the inoculation of *B. subtilis* C-3102 at a density of 10^7 cfu · g^{-1} soil. This increased grain production was caused by higher numbers of grain due to higher tiller and panicle numbers which were supported by higher photosynthetic activities represented by higher SPAD values. Enhanced plant growth was also observed in barley (Experiment 2), in which grain production was increased by 18% by inoculating *B. subtilis* C-3102 at a density of 10^7 cfu · g^{-1} of soil. Both in rice and barley, enhanced growth was not observed in the

B. subtilis C-3102 treatment at an inoculation density of 10^5 cfu · g^{-1} soil. *Bacillus subtilis* C-3102 was detected at transplanting almost at the calculated values (0.5×10^7 and 1.4×10^5 cfu · g^{-1} for the treatments of 10^7 and 10^5). Inoculated *B. subtilis* C-3102 survived in the soil even at harvest, ranging from 8×10^4 to 250×10^4 cfu · g^{-1} in Experiment 1 and from 83×10^4 to 1000×10^4 cfu · g^{-1} in Experiment 2. In Experiment 1, *B. subtilis* C-3102 was detected in root at harvest only in the treatment 10^7 and by more than ten times at the higher inoculation than at the lower inoculation in root in Experiment 2. Thus, it was considered that higher colonization of soil and root by *B. subtilis* C-3102 caused greater dry matter production of rice and barley. In this study, the density of

B. subtilis C-3102 was measured by the dilution plate method using heat-treated soil or root suspensions, indicating that only spores were counted and the vegetative cells were not counted. Next, colonies with the same appearance as *B. subtilis* C-3102 were picked up and their identification was confirmed by colony PCR, based on the findings that only *B. subtilis* C-3102 strain out of 18 *B. subtilis* strains tested reacted to both species primer sets to *B. subtilis* and *B. amyloliquefaciens*. In non-inoculated treatments, colonies with both positive reactions in the PCR assay were markedly and consistently low compared with the treatments inoculated with *B. subtilis* C-3102. Thus, it was considered that the present method suitably quantified the number of *B. subtilis* C-3102 although we cannot deny the possibility of underestimating the number of live *B. subtilis* C-3102 cells.

Inoculation of *B. subtilis* C-3102 was done using a commercial product which consists of $CaCO_3$ (73% of the total weight), soybean extract (a culture medium) (27%) and living cells. Inoculation of *B. subtilis* C-3102 at 10^7 cfu · g^{-1} of soil indicates that 1 g of $CaCO_3$ and soybean extract is added to 1 kg soil and therefore, its neutralizing and nutritional effect must be considered. Then, the growth promotion effect of the *B. subtilis* C-3102 mixture, autoclaved mixture and carrier materials were compared (Experiment 3). The results showed that the addition of both the autoclaved mixture and the carrier materials significantly increased the number of panicles and dry matter production of straw and grain, suggesting that the carrier materials have a rice growth promoting effect possibly through neutralizing and nutritional effects. In addition, the SPAD values were significantly higher during the period from July 10 to and August 22 in the autoclaved mixture than in the carrier materials, suggesting that the dead cells might have some effect on the plant physiology. Dry matter production of grain was the highest in the live mixture, although a significant difference was observed only between the mixture and the carrier materials, not between the live mixture and autoclaved mixture, suggesting that the living cells might have an additional growth promoting effect, supported by the result that tiller numbers and SPAD values were significantly the highest in the live mixture from July 19 to August 6. To evaluate the effect of the living cells of *B. subtilis* C-3102, Experiment 4 was carried out by inoculating only the cultured cells into soil after removing the culture medium. The results showed that the inoculation of *B. subtilis* C-3102 increased the dry matter production of straw and grain by 24% and 35%, respectively, confirming the growth promoting effect of the living cells.

Many papers have reported enhanced plant growth by bacterial inoculation into soil (Kumar *et al.* 2012a; Habibi *et al.* 2014; Qiao *et al.* 2014; Islam *et al.* 2016).

Nitrogen fixing and P solubilizing abilities were involved in plant growth promoting mechanisms by the *Bacillus* strains in the studies, suggesting that a nutritional supplementary effect, in particular N, may be a key factor. In this study, SPAD values, an indicator of N-use efficiency (Cabangon *et al.* 2011), were significantly increased in the early stages in Experiment 1, the middle to late stages in Experiment 3 and throughout all of Experiment 4 by the inoculation of *B. subtilis* C-3102, indicating better N status in the rice plants by *B. subtilis* C-3102. Although a compound fertilizer consisting of N, P and K was added in this study, extra N nutrition might be provided by the inoculation. Since *B. subtilis* C-3102 has no N fixing ability, a plausible explanation is that the soil protease activity was significantly increased by the inoculation throughout the growing periods of Experiments 3 and 4. The strain NBRC109335 with the second highest protease activity also increased the soil protease activity by a similar degree as *B. subtilis* C-3102 in the early stage, but not 6 weeks after transplanting. Increased soil protease activity by *B. subtilis* C-3102 throughout the growing period might provide more available N to plants. Both the autoclaved mixture and the carrier material increased the soil protease activity in Experiment 3 except for 1 week after transplanting. In general, soil protease activity is the highest at neutral pH (Ladd and Butler 1972) or active in neutral to alkaline pH ranges of soil (Sanghavi *et al.* 2016; Sharma *et al.* 2017) and thus $CaCO_3$ included in the *B. subtilis* C-3102 mixture might enhance soil protease activity, through which dry matter production of grain might be increased.

No phosphate solubilizing ability was observed in *B. subtilis* C-3102 and no difference was observed in the soil of available phosphate content between inoculated and non-inoculated soils in Experiment 1, suggesting that phosphate availability is not involved in the plant growth promotion effect by *B. subtilis* C-3102.

Bacillus subtilis C-3102 produces IAA, a well-known phytohormone to enhance plant growth, and thus this property might also be involved in plant-growth promotion by the strain. This hypothesis was tested using two *B. subtilis* strains (NBRC109335 and NBRC104449) with similar IAA producing abilities. The results showed no plant growth promoting effect in the dry matter production of straw and grain by the strains and thus IAA production may contribute little to the plant growth promoting effect of *B. subtilis* C-3102, although a possibility remains that IAA is effective, but NBRC109335 and NBRC104449 failed to colonize soil and root. In this study, *B. subtilis* C-3102 had the unique colony appearance and specific reactions in the PCR assay, making it possible to count the number of this strain specifically. But the NBRC strains have no such property and thus it was impossible to evaluate their colonization abilities in this study.

Siderophore production is positive in *B. subtilis* C-3102 and its possible contribution to plant growth promotion has been reported (Chen *et al.* 2010; Yu *et al.* 2011; Kumar *et al.* 2012b; Lin *et al.* 2014). In this study, no growth promoting effect was observed by inoculating either a high siderophore producing strain NBRC104449 or a no siderophore producing strain NBRC101240. Fe deficiency does not occur under flooded conditions in general, because the solubility of Fe^{2+} increases with reduced conditions (Robin *et al.* 2008) and thus siderophore producing ability will contribute little to growth promoting effects of rice grown under flooded conditions.

In this study, four parameters were focused on as possible causes of plant growth promoting effect by *B. subtilis* C-3102: protease, IAA, phosphate solubilization and siderophore. This study suggested that the largest contribution was protease activity. However, other features of *B. subtilis* C-3102 might be involved in growth promotion. According to a study dealing with *B. subtilis* C-3102 as a feed additive for broilers, *B. subtilis* C-3102 reduced the number of pathogenic bacteria, such as *Salmonella*, *Campylobacter* and coliforms, and increased the body weights of broilers (Fritts *et al.* 2000). *Bacillus subtilis* C-3102 also suppressed the growth of Gram-positive soil bacterium, *Arthrobacter globiformis*, on nutrient agar medium (data not shown). It has been frequently reported that deleterious rhizobacteria (DRB) decrease plant growth (Suslow and Schroth 1982; Suarez *et al.* 2014). For example, co-inoculation of sugar beet seed with strains of PGPR and DRB resulted in the inhibition of DRB colonization in roots, and thus increased plant growth (Suslow and Schroth 1982). Therefore, the suppression of DRB might be another cause of plant growth promotion by C-3102.

In conclusion, this study demonstrated that *B. subtilis* C-3102 promoted growth of both rice, cultivated under flooded conditions, and barley, cultivated under upland conditions. The plant growth promoting effect was likely due to higher soil protease activity brought by the strain. However, further study is needed to elucidate the detailed molecular mechanism of plant growth promotion and field tests are required for future application, including other crops.

Acknowledgements

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