Summary of Doctoral Thesis

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Title	Compatible bioinoculants for improved plant growth-promotion

Introduction and purpose

Crop productivity is an answer to the increasing demand for food to feed the inevitably rising population. But, in order to reach and maintain high yields, plants should be provided with the necessary nutrients for their growth and development. In this regard, artificial sources of nutrients commercially packed as fertilizers that are specific for plant growth promotion have been extensively utilized, which usually results in the excessive nutrient accumulation in soil, thus, leading to environmental pollution. Because of this, farmers have resorted to alternative agricultural practices such as the application of bioinoculants containing beneficial microorganisms (*i.e.* plant growth promoting bacteria and mycorrhiza) in order to provide the hardly acquired moisture, minerals, and nutrients for plant nutrition without imposing harm to the environment.

Bioinoculants may be formulated with only one or a variety of strains that are proven to deliver positive results to plant growth. However, the efficiency of bioinoculants in plant growth promotion greatly depends on factors such as host specificity, biochemical productivity, stress tolerance, and compatibility especially for those containing a consortium of different strains. This study confirmed that the four new bacterial strains isolated from potato roots (R170, R181, R182) or tubers (T168) are efficient plant growth promoters either as single inoculants, in co-inoculation with each other (R170 with T168 or R182) or with mycorrhiza (+R170 or +R182). Thus, this study aimed to contribute to the development of reliable and efficient bioinoculants for the ultimate improvement of the growth of potato seedlings.

Materials and methods

Microbial strains and medium

Nineteen representative strains with high affinity for potato roots or tubers (cv. Matilda, one of the common varieties in Hokkaido, Japan) isolated previously (Someya *et al.* 2013), were studied for their plant growth promotion (Kenkyuseika, vol. 539. 2015), and the four best PGPB strains, namely, *Sphingomonas* sp. T168 (accession number AB730532), *Streptomyces* sp. R170 (AB730341), *Streptomyces* sp. R181 (AB730352), and *Methylibium* sp. R182 (AB730353) selected among them were used throughout the experiment. Strains T168, R170, R181, and R182 belong to AP6, AC4, AC1, and BP12 of OTU-group shown in previous study (Someya *et al.* 2013), respectively. All strains were grown in R2A medium.

Mycorrhizal spores were isolated from a soil inoculant (*MYKOVAM*®) provided by the National Institute of Molecular Biology and Biotechnology of the University of the Philippines Los Baños. The isolation of mycorrhizal spores from *MYKOVAM*® that contains 8 different species, was conducted following the standard methods of wet sieving and decanting as reported by Gerdemann and Nicolson (1963). Among the spores isolated, only a single inoculum, which has been previously identified as *Gigaspora margarita* (Aggangan *et al.* 2013) and verified in this study by morphological characterization following the manual of Schenck and Perez (1990), was cultured in a soil:sand medium using wheat (cv. Kitahonami) as host plant, as well as in MSR agar medium prepared according to the methods reported by Declerck *et al.* (2005). Prior to the *in vitro* propagation of mycorrhiza on MSR agar, spores were carefully disinfected by chloramine T solution (containing 2–3 drops of Tween 20), streptomycin, and gentamycin following the methods reported by Mertz et al. (1979).

Assessment for biochemical and enzyme activities

Bacterial production of indole-3-acetic acid (IAA), siderophore, and biofilm in single and combined inoculations were determined following the Salkowski (Gopalakrishnan *et al.* 2012), Chrome-Azurol S (CAS) agar diffusion (Shin *et al.* 2001), and microtiter plate (Yuttavanichakul *et al.* 2012) assays, respectively. Whereas, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, cellulase, protease, lipase, and chitinase activities were verified in single strain inoculation following the methods reported by Penrose and Glick (2003), Crabbe *et al.* (1994) Bhattacharya *et al.* (2009), and O'Brien and Colwell (1987), respectively. IAA production of bacterial cells was determined by optical density at 530 nm. Siderophore, cellulase, protease, lipase, and chitinase production activities were evaluated by halo formation on agar medium. The amount of biofilms produced was determined by obtaining absorbance values of biofilms left by loosely associated bacteria at 595 nm, while the ACC-deaminase activity was determined by the production of α-ketobutyrate (AKB) generated by the cleavage of ACC expressed as nmol AKB (mg wet weight of cell)$^{-1}$ per hour.

Mycorrhizal phosphatase and siderophore production was tested for single and combined inoculation with PGPB following the methods reported by Tabatabai (1994), and Scwhyn and Neilands (1987). Mycorrhiza acid and alkaline phosphatase activities in roots were calculated from the standard curve of *p*-nitrophenol using absorbance values measured at 450 nm.

Evaluation of the bacterial strains for stress tolerance

Bacterial strains were cultured in R2A broth in L-tubes adjusted to different levels of $AlCl_3$ (0.0001%- 1%, w/v), NaCl (1.0%- 5.0%, w/v), pH (4.0- 10.0) and temperature (10° - 40° C), and the growth was monitored at OD_{660} by a biophotorecorder. For the aluminum, salts, and pH stress test, each culture was incubated with shaking at 30 rpm for 120 hr at 30° C, and for the temperature stress test, each culture was incubated at temperature between 10 and 40° C under the same conditions as those used for the shaking and incubation periods mentioned above.

Microbial inoculation and evaluation for plant growth promotion

To evaluate the compatibility of strains in formulating a bioinoculant for plant growth promotion, we focused on the combination of R170, which exhibited the highest potential as a bioinoculant with T168 or R182, with a parallel combination using R181 instead of R170. We directly inoculated single (1 mL) and combined cell suspensions (2 mL; 1 mL from each strain) on potato seeds derived from the open pollination of cv. Hokkaikogane sown on pots containing approx. 100 g of sterilized seedling-raising culture soil. Pots were covered with aluminum foil and placed in a growth chamber under light and dark conditions. The cover was removed upon the germination of seeds, and 30 days after the inoculation, the plant growth parameters such as the plant weight (mg) and germination rate were measured. The plant dry weight was measured after oven drying at 60° C for 3 days.

Mycorrhiza (1 mL) and bacterial (1 mL) suspensions containing approx. 100 spores and 1 x 10^8 CFU, respectively, were directly inoculated on potato seeds sown on pots containing approx. 100 g of sterilized soil. Controls were either inoculated with 1 ml of mycorrhizal spore suspension or not inoculated. Pots were covered with aluminum foil and placed in a growth chamber under light and dark conditions. The cover was removed upon the germination of seeds, and 30 days after the inoculation, the plant growth parameters such as plant weights (mg) and germination rates were measured. Plant dry weights were measured after oven drying at 60° C for 3 days.

Localization of co-inoculated PGPB to plant roots

14-days-old potato seedlings were grown on plant agar (0.3%) containing a 500-times dilution of HYPONeX® 6-10-5 and inoculated with a single or combined PGPB. The tissue localization of each strain in a combination in the plant roots was examined by GUS and/or Gram staining. For the plants inoculated with GUS-marked strains, bacterial cells were stained by immersing plant samples in a GUS-staining solution with continuous deaeration in a desiccator connected to a vacuum pump for 30 min, and then allowed to incubate on plants at 30° C for 3 days. Gram staining was done according to the instructions of Favor G "*Nissui*" (Nissui Pharmaceutical Co., Tokyo) with few modifications.

Briefly, plants inoculated with strain R170 or R181 were soaked in Victoria blue solution for 1 min, washed with distilled water, submerged in a destaining reagent for 5 min, and then washed again with distilled water to remove excess stain. The tissue localization of bacteria in the plant roots was examined in 70% glycerol under a light microscope (IX70 Inverted Microscope, Olympus, Tokyo) until 28 days after the inoculation. Photomicrographs were taken using a high-sensitivity CCD camera.

Mycorrhiza root infection and spore germination

Potato seedlings were inoculated with either mycorrhiza alone or with PGPB. Mycorrhizal inoculant was prepared by suspending the spores harvested from MSR agar to sterilized distilled water. For mycorrhiza, 100 µL of spore suspension containing approx. 10 spores were directly inoculated to the roots, whereas for mycorrhiza with PGPB, 200 µL (1:1 of mycorrhiza and PGPB) were inoculated. The concentration of bacteria used was $1x10^8$ CFU per ml. Twenty eight days after inoculation, mycorrhiza root infection was assessed following the grid-line intersection method (Giovannetti and Mosse. 1980). Prior to microscope observation, roots were cleared of cell contents following the manual instructions of SUNJin Lab using *RapiClear*® 1.55 (Funakoshi Co. Ltd., Tokyo), immediately followed by staining using 0.05% trypan blue in glycerol and incubated for 24 hrs at 30°C, and then de-stained with 85% lactic acid. Root infection was assessed by the presence of either one of the following structures: hyphae, vesicles, arbuscules, and spores which were visible after staining. The number of infected and uninfected roots that intersect gridlines were counted. Percent mycorrhizal infection was calculated by the ratio of the total number of roots infected against the total number of roots.

For the assessment of spore germination, mycorrhizal cultures on MSR agar were treated with the 4 PGPB strains each having cell concentrations of $1x10^8$ CFU ml^{-1}. From an initial spore density of 8, the total number of spores on MSR agar was counted after 14 days of incubation. Percent spore germination was calculated by getting the ratio of the final spore density (final number of spores – initial number of spores) against the initial spore density.

Cross-streak test between co-inoculated PGPB strains

Each of the co-inoculated PGPB was grown in R2A agar medium at 30° C for at least 3 days and then streaked perpendicularly on freshly prepared R2A agar medium; *i.e.*, after the first strain was allowed to grow at 30° C for 3 days, the second strain was streaked at an angle of approx. 90° going outward from the emerged colonies of the first strain. The second colony was allowed to incubate at 30° C for another 3 days. Strain combinations that showed inhibition zones at the intersection of the paired strains were noted.

Ability of mycorrhiza to biologically control a pathogen

The biological control activity (BCA) of mycorrhiza co-inoculated with PGPB against a pathogen was performed according to the methods reported by Schelkle and Peterson (1997) with the following modifications: mycorrhizal plugs (6 mm dia.), taken from the periphery of growing colonies on MSR agar, were placed at the edge of a petri plate containing a mixture of MSR and PDA (1:1) buffered with 3.3% 2-(N-morpholino)ethanesulfonic acid (MES), and then incubated in the dark at 27°C for 12 hrs. Subsequently, plugs (6 mm dia.) of *Rhizoctonia solani* (MAFF no. 305250) maintained on potato dextrose agar (PDA) were placed on the opposite side of a 12-hr culture of mycorrhiza concomitantly inoculated with 100 µL of bacterial solution. For the control, 100 µL of 0.1 M $MgSO_4$ ●$7H_2O$ was inoculated to the mycorrhiza alone. All treatments were replicated three times. After 14 days of incubation in the dark at 27°C, straight line measurements of inhibition zones between the two growing colonies were performed.

Statistical analysis

All experiments were performed with a minimum of three replicates for each treatment. The statistical analysis was carried out using SPSS Statistics for Windows v.22.0. Data were either subjected to an analysis of variance (ANOVA) or Student's t-test. A post-hoc comparison of mean values among treatments was performed using Tukey's honestly significant difference (HSD) test at

the 5% confidence level.

Results and Discussions

PGPB as potential bioinoculants

Results confirmed that the four PGPB strains with high affinity for potato roots had both the ability to produce plant growth-promoting substances as well as tolerance to environmental stress, and that these strains could be potential bioinoculants. In fact, the inoculation test with these bacteria (T168, R170, R181, and R182), showed significant increases in the dry weight of potato seedlings compared with the control.

In the evaluation of potential bioinoculants, strain R170 was identified to be the most suitable candidate due to its ability of producing the highest level of important plant growth promoting substances (IAA, siderophore, biofilm and ACC deaminase) among the four PGPB, and capability of producing hydrolytic enzymes (β-1,3-glucanase, cellulase, protease and lipase).

The usefulness of R170 as a potential bioinoculant may also be evidenced by its higher tolerance against NaCl and $AlCl_3$, and growth in a wider range of pH compared with the other three strains. However, the efficiency of a single-strain bioinoculant could not be confirmed unless it is applied in the field and exposed to many external factors and its interaction with other soil microorganisms.

Mycorrhiza as bioinoculant

Mycorrhizal strains (*Gigaspora margarita*) isolated from a commercial soil inoculant from the Philippines (*MYKOVAM*®) was confirmed to infect the roots of a local cultivar of potato seedlings grown in Japan (cv. Hokkaikogane) and effective in promoting its growth. The enhanced growth of seedlings may be attributed to the ability of mycorrhiza to solubilize insoluble P in the medium and provide them to plants for absorption which is associated with the production of phosphatases (Schachtman *et al.* 1998, Smith *et al.* 2011, Zheng *et al.* 2011). This was manifested by the higher acid phosphatase (AP) activity of mycorrhiza in the roots compared with the uninoculated control, which can be related with the significantly heavier fresh and dry weight of seedlings inoculated with mycorrhiza in reference to the uninoculated control.

Another evidence that demonstrates the growth promotion of potato seedlings may be the production of mycorrhizal siderophores which might have probably enhanced the iron acquisition in the plants (Aliasgharzad *et al.* 2009). Nevertheless, prior to the execution of plant growth promoting activities, microbial inoculants should have a stable infection to the host plant in order to secure an easy exchange of nutrients between the symbionts. In this regard, the high root infection of mycorrhiza reported in this particular study may be related with the viability of the spores revealed by their capacity to germinate. However, sporulation was only confirmed on an artificial medium (MSR agar), thus, the viability of spores that are already associated with the roots should be examined in further study.

Moreover, it has been reported that mycorrhiza can serve as a biological control agent against harmful pathogens (Schelkle and Peterson, 1997; Tahat *et al.* 2012). In our study, mycorrhiza demonstrated this ability confirmed by the production of the inhibition zones between mycorrhiza and *R. solani*. The manifested deformation and degradation of the pathogen's hyphae shown on dual culture on agar indicated that mycorrhiza can effectively suppress the growth of *R. solani.*

Hence, the inhibition of *R. solani* by mycorrhiza may also be attributed to the siderophore activity of mycorrhiza which may have deprived the pathogen's nutrition because mycorrhiza have already efficiently utilized the nutrients from the medium (particularly iron), thus, leaving the pathogen with less nutrients to use. Moreover, the utilization of microbial siderophores is specific only to the microbes that produce them (Schelkle and Peterson, 1997).

Therefore, our results confirmed that mycorrhiza can be an effective bioinoculant for potato seedlings (cv. Hokkaikogane), manifested by its efficient infection to the roots, acid phosphatase and siderophore production activities, resulting in the significant increase in plant growth.

Compatible strains for plant growth promotion

Our results proved that the compatibility between strain inoculants is crucial for enhancing plant growth. This was confirmed by the synergistic interaction between compatible PGPB strains, as well

as with the co-inoculation of PGPB with mycorrhiza which was primarily evidenced by the significant increase in the weights of potato seedlings (R170 with T168 or R182; R170 with mycorrhiza) compared with the uninoculated control and with the single strain inoculation. This improvement in plant growth can be related with the enhanced biochemical productivity of co-inoculated strains which may be attributed to the ability of strains to complement each other's plant growth-promoting (PGP) activities. The enhanced PGP activities of co-inoculants were made possible by the efficient infection and co-existence of strains to the host plants which were specifically observed in the combination of R170 with T168 or R182 showing co-localization of strains to the host plant. Likewise, R170 was proven to advance the infection of mycorrhiza to the roots of potato seedlings which may be related with the enhanced spore germination on MSR agar upon the co-inoculation of R170. Whereas, the low mycorrhizal root infection for T168 or R181 co-inoculations may be due to the decreased capacity of mycorrhizal sporulation on MSR agar. Thus, the localization of PGPB with mycorrhiza was not examined. Further investigations are necessary.

Synergistic interaction between co-inoculants has also been evidenced by the improved IAA and siderophore productions noted for the inoculation of R170 with T168 or R182. Probably, the improved production of IAA and siderophores in combined strains inoculation could be due to the increased cell number and/or producing activity of either or both bacteria. Thus, further investigations are needed.
Similarly, with the co-inoculation of R170 and mycorrhiza, a significant improvement in the mycorrhizal AP activity was recorded, whereas a synergistic increase on the siderophore production was observed. The enhanced plant growth promotion may be brought about by the increased AP activity of mycorrhiza. Thus, AP activity may be attributed to the improved root infection of mycorrhiza after the co-inoculation of PGPB. In contrast, the improved production of microbial siderophores may be due to the synergistic effect of co-inoculating R170 with mycorrhiza, and hence, may imply an enhanced iron acquisition in plants resulting in improved plant growth.

While the compatibility of R170 with T168 or R182 was verified by the lack of inhibition on dual culture, which resulted in the co-localization of strains on the roots, the incompatibility of R181 with T168 or R182, on the other hand, could be attributed to the growth competition of R181 against other bacteria, manifested by the inhibition of T168 at the base of lateral roots and a weakened infection of R182 to the root hairs. Furthermore, the dominance of R181 over T168 or R182 was manifested by the presence of inhibition zones at the intersection of two colonies, which may be related with the production of toxins in the hyphae of R181. Thus, a thorough assessment on this assumption for R181 is recommended for further study.

Reports showed that mycorrhizas can serve as biological control agents against harmful pathogens (Schelkle and Peterson, 1997; Tahat *et al.* 2012). In the same manner, our study confirmed that mycorrhiza can suppress the growth of *R. solani* which can be enhanced by the co-inoculation of R182, R170, and T168. Biological control was demonstrated by the inhibition zones produced between mycorrhiza and *R. solani*. In this regard, the hydrolytic enzymes present in PGPB, which include β-1,3-glucanase (T168, R170, R181, R182), cellulase (R170, R181, R182), protease (R170, R181, R182), chitinase (R182), and lipase (R170) that are known for cell wall degrading properties (Kubicek *et al.* 2014), may have improved the biological control activity of mycorrhiza. Additionally, Schelkle and Peterson (1997) cited that the suppression of pathogens may also be due to the competition for nutrients and space in the rhizosphere which is also related with the siderophore production of PGPB and mycorrhiza as well.

Conclusion and consideration

With the results shown above, we can therefore conclude that the compatibility of strains in combined inoculation is important in promoting plant growth. Particularly, strain R170 proves to be a promising strain (particularly in combination with other compatible strains such as T168, R182, or mycorrhiza) for the formulation of efficient bioinoculants for potato seedlings. Furthermore, the use of R170 with compatible strains may eventually reduce the utilization of chemical fertilizers.

The potential of R170 as bioinoculant for the growth promotion of potato seedlings was evidenced by its biochemical productivity, tolerance to environmental stresses, and synergistic effect with compatible PGPB and mycorrhiza strains.

Our results hope to provide additional information that will contribute to the development of potential bioinoculants containing effective formulations of compatible strains for plant growth promotion which might possibly reduce the utilization of chemical fertilizers.

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