College of Tropical Agriculture and Human Resources
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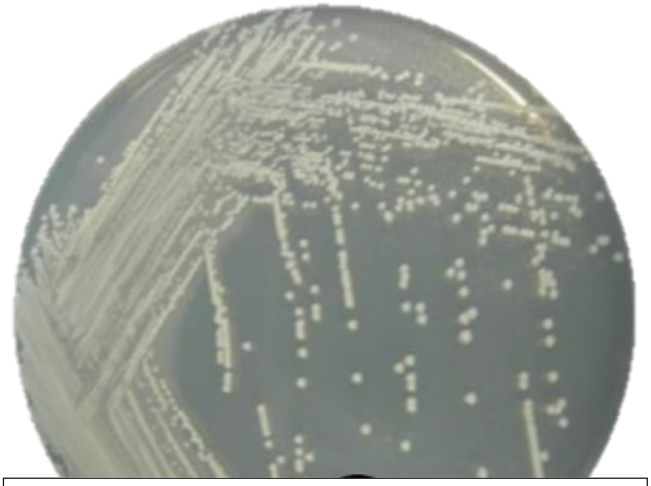
Natural Farming: The bacteria in 10 different sources of indigenous micro-organisms (IMO4) from natural farming operators in Hawai'i

Abstract

Hawai'i has a long history of agriculture, however, the monocultures of two major plantation crops has resulted in a decline of soil fertility and organic matter for plant growth. Synthetic fertilizers, pesticides, and the burning of agricultural plant residues were commonly practiced. Advocates for alternative agricultural practices promoted the use of indigenous micro-organisms (IMO) as a potential bio-fertilizer that naturally adds nutrients into the soil. There is a growing interest in IMOs among small farmers.

This research was aimed at expanding the understanding of IMOs by identifying indigenous bacteria from 10 difference sources in different sites of Hawai'i. Ten IMO4 samples collected from natural farming operators were subjected to serial dilutions and plating on selective agar that supports bacteria growth. Bacterial colonies were enumerated, and isolates were randomly selected and purified via the streak plate method. The 16S rRNA gene of each isolate was amplified using oligonucleotide primers and polymerase chain reaction (PCR) procedures. The amplified genes were subjected to DNA sequencing to identify the bacteria species.

The results show bacteria counts were similar across most locations, at approximately 8.00 log CFU/g. However, the sample from "BL" site exhibited the lowest counts across all three media, at 5.78 log CFU/g, 6.56 log CFU/g, and 6.24 log CFU/g on azospirillum agar, MRS agar, and phosphorus-solubilizing agar, respectively. Amongst the bacteria identified at 10 locations, Bacillus strains accounted for 90% of total strains. The *Bacillus* isolates consisted of 54% of *B. subtilis*, 11% *B. velezensis* and 11% *B. amyloliquefaciens*.



Bacteria colonies on specific agar.

Introduction

Hawai'i's population has a growth rate of 0.5% per year and is expected to top 1.65 million by 2045 (DBEDT 2018). Hawai'i continues to rely on imported foods and goods to sustain its population growth. Even in the agriculture sector, Hawai'i relies on most of the inputs for farming; e.g seeds, fertilizers, herbicides, and pesticides. Several white papers and policies addressed the importance for Hawai'i to be more self-sufficient to achieve food security (Office of State Planning, 2008; Sustainable Hawai'i Initiative, 2019). Baer-Nawrocka & Sadowski (2019) reported that food production and food security are dependent of natural and economic factors. While Hawai'i is blessed with a climate where crops can grow all year round, most of the inputs are shipped in. Hence, a more affordable food system and one which is more reliable could be attained if

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Hawai'i is more self-sufficient in some of the farm inputs, e.g. fertilizer.

Hawai'i's long history of plantation agriculture where monocrops of sugar or pineapple were grown, where crop residues (cane and pineapple) were burnt, and the heavy use of herbicides, etc. led to a decline in soil fertility and nutrients that are essential for supporting crop growth. Chemical fertilizers, pesticides, and herbicides are expensive and deteriorate environmental and agricultural quality, as suggested by Kumar & Gopal (2015). Nutrient loss in plants from harvest and erosion reduces the functionality and agriculture value of the land, which creates an issue with the world's sustainable food supply (Hefferon, 2015).

Natural soil amendments, such as IMOs, are growing in popularity as the demand by consumers for more natural and organic produce is increasing (Greene, 2019). IMOs are micro-organisms that are naturally occurring in a specific area that could be used as bio-fertilizers. Beneficial bacteria and fungi colonize several parts on plants, such as the rhizosphere, rhizoplane, and intercellular spaces (Kumar & Gopal, 2015). Moreover, IMOs as "bio-fertilizers" could increase nutrient availability in soil for plants due to the production of secondary metabolites, which are essential nutrients for plants. Secondary metabolites are produced through nitrogen fixation and solubilization of phosphorous (Vessey & Vessey, 2003). Plants may have the difficulty of absorbing certain nutrients directly, due to a lack of necessary transport mechanisms. IMOs convert absorbable nutrients for plants by utilizing carbohydrates from plants (Blagodatskaya & Kuzyakov, 2013; Ortíz-Castro, Contreras-Cornejo, Macías-Rodríguez, & López-Bucio, 2009; Rashid et al., 2016). IMOs are effective in enhancing soil fertility for farming and prevention of plant diseases. IMOs found in soil have the potential to be an affordable and effective fertilizer option for Hawai'i.

While there is a publication on how IMOs can be developed (Park and DuPonte, 2008, followed by several YouTube demonstrations), there is no publication on what is inside IMOs. The authors suggested these natural/native mirco-organisms were beneficial to plants. These bacteria may be involved in nitrogen fixation, phosphate solubilization, and other activities that benefit plants. According to Sharma et al., (2013), nitrogen is the most important mineral nutrient in terms of measurable plant requirements, followed by phosphorus.

Hence, we developed a simple study with the objectives to identify and quantify bacteria in IMOs. The primary goal was to specifically target and identify *Lactobacillus species*, phosphorus-solubilizing, and nitrogen-fixing bacteria. In addition, potassium (K), nitrogen (N) and phosphorus (P) are three nutrients vital for plant growth and development (Scholberg et al., 2000; Wang et al., 2018).

Materials and Methods

Soil sample preparation - The samples were prepared from soils from different locations and different topographic elevations. The samples were prepared by different individuals who are considered "Korean Natural Farming" practitioners at their respective sites. It is likely that they followed the method outlined by Park and DuPonte (2008). For a clearer understanding of what is IMO4, refer to the publication by Keliikuli et al (2019).

The IMO samples were collected from the following locations: Kailua-Kona (two samples; KK-1 and KK-2), the Hilo area (H-100, H-200, H-520, H-1000, H-2500), Lua-pāhoehoe (BL), Panaewa (AP-1), and Kula, Maui (KM). The IMO samples from the Hilo area were taken from soils of different elevation; 35m, 160m, 488m 586m, 812m. The IMO4 were prepared by the farmer at the respective sites. All samples were prepared and collected between the months of October 2014 to January 2015.

For each sample, 5 g of soil were sifted through a 2 mm mesh sieve and placed into sterile containers, containing 45 mL of 0.1% (w/v) peptone, for dilution and plating (Keliikuli, et al., 2021).

Selective Media Preparation - Many variations of selective media could have been used to target certain groups of bacteria present in the soil sample. Three selective media were used to culture the soil samples: 1) MRS (De Man, Rogosa and Sharpe) agar, 2) azospirillum agar, and 3) phosphorus-solubilizing agar. The MRS medium contained the following ingredients l-1: Difco Lactobacilli MRS Broth, 55 g and Difco Agar, 15 g (BD™, Franklin Lakes, New Jersey). This medium mixture has been known to support lactic acid bacteria (LAB) growth, as several of the practitioners had suggested that IMO4 is rich in LAB. The azospirillum agar contained l-1: K2HPO4, 5 g; MgSO4·7H20, 0.975 g; NaCl, 1 g; yeast extract, 0.5 g; and Difco Agar, 15 g; the pH was adjusted with 1M HCl to 6.8 prior to autoclaving (Hurst et al., 2000). The phosphorus-solubilizing agar contained l-1: Difco Plate Count agar (PCA), 23.2 g; Ca(PO4)2, 5 g; and Difco agar, 25 g (Atlas, 2010). A nutrient broth of all three selective agar was also prepared (devoid of agar) for growth of purified isolates.

Serial Dilution Preparation and Plating - From the sifted samples, 5 g of soil was added to a container containing 45 ml of 0.1% peptone water (10-1 dilution). The sample was homogenized with a vortex mixer for approximately 5 minutes. One mL of the sample was placed into a tube containing 9 mL of 0.1% peptone water. This process was repeated until the samples were serially diluted a total of five times (10^2 to 10^5). Dilutions (0.1 mL) of 10^4 and 10^5 were plated onto the selective agar described above. The technique used to inoculate the plates was the spread-plate technique (Balows, 1992). The plates were incubated at 35°C for approximately 16 h.

Streak Plate Method - The colonies appearing on the agar were counted and recorded to determine colony forming units (CFU). Bacterial colonies (8-20 colonies per plate) were randomly selected using a grid with 1 cm x 1 cm blocks. Three blocks on the grid (within the area of the plate) were selected and consistently used for each plate (Keliikuli, et al., 2021). Each bacterial colony that fell within the three boxes was sub-cultured via streak-plate method, to obtain isolated colonies.

Identification of Isolates

Partial sequencing of the 16S rRNA genes of isolates was carried out as described by Xue et al., (2018), after PCR amplification of the 16S rRNA gene using oligonucleotide primers 16S1-F (5'-GGAGAGTTTGATCCTGGCTCAG-3') and 16S1-R (TATTACCGCGGCTGCTGGCAC). The amplified samples were submitted to the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) laboratory at the University of Hawai'i-Manoa and subjected to high throughput DNA sequencing. ChromasPro was used to view the DNA sequence. The sequences were compared with those in the GenBank databases by using the BLAST program (www.ncbi.nlm.nih.gov/blast) to identify the isolates.

Results and Discussion

Figure 1 shows the total colony forming units (cfu/g) of the 10 IMO samples in three media (azospirillum agar, MRS agar, and phosphourous-solubilizing agar). The KK samples showed the highest amount of IMO bacteria colonies (8.67 log cfu/g) in azospirillum agar. Samples from KK-1, KK-2, and AP exhibited the highest overall cfu counts, while BL had the lowest cfu counts. The counts of the ten soils were similar except for the BL samples, which was approximately 2 logs lower. We are uncertain why BL had lower cfu/g unless further investigation were carried out, e.g. an interview with the farmer. However, the composition of IMO4 in this farm was not different from the others.

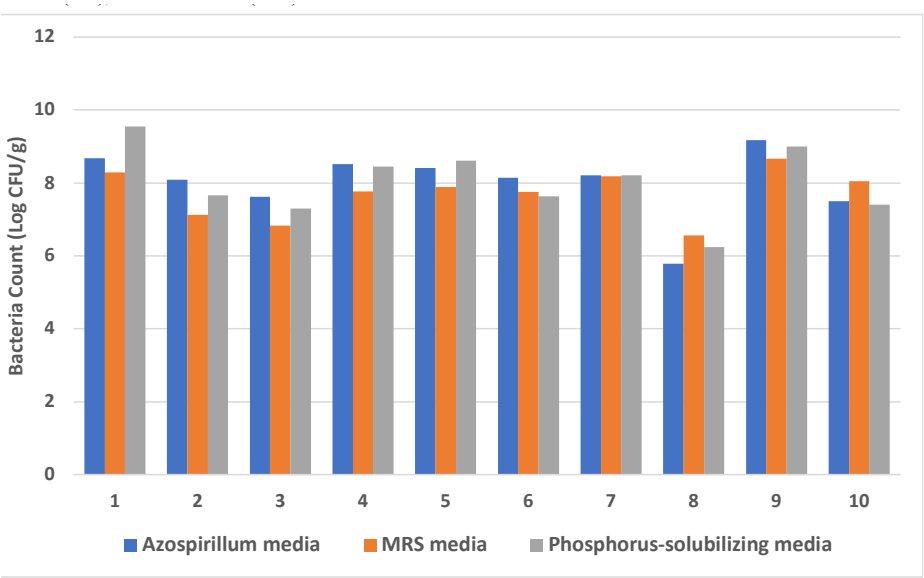

Figure 1. Distribution of bacteria colonies from 10 different sources of IMO4 when cultured in Azospirillum agar, MRS agar and phosphorus-solubilizing agar. 1. Kailua-Kona-1 (KK-1), Kailua-Kona-2 (KK-2), the Hilo area: 3(H-100), 4(H-200), 5(H-500), 6(H-1,000), 7(H-2,500), 8-Luapāhoehoe (BL), 9-Panaewa (AP), and Makawao (KM).

Samples from the H group were prepared by a single practitioner but at sites of different elevations. H-100 means it was taken at 30.5m above sea level. H-500 means it was 152.4 m above the sea level and H-2,500 mean it was taken at 762m above the sea level. The data showed that elevation did not has any effect on the bacteria yield (cfu) per gram. Although the higher elevation generally has a cooler climate, the soil samples were of similar classification.

Most of the soils from this study were Andisols or Histosols based on a report by Deenik (2014) and Hue, et al. (2007). Based on these soil maps, the differences in cfu counts of the IMOs are more likely due to the processing method of the practitioner.

Figure 2 shows that 90% of bacteria in the soils are *Bacillus* strains. *Bacillus* species are gram-positive bacteria that are typically found in soil and the human gastrointestinal tract. Bacillus are able to grow on selective agar (MRS), as they have similar metabolism with *Lactobacillus* species. *Bacillus* are able to metabolize phosphorus (through solubilization) and nitrogen (through nitrogen fixation). They are found in the upper layers of soil and can grow in high elevations (Gingichashvili et al., 2017). Soils with higher amount of *Bacillus* bacteria would provide a more effective medium for plant growth due to their effects of promoting plant growth. (Radhakrishnan, et al., 2017; Akinrinlola et al., 2018).

A primary reason why *Bacillus* is the main bacteria in soil is due to its spore forming ability. *Bacillus* bacteria forms endospores during environmental stresses in soil, such as during nutrient depletion, extremely low moisture, and presence of pesticides.

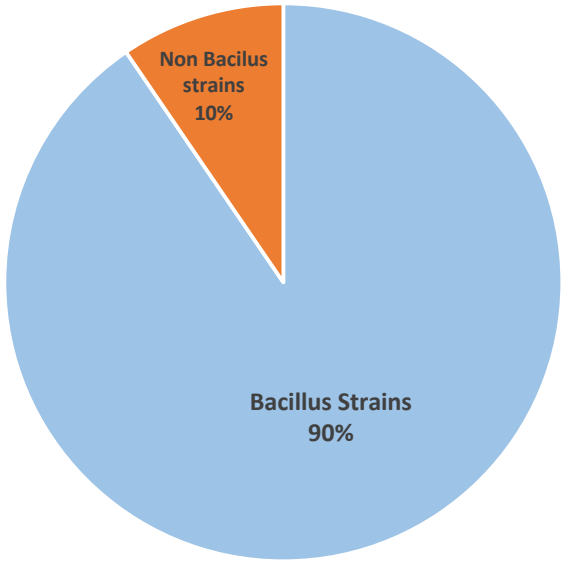

Figure 2. The taxonomy of bacteria genus found in the 10 different IMO 4 samples.

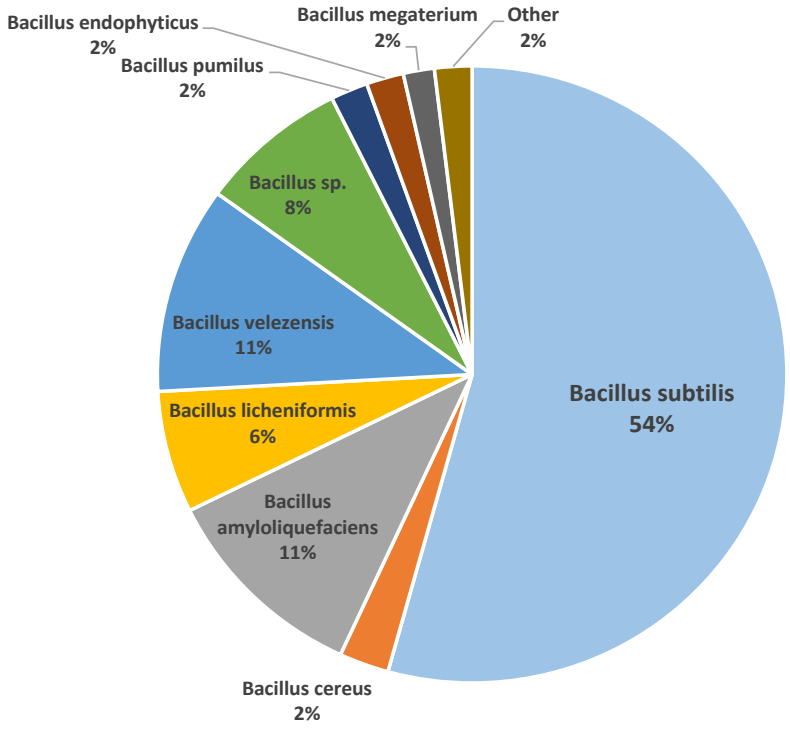

Figure 3. The specie of bacillus bacteria found in the IMO4 samples.

Spore formation is a strategy for some bacteria, especially *Bacillus* species, to temporarily avoid unfavorable environmental conditions and exist in dormancy until environmental conditions becomes favorable (Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000). Because Bacillus are shown here to be the main genus in IMO4 (the final stages of IMOs preparation) for all the local soils, it could be an ideal natural fertilizer for Hawai'i.

Some non-*Bacillus* strains (10% of total strains) that were isolated were: *Rummeliibacillus stabekisii, Enterobacter,* and *Rhizobium* sp. While other researchers suggest there are lactic acid bacteria in IMO4, none was identified in the current study.

Figure 3 shows the breakdown of *Bacillus* species found in the IMO4 samples. *Bacillus subtilis* species were most abundant, consisting of 54% of Bacillus species that were identified. Other *Bacillus* species were *Bacillus amyloliquefaciens* (11%), *Bacillus velezensis* (11%), and *Bacillus licheniformis* (6%). This information confirms the earlier findings in our laboratory that showed the presence of *Bacillus* species in KNF treated soils (Keliikuli, et al., 2021).

Bacillus species have been found to promote plant growth using various mechanisms, such as solubilizing iron by using siderophores that bind Fe3+ to be reduced to Fe2+, that plants use as minerals (Radhakrishnan, Hashem, & Abd Allah, 2017). Additionally, *Bacillus* species secrete 1-aminocyclopropane-1-carboxylate (ACC) deaminase to inhibit ethylene synthesis in crops and promote plant growth through the synthesis of indole-3-acetic acid, a plant hormone of the auxin class (Glick, 2014). *Bacillus subtilis* has been reported to be a growth-promoting rhizobacterium as it can solubilize phosphorus, fix nitrogen, and assist plants to cope with stress conditions by suppressing harmful bacteria (Hashem, et al., 2019).

The four highest *Bacillus* species that were isolated are plant growth-promoting rhizobacteria, which are bacteria that colonize plant roots and promote plant growth. *B. amyloliquefaciens* efficiently colonizes the rhizosphere to stimulate plant growth and/or inhibit root pathogens, such as *Ralstonia solanacearum, Pythium ultimum*, and *Fusarium oxysporum*, through its antibacterial activity. These pathogens cause diseases in plants, such as *P. ultimum* causing gray mold, which lowers plant yield. Root colonization of *B. subtilis* is achieved through the formation of biofilms to provide plants with a protective barrier against pathogens (Molohon et al., 2011; Wu, Wu, Chen, et al., 2015). *B. amyloliquefaciens* also allows plants to tolerate high salt concentrations and reduce salt concentration in plant tissues to increase plant yield (Chen et al., 2016).

B. velezensis synthesize difficidin and bacilysin, antibacterial agents that controls pathogens that causes rice disease, such as bacterial blight and bacterial leaf streak (Wu, Wu, Qiao, Gao, & Borriss, 2015). Bacillomycin-D and fengycin, produced by *B. velezensis*, exhibit defense mechanisms against *Ralstonia solanacearum*, an etiological agent of tomato wilting (Cao et al., 2018). *B. velezensis* has also been found to produce antifungal molecules and nematocidal molecules, molecules which inhibit the activity and/or survival of plant-parasitic nematodes (Rabbee et al., 2019).

Like many plant growth-promoting rhizobacteria *Bacillus* species, *B. licheniformis* produces the enzyme, amylase, which promotes early germination and is effective in mass production of plants. Amylase activation in *B. licheniformis* is triggered by developing a relationship with the host seed to produce gibberellin, plant hormones that stimulate elongation, germination, and flowering; *B. licheniformis* produces high amounts of active gibberellins for plant germination. Moreover, *B. licheniformis* exhibits a high nickel tolerance in plants (Jamil et al., 2014). Nickel is a vital nutrient for plants; however, at high concentrations, nickel inhibits many physiological, anatomical and morphological processes (Hassan et al., 2019).

Overall, many *Bacillus* species found in plants are effective in providing nutrients and promoting plant growth.

Summary

This study focused on identifying phosphorus-solubilizing, nitrogen-fixing bacteria, in preparations of IMO4. It was established that predominate bacteria in IMO4 from different sites and prepared by different farmers are *Bacillus* species. No *Lactobacillus* species was found in the soil. *Bacillus subtilis* was the major species in IMO4. This top four Bacillus species isolated from the 10 different samples could potentially be used as biofertilizers. Commercialization of these IMO4 may provide a cheaper organic alternative fertilizer for Hawai'i producers. The use of IMOs as fertilizers should be investigated in futures studies because of the potential to reduce the need for pesticides and herbicides.

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