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RESEARCH ARTICLE

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Characterization of Soil Microorganism from Humus and Indigenous Microorganism Amendments

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ABSTRACT

This study was conducted to understand the dynamics of microbial communities of soil microorganisms, and their distribution and abundance in the indigenous microorganisms (IMOs) manipulated from humus collected from the forest near the crop field. The soil microorganisms originated from humus and artificially cultured microbial-based soil amendments were characterized by molecular and biochemical analyses. The bacterial population ($2 \times 10^6 \sim 13 \times 10^6$ CFU/g sample) was approximately 100-fold abundant than the fungal population ($2 \times 10^4 \sim 8 \times 10^4$ CFU/g sample). The 16S rDNA and ITS sequence analyses showed that the bacterial and fungal communities in humus and IMOs were mainly composed of *Bacillus* and *Pseudomonas*, and *Trichoderma* and *Aspergillus* species, respectively. Some of the bacterial isolates from the humus and IMOs showed strong inhibitory activity against soil-borne pathogenic fungi *Fusarium oxysporum* and *Sclerotinia sclerotiorum*. These bacteria also showed the siderophore production activity as well as phosphate solubilizing activity, which are requisite traits for biological control of plant pathogenic fungi. These results suggest that humus and IMOs could be a useful resource for sustainable agriculture.

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1. Introduction

Understanding the dynamics of the soil microorganism community is important to know their distribution, structure, and abundance. Furthermore, knowing about their arrangement and shape in structure and response of changing depending on time and space is also considered to be an important aspect [1]. Soils have a unique property of accumulating heavy metals and minerals in itself and these properties vary from type to type. The key factor to this property is the soil organic matter which shows its ability in the release, retention, and availability of heavy metals [2]. In the present time, the safe and protective environment is the foremost importance to all mankind. That is why scientists are trying to develop a kind of technology that can be readily naturally available for bringing improvement and enhancement in agricultural management. Indigenous microorganisms (IMOs) are generated from humus collected from the forest near crop field [3] and are considered as one of the great technologies applied for sustainable agriculture named as Korean Natural Science Farming [3,4]. The IMOs are mostly applied for sustainable agriculture for artificially fortified microbial soil amendments in the Asian countries like Korea and Japan. It is also widely practiced in Hawaii, USA [5]. The use of IMOs to get economic, social, and environmental benefits is inherently attractive and is a spectacular evolution from traditional technologies to modern techniques to provide an efficient way to protect the environment and new methods of environmental monitoring [6]. IMOs are a group of an innate microbial consortium that inhabits the soil and the surfaces of all living things in the soil having the potentiality in biodegradation, bioleaching, biocomposting, nitrogen fixation, improving soil fertility, and the production of plant growth hormones. Humus and IMOs could be very diverse and microbe-rich amendments for agricultural soil.

As microbes are the carbon and nutrient mediators and, by reacting with abiotic conditions in the soil, they result in the formation of humus [7]. The fixation of nitrogen in the plant root zone and the plant cellular metabolism in photosynthesis and respiration are also affected by humus. Previous reports have suggested that microbes have the ability

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to promote plant growth through their microbial products that are mostly secreted by rhizospheric microbes [8]. Such type of bacteria is known as plant growth-promoting rhizobacteria (PGPR) or plant growth-promoting bacteria (PGPB) [8]. Most of the soil microbes are still unidentified, and their function is not well known, although the analysis of metagenomic sequencing reveals microbial identity and few functional genetic information. With a wide variety of physiological conditions, molecular interactions between different microbial species and their environments are strongly influenced by the fate of soil carbon, but still, the details about these interactions of the microorganism are largely unknown [9].

In terms of agriculture purposes, PGPR could be readily used as bio-fertilizers which are alternatives to many chemical fertilizers and pesticides that are causing harm to the environment. This can also help to get new insights for enhancing plant growth and yield by suppressing disease [10]. The beneficial microbes which are associated with the root zone, develop biofilms to increase plant growth [11]. Application of PGPR as bio-fertilizer or bio-pesticides can enhance the availability of nutrients in rhizospheres to plants and can suppress the activity of disease-causing soil-borne pathogenic fungi and other harmful biotic factors that cause harm to plants. With the use of environment-friendly organisms, plant growth can become better and the plant pest defense system can be induced [12].

PGPR plays a significant role in reducing the biotic and abiotic stress either by direct or indirect relationships with plant roots. The ultimate source of biotic stress on plants are mostly the microorganism like the microorganisms, including viruses, bacteria, nematodes, arachnids, weeds, etc. Additionally, they are also involved in causing the losses of pre- and post-harvest yields [13]. The prevailing genera of PGPR are mostly composed of *Pseudomonas*, *Bacillus*, and different fungal species such as *Trichoderma* and *Aspergillus*, etc. Plant growth can be enhanced in many ways through the interaction between plant and microorganism, such as induction of systemic resistance, antibiosis, siderophore production, and phosphate solubility, etc. The fungal species of *Trichoderma*, *Aspergillus*, and *Penicillium* can also inhibit the growth of many soil-borne pathogenic fungi through mycoparasitism, production of antibiotics, competition for nutrients, and induction of systemic resistance in plants [14].

In this study, we characterized the microorganisms that present in humus and different stages of IMOs in terms of their physiological and biochemical traits, such as siderophore production, phosphate solubilization, and antifungal activity.

2. Materials and methods

2.1. Humus sampling and IMO preparation

The humus samples were collected from the deciduous leaves of oak and pine trees near greenhouse of the Konkuk University (Seoul, Korea). IMO1 was prepared using a plastic container filled with hard steamed rice and covered by white paper (Hanji). There should be air space between the cooked rice and Korean traditional paper. At 7 days after incubation, the cooked rice in the container was fully grown with white molds. This process is called the cultivation of indigenous microorganisms and named as IMO1. For the preparation of IMO2, IMO1 was mixed with brown sugar in a 1:1 ratio, that is, 20 g of IMO1 was mixed with 20 g of brown sugar. The mixture was stirred thoroughly. The plastic jar was filled up to 2/3 full leaving 1/3 for airspace. At 7 days after incubation of IMO2, the steamed rice color changed into brown due to adding the sugar. For IMO3, IMO2 was diluted with water in 1:1000 ratios and the dilute was mixed with rice bran and maintained at 50 °C. IMO4 was made by mixing IMO3 with field soil.

2.2. Isolation of bacterial and fungal colonies

One gram of humus or IMO sample was serially diluted up to 10^{-5} in sterile distilled water. The diluted samples were then mixed into tryptic soy agar (TSA) for bacteria and potato dextrose agar (PDA, BD Difco, Franklin Lakes, NJ) for fungus, respectively. The plates were incubated at 30 °C (bacteria) and 25 °C (fungi) for 2–3 days. The bacterial colonies were counted and were subjected to further streaking for single colony purification on TSA (supplemented with 5 mL of glycerol). Similarly, the fungal colonies were punched down with a 5 mm cork borer in the PDA.

2.3. Gram staining and starch hydrolysis

Gram staining was performed with Gram Staining kit and protocol instructed in the protocol of Materials and Data Sheet (Merk, Darmstadt, Germany). Starch hydrolysis was conducted using 1% of starch agar streaking with fresh subculture isolate and incubating it for 36 h. After incubation, 1% of Gram Iodine was poured down. A clear zone around streaked culture showed positive activity.

2.4. Antibiotic activity

Antifungal activity was performed using Dual culture techniques by streaking bacterial isolate at 3 cm distance using small (5 cm) petri dish against

soil-borne fungal pathogens *Fusarium oxysporum,* and *Sclerotinia sclerotiorum* at a distance of 1.0 cm from the edge of the plate and then incubated at 25 °C for 24 h. The active mycelium of fungal pathogens was cut down using 2 mm cork borer and transferred to another side of the PDA plates streaked with bacterial isolates. For the antifungal activity of fungi, the mycelium of each fungal pathogen was used from a freshly grown cultured one. The inhibition zone was evaluated by the radius of the fungal colony compared to the control culture. Inhibition zone of above 1 cm was considered as strong (+++); around 1 cm was moderate (++); and 0.5 cm was good (+); non inhibition zone was (−), and ± Not determined due to unclear results.

2.5. Isolation of siderophore producing bacteria

The production of siderophores is indicated by the typical color described in the literature for the reaction involving the removal of iron from chrome azurol sulfonate [15] by the siderophores [16,17]). CAS blue agar was prepared using previous methods [16,18]. In brief, the CAS indicator solution was prepared by dissolving 60.5 mg CAS in 50 mL distilled water in a glass beaker and then mixed with 10 mL of iron (III) solution containing (1 mM $FeCl_3.6H_20$ in 10 mM HCl). 40 mL of hexadecyltrimethylammonium bromide (HDTMA, 72.9 mg) solution was mixed to the CAS indicator solution and then the mixture was autoclaved for 20 min and cool down to 50 °C. At the same time, PIPES agar medium (pH 6.5–6.8) containing 15.6 g of PIPES and 15 g agar in 500 mL of DW was also autoclaved. The two solutions were mixed together with the addition of 30 mL of filter-sterilized 10% casamino acid solution and then the mixture was poured into Petri dish. Siderophore production was performed growing the isolates in TSB for 36 h. (OD 600 nm = 0.8). Approximately 50 µL of the culture was added to a 10 mm paper disk and placed on the center of plates containing CAS Blue Agar, kept for incubation in dark at 28 °C for 7 days. *Pseudomonas aeruginosa* was used as positive control (KACC 11085) as it is a well-known nitrogen-fixing and siderophore producing PGPB.

2.6. Biochemical and physiological characterization

The isolated bacteria were subjected to biochemical characterization and phenotypical analysis. Different enzymatic activity shows the ecology, physiology, or natural habitat of microorganisms. The isolates were tested on gram staining, starch hydrolysis, siderophore production, phosphate solubilization and were identified by using API 20E systems (BioMerieux, Marcy L'Etoile, France). API 20E kit is to determine the enzymatic activity of microorganisms. This system consisted of 19 enzymatic reactions for rapid detection [17].

2.7. Phosphate-solubilizing activity

The positive isolates showing siderophore producing activity was further tested for the phosphate solubilizing activity. For this purpose, the isolates were tested on the NBRIP medium [19] containing 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2{\cdot}6H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.2 g KCl, and 0.1 g $(NH_4)_2SO_4$, and 10 g agar per liter (pH 7). All the ingredients were mixed and were autoclaved for 15 min at 121 °C. The media was cooled down to 50 °C for further use and poured in the plates for solidifying. Phosphate-solubilizing abilities of the isolates were tested by inoculating each isolate on NBRIP agar and estimated with absorbance at 600 nm. *Pseudomonas aeruginosa* was used as a positive control (KACC 11085). About 40 µL bacterial isolate grown on TSB was spread on the agar plate and kept on incubation for at least 6–7 days at 30 ± 2 °C after the incubation clear zones around grown bacteria were recorded. The positive microorganisms were able to produce a halo zone showing the production of organic acid into the surrounding medium [20,21].

2.8. Phylogenetic analysis of selected bacterial and fungal isolates

Twenty-eight selected isolates of bacteria having the most positive results from the different tests were subjected to PCR analysis. For analysis of bacteria diversity, partial 16S rRNA universal gene sequence fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rp2 (5′-ACGGCTACCTTGTTACGACTT-3′) were used [22]. The PCR mixture having 40–50 ng of the templet DNA and primers of 10 pmol concentration, PCR master mix containing Taq DNA polymerase, dNTPs, Tris–HCl, $MgCl_2$ stabilizer, were used according to the manufacturer's instructions (SolGent Co., Daejeon, Korea). The reaction conditions for 16S rDNA amplification were, 1 min at 94 °C, 1 min at 55 °C, and 1 min 50 s at 72 °C for 30 cycles. Similarly, for fungal isolates, PCR was carried out using ITS1 (TCCGTAGGTGA ACCTGCGG) and ITS4 (TCCTCCGCTTATTGA TATGC) primer. The conditions for PCR were set to 32 cycles of 95 °C for 5 min of denaturation and 55 °C of annealing temperature for 30 s, extension at 72 °C for 1 min followed by holding at 72 °C for 10 min and final temperature at 4 °C forever. The expected size

Table 1. Microbial population of humus and indigenous microorganisms (IMOs).

	Population (CFU[a]) of microorganism (Mean ± SEM[b])	
Sample	Fungi × 10^4	Bacteria × 10^6
Humus	5.0 ± 1.5	10.8 ± 2.5
IMO1	7.46 ± 1.7	6.0 ± 2.4
IMO2	2.09 ± 1.11	1.87 ± 1.74
IMO3	7.65 ± 1.34	13.41 ± 2.22
IMO4	4.8 ± 1.9	4.4 ± 0.5

[a]CFU: colony forming unit; [b]Standard error of the mean of three replications.

Table 2. The diversity and abundance of different fungal species isolated from humus and IMOs samples.

Fungal genus	Humus	IMOs
Aspergillus	34 ± 11.3	28 ± 9.3
Penicillium	25 ± 8.3	11 ± 3.6
Trichoderma	23 ± 7.6	11 ± 3.6
Rhizopus	15 ± 5	8 ± 2.6
Fusarium	11 ± 3.6	5 ± 1.6
Alternaria	22 ± 7.3	15 ± 5
Botritis	7 ± 2.3	12 ± 4
Unknown	24 ± 8	14 ± 4.6
Total	161	104

of the amplicon was 1.45 kbp (bacteria) and 550 bp (fungi). The PCR product was analyzed by Macrogen (Seoul, Korea) and nucleotide sequences from the PCR products were analyzed with those known bacterial and fungal isolates from NCBI database search (http://blast.ncbi.nlm.nih.gov/). The phylogenetic trees were constructed using the neighbor-joining method in (CLC Main Work Bench 8) software.

3. Results and discussion

3.1. Microbial population of humus and IMOs

The mean total number of fungal counts for humus was 5.0 × 10^4 colony forming units per gram (CFU/gram) of the sample (Table 1). IMO1, IMO2, IMO3, and IMO4 also showed similar fungal numbers as 7.46, 2.09, 7.65, and 4.8 × 10^4 CFU/g, respectively (Table 1). The number of bacteria was higher by showing 10.8, 6.0, 1.87, 13.41, and 4.4 × 10^6 CFU/g for humus IMO1, IMO2, IMO3, and IMO4, respectively, compared with the fungal population. The results are in line with the previous study by Murphy et al. [23] in which they reported a higher bacterial population than fungal one in the soil. Microbial activities of soil are crucial for sustainable agriculture. Soil fertility is mainly affected by soil mineralization, the transformation of different nutrients, and the microbial population [24] For improving the quality of soil and crop growth enhancement, IMOs can be added for sustainable agriculture. For this purpose, it is much important to know what kinds of microorganisms are present in the IMOs and humus.

Approximately two hundred and sixty fungi were isolated using a single colony isolation technique from humus and IMOs for the identification. Morphology analysis under the inverted microscope revealed that *Aspergillus*, *Rhizopus*, *Trichoderma*, *Alternaria,* and *Penicillium* species were abundant in both the humus and IMOs (Table 2). Subsequent molecular analysis confirmed the morphological analysis (Supplementary Table S1). According to Montealegre et al. [25] direct input of *Trichoderma* into the soil could be used for better seed treatment and making compost. It could also be combined with organic fertilizers. In this case, different species like *Trichoderma asperellum*, *Trichoderma polysporum*, and *Trichoderma viride* were the commonly used biocontrol agents.

3.2. Antagonistic activity

For the selection of good isolates from the bacterial community, the isolates were tested for the antagonistic activity against soil-borne pathogens. Approximately three hundred samples of bacteria were tested out of which two hundred and three were positive in antagonism against soil-borne fungal pathogens by inhibiting their growth. However, some of the isolates failed to show the inhibition in the dual culture technique or dual plate assay using *F. oxysporum* and *S. sclerotiorum* as representative fungal pathogens (Supplementary Figure S1; Table 3). The isolates *Bacillus*-CCJH38 and *Micrococcus*-CCJHP47 showed strong antifungal activities against *F. oxysporum*, followed by *Bacillus*-CCJ74, *Bacillus*-CCJ73, and *Bacillus*-CCJHP38, while *Methylobacterium*-CCJHP43 and *Bacillus*-CCJHP25 failed to show any inhibition. The isolates *Bacillus*-CCJ74, *Bacillus*-CCJ73, and *Micrococcus*-CCJHP47 showed strong activities against *S. sclerotiorum* while fewer isolates show moderate activity. Findings agree with many reports regarding the antagonistic activity of bacteria having an effective activity against phytopathogenic fungus [26,27]. Report by Elamvazhuthi and Subramanian [27] also focuses on screening the potential *Bacillus* species against *Phytophthora capsici* for its biocontrol activity which showed to be much effective in using dual culture assay. The bacterial samples were isolated from paddy soils which showed strong antagonistic activity against *Rhizoctonia solani*, *Helminthosporium oryzae*, *Curvularia lunata*, and *Fusarium oxysporum* by using dual plate assay [28]. Moreover, it was reported that *Bacillus* species tried in dual culture assay could produce certain volatile antifungal metabolites that stops the growth of pathogenic fungi *Rhizoctonia solani*, as they did not see the contact between fungal mycelia and the bacteria streak against it in dual culture assay using the plates separated by septum [26].

Table 3. Biological activities of the isolated bacteria from humus and IMOs.

Strains	Biological activity				Antifungal activity	
	Gram staining	Siderophore production	Phosphate solubilization	Starch Hydrolysis	*F. oxysporum*	*S. sclerotium*
Pseudomonas aeruginosa (KACC 11085)	+	+	+	+	++[a]	++
Bacillus-CCJ74	+	+	+	+	++	+++
Bacillus-CCJ617	+	+	+	+	++	−
Bacillus-CCJ83	+	+	−	−	+	−
Bacillus-CCJ73	+	+	+	−	++	+++
Bacillus-CCJ822	+	+	+	+	++	−
Bacillus-CCJHP43	−	−	−	+	−	−
Pseudomonas-CCJ18	+	+	+	+	++	++
Pseudomonas-CCJ20A	+	+	+	+	+	+
Pseudomonas-CCJ18B	+	+	−	−	+	−
Bacillus-CCJH38	+	+	+	+	+++	+
Bacillus-CCJHP38	−	−	+	−	+	−
Bacillus-CCJHP25	+	+	+	+	−	−
Bacillus-CCJHP381	+	+	+	+	+	+
Bacillus-CCJIMT213	+	−	−	+	−	−
Micrococcus-CCJHP47	±[b]	+	±	±	+++	+++
Methylobacterium-CCJHP433	±	±	±	±	++	++

[a]Inhibition zone of above 1 cm was considered as strong (+++) superscript; around 1 cm was moderate (++), 0.5 cm was good (+) and non inhibition zone was (−), respectively; [b]Not clear to determine.

3.3. Detection of siderophore production

Based on the antibiosis activity of bacteria against the soil-borne fungal pathogen, the isolates which showed positive antagonism were selected for further experimentation. In this case, three hundred bacteria samples were isolated from humus and IMOs. In which two hundred three were successfully characterized by various biological and biochemical activities. Sixteen different isolates showing the best activities in all of the experiments were selected for further study. Among 16 selected isolates, the *CCJHP38* and *CCJIMT213* isolates failed to produce siderophores by CAS-blue agar assay (Table 3). While production in isolate *CCJHP477* and *CCJHP433* were almost negligible amounts. All other positive isolates were able to produce the color change from blue to orange around the inoculated part (Supplementary Figure S2). *Pseudomonas aeruginosa* (KACC 11085; Supplementary Figure S2(a)) was used as a positive control for the siderophore producing activity. Bacteria have been reported to produce siderophore and the siderophores are known to prevent phytopathogens from accumulating iron and other essential minerals, which are the limiting factors of the fungal proliferation [29]. It is a well-known phenomenon that siderophores directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of essential minerals, and that suppresses the growth of plant pathogens such as *F. oxysporum* and *R. solani* involved in a stress factor in inducing host resistance [17,30–33].

3.4. Phosphate solubilizing activity

The collected IMO samples were plated in the NBRIP agar plate for the identification of phosphate solubility. Almost all the isolates were found to be potent phosphate solubilizers by showing clear and halo zone around the colony (Supplementary Figure S3; Table 3). The formation of the halo zone is because of the production of organic acids and polysaccharide, or phosphate solubilizing enzyme by the isolates. Chaiharn and Lumyong [34] showed that PGPB, belonging to the genera of *Pseudomonas* and *Bacillus*, showed resistance to water stress, and the promotion of plant growth in different green plants such as rice and wheat by phosphate solubilization. Ahmad et al. [35] concluded that bacteria having the ability of phosphate solubilization could improve the phosphate solubility of fixed soil, and as a result could give the ability of higher crop yields. These kinds of microbes having the solubilization activity were isolated from time to time from various plant environments, that is, rice [36], wheat [37], soybean [26,38], and mustard [39].

3.5. Biochemical characterization

By using the API 20E strip, the different enzyme activity of the isolates was performed. According to the result (Supplementary Tables S2 and S3, Supplementary Figure S4) almost all isolates are positive in terms of the production of acetone, beta-galactosidase, arginine dihydrolase, urea hydrolysis, and amino acids. While in terms of citrate utilization, hydrogen sulfide production and in some cases failed to produce the indole. However, most of the isolates were not good in the production of fermentation and oxidation. Soil microorganisms are also involved in performing the acquisitions of carbon, nitrogen, phosphorus, etc., and other plant growth and circulation supporting nutrients [1]. It is well known about acetone and other volatile organic

compounds (VOCs) that are involved in plant growth and development [17,40]. They also concluded that these VOCs are mostly involved in the regulation of growth and plant development as it has been observed that volatile compounds present in bacteria could act as an activating agent for the growth promotion in *Arabidopsis* [40].

3.6. Phylogenetic analysis for bacterial and fungal isolates

Through the 16S rDNA and ITS analysis, these 19 isolates of bacteria and 6 isolates of fungi showed a high similarity results with the corresponding strains. The obtained PCR sequences were aligned using CLC Main WorkBench 8.1.3 software (QIAGEN BioInformatics) and the contig results were searched in NCBI blast showing that these isolates were confirmed to be species of *Streptomyces*, *Pseudomonas, Bacillus*, and *Micrococcus*. Out of which the *Pseudomonas* and *Bacillus* were abundant. With regard to fungi majority of the isolates were belonging to the group of *Aspergillus* and *Trichoderma*. In both cases, the isolates showed 99–100% of the similarity with the corresponding strain (Supplementary Tables S1 and S4). For the phylogenetic analysis, the aligned sequence was matched with isolates showings high similarity and tree was constructed using CLC Main Workbench 8.1.3 software (QIAGEN BioInformatics; Supplementary Figures S6–S11). In the last few years, the great increase has occurred in the number of these PGPB which upon analysis many of them belong to the species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus* and *Serratia* [17,39–41].

Disclosure statement

No potential conflict of interest was reported by the author(s).

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