Tropical Legumes for Improvements of Red Soils and Crop Production

(赤色土壌の改良と作物生産のための熱帯マメ科植物に関する研究)

MD. SAGIRUL ISLAM MAJUMDER

2020

Tropical Legumes for Improvements of Red Soils and Crop Production

(赤色土壌の改良と作物生産のための熱帯マメ科植物に関する研究)

A dissertation submitted to

The United Graduate School of Agricultural Sciences,

Kagoshima University, Japan

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

IN

AGRICULTURE

MARCH 2020

BY

MD. SAGIRUL ISLAM MAJUMDER

SCIENCE OF BIORESOURCE PRODUCTION

THE UNITED GRADUATE SCHOOL OF AGRICULTURAL SCIENCES

KAGOSHIMA UNIVERSITY, JAPAN

A dissertation entitled "**Tropical Legumes for Improvements of Red Soils and Crop Production**", prepared and submitted by **Md. Sagirul Islam Majumder** in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Agriculture is hereby accepted and approved.

Dissertation Committee

Dr. Md. Amzad Hossain
Professor
Crop Production Science
Faculty of Agriculture
University of the Ryukyus
Japan
(Major Advisor)

Dr. Hikaru AKAMINE
Associate Professor
Turfgrass science
Faculty of Agriculture
University of the Ryukyus
Japan
(Advisor)

Dr. Michio ONJO
Professor
Tropical crop science
Faculty of Agriculture
Kagoshima University
Japan
(Advisor)

Dr. Ayako SANO
Professor
Mycology
Faculty of Agriculture
University of the Ryukyus
Japan
(Advisor)

Dr. Jun-Ichi SAKAGAMI
Professor
Tropical Crop Science
Faculty of Agriculture
Kagoshima University
Japan
(Advisor)

LIST OF CONTENTS **Page**

Abstract in English

Tropical Legumes for Improvements of Red Soils and Crop Production

(赤色土壌の改良と作物生産のための熱帯マメ科植物に関する研究)

Most of the red soil in tropical and subtropical regions has low organic matter, nutrients, water holding capacity and high phosphate fixing capacity. Therefore, chemical fertilizers are used increasingly worldwide for efficient crop production, which causes soil degradation and environmental pollution. Thus, the legumes could be used in agriculture to reduce chemical fertilizer application and to improve soil physio-chemical properties, soil nutrients, biological properties and crop yield. A series of experiments has been conducted in laboratory and greenhouse to evaluate growth characteristics, biomass production and nutrient status of tropical and subtropical legumes hairy vetch (*Vicia villosa*), chickpea (*Cicer arietinum*), grasspea (*Lathyrus sativus*), dhaincha (*Sesbania aculeata*), mungbean (*Vigna radiata*), lentil (*Lens culinaris*) and soybean (*Glycine max*), and their effects on crop production to select some legumes as green manure in red soil management.

The legumes were cultivated from March to May (spring), June to August (summer), and November to January (winter) in cultured soil and red soil. In cultured soil, the highest plant biomass was obtained from dhaincha (41.84g pot^{-1}) followed by chickpea (16.45g pot^{-1}), grasspea (9.8g pot^{-1}), soybean (8.92g pot^{-1}) and mungbean (8.47g pot^{-1}) when planted in March. Dhaincha and mungbean produced 117g pot^{-1} and 41.43 g pot^{-1}, respectively when planted in June. Dhaincha produced 3 times and mungbean produced 5 times higher biomass in June plantation as compared to that in March plantation.

In red soil, the highest plant biomass was obtained from soybean (4.35g pot^{-1}) followed by dhaincha (2.84g pot^{-1}), chickpea (2.80g pot^{-1}), grasspea (2.75g pot^{-1}) and mungbean (1.87g pot^{-1}) in March plantation. Dhaincha and mungbean produced 16.41g pot^{-1} and 18.96g pot^{-1} biomass, respectively in June plantation. In November plantation, chickpea and grasspea produced dry biomass 8.413 g pot^{-1} and 10.56 g pot^{-1}, respectively. Dhaincha produced 10 times, and mungbean produced 6 times of

biomass in June plantation than in March plantation. Chickpea produced 3 times and grasspea produced 4 times higher biomass in November plantation than in March plantation.

Total nitrogen in dhaincha, grasspea, soybean, mungbean and chickpea was 4.73, 3.77, 2.73, 2.68 and 2.67%, respectively; phosphorous was 0.18, 0.08, 0.01, 0.06 and 0.04 mg g^{-1}, accordingly; and potassium was 2.48, 2.59, 1.87, 3.12 and 3.34 mg g^{-1}, accordingly.

Effects of soybean, mungbean and dhaincha on soil and turmeric cultivation were evalauted. The soil with green manure plant was found to be loose and had lower soil bulk desity and higher soil moisture than untreated soil. Turmeric plants treated with the legumes survived longer and resulted in increased 155-216% shoot biomass and 89-143% yield. The highest turmeric yield was obtained with mungbean (213 g $plant^{-1}$) followed by dhaincha (176.9 g $plant^{-1}$) and soybean (166.4 g $plant^{-1}$).

Hairy vetch effects on red soil and turmeric were evaluated. The soil with hairy vetch was loose and had higher soil moisture and lower bulk density. All the turmeric plants treated with hairy vetch survived longer and had significantly higher growth parameters and yield. Hairy vetch provided organic matter and nutrients, and improved soil microbial activities. Bacteria and fungi in the soil amended with hairy vetch were 3.7-fold and 4-fold higher, respectively, compared to control soil. Two isolates, *SI-17URAgr and SI-19URAgr were* identified as *Talaromyces pinophilus*, which produced considerable organic acids and solubilized phosphorous and zinc.

This study showed that all the legumes used in the experiments could be cultivated in subtropical Okinawa, which were different in biomass production with the season. However, dhaincha and mungbean grew best in summer, and chickpea and grasspea in winter. Hairy vetch grows well in winter. These five legumes provided better organic matter and nutrients, and improved soil physio-chemical properties and microbial activities, which integratedly contributed to increased plant growth and yield. These five legumes could be used as green manures to improve soil and crop production.

Abstract in Japanese

赤色土壌の改良と作物生産のための熱帯マメ科植物に関する研究

(Tropical Legumes for Improvements of Red Soils and Crop Production)

熱帯、亜熱帯に分布する赤色土壌の多くは、有機物や栄養分の含有量が少なく、保水力も低く、リン酸固定能が高い特徴がある。従って、効率的な作物生産を目的に益々化学肥料が世界中で使用され、土壌劣化や環境汚染を引き起こしている。そこで、マメ科植物が、農業において化学肥料使用を減じ、土壌の物理性、化学性、生物性、栄養状態、作物生産の改善に使用出来ると考えた。熱帯や亜熱帯のマメ科植物、ヘアリーベッチ、ヒヨコマメ、グラスピー、ダインチャ、リョクトウ、レンズマメ、大豆の生育特徴、バイオマス生産、栄養分、作物生産への効果の評価や、赤色土壌の改良に使用できる緑肥を選定する為、実験室とグリーンハウスで実験を行った。

まず、マメ科植物は3月～5月（春季）、6月～8月（夏季）、11月～1月（冬季）に培養土と赤色土壌を使って栽培された。培養土での春季栽培では、最も高いバイオマスはダインチャ（41.84g　pot^{-1}）、次いでヒヨコマメ（16.45g　pot^{-1}）、グラスピー（9.8g pot^{-1}）、大豆（8.92g pot^{-1}）、リョクトウ（8.47g pot^{-1}）の順で得られた。夏季栽培では、ダインチャ（117g pot^{-1}）、リョクトウ（41.43g pot^{-1}）がより多くのバイオマスを生産した。ダインチャとリョクトウは、春季栽培に比べ、夏季栽培でそれぞれ3倍、5倍高いバイオマス生産を示した。

赤色土壌での春季栽培でバイオマス量は大豆（4.35g pot^{-1}）、次いでダインチャ（2.84g pot^{-1}）、ヒヨコマメ（2.80g pot^{-1}）、グラスピー（2.75g pot^{-1}）、リョクトウの（1.87g pot^{-1}）の順であった。夏季栽培では、ダインチャが（16.41g pot^{-1}）、リョクトウが（18.96g　pot^{-1}）を生産した。冬季栽培では、ヒヨコマメとグラスピーが（8.413g pot^{-1}）、（10.56g pot^{-1}）を生産した。ダインチャとリョクトウは、春季栽培に比べ夏季栽培で10倍、6倍のバイオマスを生産した。また、ヒヨコマメとグラスピーは、春季栽培と比べ冬季栽培で3倍、4倍のバイオマスを生産した。

ダインチャ、グラスピー、大豆、リョクトウ、ヒヨコマメの窒素含有量は、それぞれ（4.73%）（3.77%）、（2.73%）、（2.68%）、（2.67%）、亜リン酸は、（0.18mg g^{-1}）、（0.08 mg g^{-1}）、（0.01 mg g^{-1}）、（0.006 mg g^{-1}）、（0.04 mg g^{-1}）、カリウムは（2.48 mg g^{-1}）、（2.59 mg g^{-1}）、（1.87 mg g^{-1}）、（3.12 mg g^{-1}）、（3.34 mg g^{-1}）であった。

次いで、大豆、リョクトウ、ダインチャの土壌とウコン栽培に及ぼす緑肥としての影響の評価を行った。緑肥を施用した土壌は空隙が多く、仮比重が低く、保水力が高くなった。マメ科植物由来の緑肥を施用し栽培したウコンは、より長い期間生育し、地上部のバイオマス量が155～216%、根茎の収量が89～143%高まった。最も高い収量はリョクトウで（213g $plant^{-1}$）、ダインチャ（176.9g $plant^{-1}$）、大豆（166.4g $plant^{-1}$）の順であった。

次に赤色土壌と栽培されるウコンへのヘアリーベッチ施用の影響の評価を行った。ヘアリーベッチを施用した土壌は空隙が多く、仮比重が低く、保水力が高まった。ウコンはより長い期間生育し、生育パラメーターと収量が有意に高くなった。ヘアリーベッチは有機物、栄養を供給し、土壌生物を活性化した。ヘアリーベッチ施用によりバクテリア、土壌細菌は対照区に比べ、それぞれ3.7倍、4倍高くなった。2種の菌はSI-17URAgrとSI-19URAgrで、タラロマイセス・ピノフィラスと同定され、有機酸や可溶性リン酸、亜鉛を作り出すことが分かった。

今回の研究で、使用したマメ科植物すべてが沖縄の環境下で生育し、栽培する時期によりバイオマス生産に違いを示した。しかし、ダインチャとリョクトウは夏季栽培で最も高い生育を示し、ヒヨコマメとグラスピーは冬季栽培が適していた。ヘアリーベッチは冬によく成長します.　また実験で使用したマメ科植物は有機物、栄養、土壌の物理、化学、生物性を改善し、植物の生育や収量を高めた。特に5種類のマメ科植物は土壌改良や作物生産に緑肥として使用しうることが分かった。

GENERAL INTRODUCTION

The global demand for food will grow considerably in the coming years due to the increasing global population. The agriculture practiced in the tropics has key importance on food supply for much of the current global population and may become even more important for future generations. Now a days, land degradation is the major constraint, which led to the nutrient depletion and limited potential yield of crops toward the food security worldwide.

Soil acidification is one of the most important land degradation issues which is a significant problem in tropical and subtropical agriculture. Acid soils occupy about 40% of the total arable area in the world, most of which are found in tropical and subtropical regions (Haug, 1984). About 43% of tropical land area comprising 68, 38 and 29% of tropical America, tropical Asia and tropical Africa, respectively, are acidic (Panday *et al.,* 1994). Acid soil contains poor fertility due to a combination of mineral toxicities (Al and Mn) and deficiencies (P, Ca, Mg and Mo). Aluminium toxicity is the single most important factor, being major constraint for crop production on 67% of the total acid soil which usually contains low organic matter, nutrients and microorganisms, and has low water holding capacity and high bulk density. Phosphorus is one of the limiting nutrients in acidic soil which often have high P fixing capacity due to their high Al and Fe oxide concentrations. Most of the applied P through mineral fertilizers was gradually reacts with Fe and Al compounds in the acid soil and is transformed into relatively insoluble P compounds.

Red soils are generally acidic in nature and deficient in most essential nutrients. They are mostly found in tropical and sub-tropical areas and are the third most important soil of the world covering 13% of the land area (Haug, 1984). These soils also have low organic matter, low fertility, high phosphate fixing capacity and low water holding capacity.

Therefore, the use of lime and fertilizers accounts for a large part of the agricultural production cost. Thus, to increase the environmental and economical sustainability, it is important to make rational application of fertilizers and find viable alternatives to maintain a good physical, chemical and biological properties. This

study focuses the use of legume as practices that can help to increase the productivity of the soils, since they act as conditioners of the physical, chemical and biological properties. Legumes are more important in agriculture due to having their root nodule bacteria accumulate atmospheric nitrogen(N). Biologically fixed N is environment friendly, because NH_3 is assimilated into an organic form by the plant, and is released gradually through organic matter decomposition by bacterial mineralization. Legume also serve as a source of dietary protein, flour, vegetable oil, a component of poultry diets.

Many kinds of leguminous plants grow well in poor fertile soil in tropical and subtropical regions, which supply nutrients and organic matters, decrease soil bulk density, increase soil aeration and water holding capacity, and improve soil microbial activities (Miyamaru *et al*., 2008; Sultani *et al*., 2007; Sullivan, 2003). Liao *et al.* (2006) reported that some tropical legumes have numerous adaptive mechanisms for growing in deficient P soils, where roots may exude organic compounds to mobilize P from bound P pools in the soil.

The application of organic matter is recommended for plant cultivation because it is eco-friendly. Legume green manuring is considered to be an important technique that has the potential to reduce the dependence on mineral fertilizers and to improve soil properties and fertility level (Elfstrand *et al.,* 2007, Tejada *et al.,* 2008; Sarwar *et al.,* 2010). Green manure amendments stimulate soil microbial growth and activity, with subsequent mineralization of plant nutrients (Lundquist *et al.,* 1999, Randhawa *et al.,* 2005, Eriksen, 2005). Legume-based green manures supply N to soil. Nitrogen mineralization is more tightly linked to microbial demand for C than phosphate mineralization. In addition, mineralization of phosphate, which is mediated by phosphatases, is also driven by microbial demand for phosphate, independent of C availability (Elfstrand *et al.,* 2007, McGill and Cole, 1981). However, phosphorus (P) is an important plant nutrient and large amounts of soluble phosphate applied to soils as fertilizer are fixed in the soil, which limits its availability to plants. Thus, the long-term application of phosphate fertilizers has resulted in an accumulation of total soil phosphate, most of which is poorly soluble (Whitelaw, 1999). Microorganisms capable of solubilizing and mineralizing phosphate in soils are considered vital for promoting

phosphate bioavailability (Tao *et al.,* 2008). In addition, acid, calcareous, saline and sodic soils, and coarse-textured soils susceptible to high weathering, besides soils subjected to intensive cropping and poor drainage exhibit Zn deficiency (Singh *et al.,* 2005) and application of Zn in the form of chemical fertilizer is inappropriate due to its unavailability to plants. Green manure stimulates the activity and abundance of soil microorganisms, including arbuscular mycorrhizal fungi, which were shown to promote plant Zn uptake (Lehmann *et al.*, 2014).

A feasible alternative would be to exploit the innate capacity of certain soil microorganisms, especially, fungi, to solubilize fixed forms of phosphorus (P) or zinc (Zn) for enhanced availability and subsequent uptake by plants. Green manure provides nutrients rich in organic carbon for the microbial biomass which converts unavailable nutrients in plant residues into forms available to plant (Carsky and Suhet, 1990).

The important leguminous green manure crops in tropical and subtropical regions are hairy vetch (*Vicia villosa*), chickpea (*Cicer arietinum*), grasspea (*Lathyrus sativus*), dhaincha (*Sesbania aculeata*), mungbean (*Vigna radiata*), lentil (*Lens culinaris*), soybean (*Glycine max*), etc. Among these, grain legumes; chickpea, grasspea, lentil, mungbean, and soybean continue to occupy an important place in human nutrition as sources of protein, vitamins and minerals. From a nutritional point of view, chickpea, grasspea, lentil, mung bean, and soybean are the important grain legumes for the millions of people in semi-arid and tropical regions of many Asian and African countries. These legumes are used in various food forms after suitable processing depending on the regions of their production and consumption.

Therefore, a series of experiments was conducted in laboratory and greenhouse to : (i) evaluate the growth characteristics, biomass production and nutrient status of some tropical legume, (ii) evaluate their effects on crop production and select the best legume as green manure in red soil management, (iii) investigate the effect of the best legume as green manure on soil physicochemical and biological properties.

Chapter I

Growth and Nutrient Acquisition of Some Tropical Legumes in Subtropical Okinawa, Japan

Abstract: The study was conducted in the laboratory as well as greenhouse of the Subtropical Field Science Center, University of the Ryukyus, Japan to evaluate growth characteristics, biomass production and nutrient content of chickpea (*Cicer arietinum*), dhaincha (*Sesbania aculeata*), grasspea (*Lathyrus sativus*), lentil (*Lens culinaris*), mungbean (*Vigna radiata*) and soybean (*Glycine max*). In this study all legumes were cultivated in both cultured and red soil in different season. In March plantation using cultured soil, plant height of all legume species except dhaincha, increased rapidly until 55 days after seed sowing (DAS) thereafter slowly, while dhaincha plant increased rapidly after 55 DAS. The number of leaves in chickpea increased rapidly from 55 DAS, whereas gradually in other species from 40 DAS. In March plantation, the highest plant biomass was obtained from dhaincha (41.84g DW pot^{-1}) followed by chickpea (16.45g DW pot^{-1}), grasspea (9.8g DW pot^{-1}), soybean (8.92g DW pot^{-1}), mungbean (8.47g DW $plant^{-1}$) and Lentil (4.4g DW pot^{-1}). Total nitrogen (%) was highest in dhaincha (4.73%) followed by grasspea (3.77%). All the legume plants contain 1.53-2.51 mg Ca, 1.87-3.79 mg K, 0.5-1.47 mg Mg, 0.08-0.11 mg Na, 0.01-0.18 mg P and 0.27-1.41 mg S per gram dry weight. When these legume species examined in red soil at same time, the highest plant biomass was obtained from soybean (4.35 g DW pot^{-1}) followed by dhaincha (2.84g DW pot^{-1}), chickpea (2.80g DW pot^{-1}), grasspea (2.75g DW pot^{-1}), mungbean (1.87g DW pot^{-1}) and lentil (0.54g DW pot^{-1}). As a seasonal trial in subtropical Okinawan, when mungbean and dhaincha were cultivated in cultured soil, mungbean and dhaincha produced dry biomass 41.43 g DM pot^{-1} and 117 g DM pot^{-1}, by around 5 and 3 times higher, respectively, as comparing the march plantation. In June plantation using red soil, mungbean and dhaincha plant produced biomass 18.96g DW pot^{-1} and 16.41g DW pot^{-1} by 11 and 6 times higher, respectively, comparing the March plantation. In November plantation using red soil, grasspea and chickpea plant produced biomass 10.56g DW pot^{-1} and 8.41g DW pot^{-1}, by around 4 times and 3 times higher, respectively, comparing the March plantation. However, the

study showed that all legume species could be grown in subtropical Okinawa. The legume species were different in biomass production as well as nutrient content. All the legume species could supply nutrients and organic matter, which are important for soil improvement and crop production in Okinawa, Japan.

Key words: Tropical legume, growth, biomass production, nutrient content, subtropical Okinawa.

INTRODUCTION

Legumes are very valuable due to their symbiotic N_2 fixation and their relatively low cell wall contents and C:N ratios, resulting in rapid release of N. Obtaining N from legumes is potentially more sustainable than from mineral fertilizers (Crews and Peoples, 2004) and they have been utilized as green manure in agriculture (Badaruddin and Meyer, 1990; Mappaona *et al.,* 1994). Grain legumes can provide 20-60 kg ha^{-1} residual N (Kumar Rao *et al.*, 1998). Forage legumes are widespread and have the potential to give high yields over a range of climatic conditions. In addition to the N benefits, legume crops have many other positive effects on subsequent crops, such as decreased plant diseases and weed density, improved soil structure and exudation of beneficial compounds, such as auxins, gibberellins and cytokinins. Apart from N, legumes also fix soil Ca, P, K, S and B (Frame *et al*., 1998; Marschner, 1995; Kuusela, 2006). The amount of N bound (symbiotically fixed) by legumes depends on the plant species and genotype (Unkovich and Pate, 2000), its N-fixation capacity, the amount of biomass formed, the *Rhizobia*-plant symbiosis and the efficiency of the symbiosis (Liu *et al*., 2011) as well as on environmental factors (Giller and Cadisch, 1995; Halling *et al.,* 2004). Soil properties also influence the growth of legumes. Beneficial effects depend on the selection of appropriate legume crops. Hence, understanding of their agronomy and physiology is fundamental for their use in sustainable cropping systems. Growth and development of a crop is determined genetically as well as influenced by environmental variables (Baligar and Fageria, 2007).

Many kinds of legumes grow well in poor fertile soil in tropical and subtropical regions, which improve soil fertility, decrease soil bulk density, increase soil aeration and water holding capacity, and improve soil microbial activities (Miyamaru *et al*., 2008; Sultani *et al*., 2007; Sullivan, 2003). Liao *et al.* (2006) reported that some tropical legumes have several adaptive mechanisms for growing in deficient P soils, where roots may exude organic compounds to mobilize P from bound P pools in the soil. Therefore, legume could be possible to cultivate in southwestern Japan, sub-tropical Okinawa, where major soils are red soil, dark-red soil and grey soil which are

phosphorous deficient (Oshiro *et al.*, 2016). The red soil and dark red soil have low pH value (5.4 and 6.6 respectively), on the other hand, grey soil has high pH value (8.4).

The important legume crops in tropical and subtropical regions are chickpea (*Cicer arietinum*), grasspea (*Lathyrus sativus*), dhaincha (*Sesbania aculeata*), mungbean (*Vigna radiata*), lentil (*Lens culinaris*), soybean (*Glycine max*), etc.

Chickpea is highly valued for its nutritional quality and health benefits and ability to improve soil fertility and sustainability of the cropping systems. It is an excellent source of protein, carbohydrate, dietary fibres, polyunsaturated fatty acids, minerals and vitamins (Jukanti *et al.,* 2012). It is also considered as a high energy and protein feed in animal diets (Bampidis and Christodoulou, 2011).

Sesbania aculeata, known locally as 'dhaincha', is a leguminous crop widely available in many tropical countries of Asia and Africa. Sesbania seeds are used as the source of gum in Pakistan and India (Evans and Rotar, 1987). It can grow in poor and degraded soil. The major use of the Sesbania crop has been as fodder for livestock and green manure to improve soil fertility. The sesbania seed contains 30-36% protein and may have potential as an ingredient in animal feeds including fish.

Lentil occupies a unique position in the world agriculture which plays a significant role in human and animal nutrition, and in maintenance and improvement of soil fertility. Its cultivation enriches soil nutrient status by adding nitrogen, carbon and organic matter which promotes sustainable cereal-based systems of crop production (Sarker *et al.,* 2011). The protein content of lentil seeds is varies from 21.2 to 32.5% (Purseglove, 1968; Dimitrove, 1973).

Soybean has a tremendous value in agriculture for source of high quality plant protein and vegetable oils and also capable to fix nitrogen. It contains about 40-45% protein, 20-22% oil, 20-26% carbohydrate and a high amount of Ca, P and vitamins (Rahman, 2001).

Grasspea is a multipurpose robust grain legume crop which can grow in both drought- and flooding-prone environments and poor soils due to its hardy and penetrating root systems (Campbell 1997; Vaz Patto *et al.*, 2006b). It has a high nutritional value (protein content ranging from 25 to 30 %), being important both for human food and animal feed. In addition to its uses as food and feed, symbiosis with

rhizobia allows an efficient nitrogen fixation in the soil, lowering the inputs needed in crop rotation and making them suitable to be used as green manure in sustainable farming systems (Hanbury *et al.*, 2000).

Mungbean is one of the most important pulse crops for protein supplement in subtropical zones of the world. It is widely grown in Indian subcontinent as a short duration catch crop between two principal crops. Mungbean contains 51% carbohydrate, 24–26% protein, 4% mineral, and 3% vitamins (Afzal *et al.,* 2008). Besides providing protein in the diet, mungbean has the remarkable quality of helping the symbiotic root rhizobia to fix atmospheric nitrogen and hence to enrich soil fertility (Anjum *et al.*, 2006).

Little research have been conducted to investigate growth and quality of tropical legume as green manure in subtropical regions. Legume can grow in subtropical Okinawa having favourable climatic features, but the volume of legume produced is very small. Therefore, present study has been conducted to evaluate growth and nutrient content of chickpea, dhaincha, grasspea, lentil, mungbean and soybean in response to subtropical climatic conditions.

MATERIALS AND METHODS

A series of experiments was conducted in a plastic house at the Subtropical Field Science Center of the University of the Ryukyus, Okinawa, Japan (26^0 N, 127^0 E) from March, 2016 to January, 2017 to evaluate growth and nutrient content of six tropical legumes species as green manure in response to subtropical climatic conditions. The legume species examined were chickpea (*Cicer arietinum*), dhaincha (*Sesbania aculeata*), grasspea (*Lathyrus sativus*), lentil (*Lens culinaris*), mungbean (*Vigna radiata*), and soybean (*Glycine max*). All legume seeds were collected from different research organizations in Bangladesh. All legume species were examined in cultured soils and red soil, treated as March plantation. Four species, i.e. dhaincha and mungbean were examined in cultured soil and red soil, respectively, treated as June plantation. Chickpea and Grasspea, were examined in red soil, treated as November plantation. Typhoon is another climatic factor which have huge impact to crop production and agro-ecosystems in Okinawa. In this context, this experiment was carried out in the plastic house to avoid the damage.

Experiment 1: March Plantation (March-May 2016)

In March plantation using cultured and red soil simultaneously, thirteen seeds of chickpea and mungbean, 23 seeds of grasspea and lentil, 9 seeds of soybean, 20 seeds of dhaincha were sown in cultured soil on March, 2016. The plants were thinned to 3 plants per pot at 11 days after sowing (DAS). All legume species were harvested on May, 2016. Each species was grown in seven (7) pots (Wagner pot, size 0.05 m^2) using cultured soil (Hanasaki monogatari) at March plantation. Plant height and leaf number were recorded at 25, 40, 55, 65 days after seed sowing (DAS). The study using red soil, thirteen seeds of chickpea and mungbean, 23 seeds of grasspea and lentil, 9 seeds of soybean, 20 seeds of dhaincha were sown on March, 2016. The plants were thinned to 3 plants per pot at 14 days after sowing (DAS). All legume species were harvested on May, 2016. Each species was grown in seven (7) pots (Wagner pot, size 0.02 m^2) using red soil at March plantation. Irrigation were provided in daily as per required.

Experiment 2: June Plantation (June-August 2016)

Ten seeds of dhaincha and mungbean were sown in both cultured and red soil, simultaneously, on June, 2016. The plants were thinned to 5 plants per pot at 14 days after seed sowing (DAS). Both legumes were harvested on August, 2016. Each species was grown in eight (8) pots (Wagner pot, size 0.05 m^2 and 0.02 m^2, respectively) using cultured and red soil, respectively, at June plantation.

Experiment 3: November Plantation (November 2016-Janury, 2017)

Thirteen seeds of chickpea and 23 seeds of grasspea were sown on November, 2017. The plants were thinned to 5 plants per pot at 14 days after seed sowing (DAS). Both legumes were harvested on January 2017. Each species was grown in eight (8) pots (Wagner pot, size 0.02 m^2) using red soil at November plantation.

Plant and Soil Sample Preparation for Chemical Analysis

Chopped legume plant was dried at 80 ^{0}C or 48 h using the forced convection oven (DRLF23WA, Advantec) for dry weight measurement and chemical analysis. Samples were prepared for chemical analysis according to method as described by Ohshiro *et al*., 2016. Plant powder of 0.25 g was taken into a 50 ml beaker, and the beaker was filled with 0.5% nitric acid (HNO_3). For extracting elements, beakers were kept into water bath adjusted to 80 ^{0}C for 24 h and the solution was filtered sequentially with paper No. 2 (Advantec Co. Ltd.) and disposable syringe filter (0.45 µm). The soil and plant solution was diluted as necessary by the addition of deionized water for determining the contents of mineral elements. The contents of Ca, K, Mg, Na, P, and S in soil and plant were determined by using a Multiple Inductivity Coupled Plasma Emission Spectrometer (ICPE-9000, Shimadzu Co. Ltd) and the total C and N contents were determined by using gas chromatograph (NC-220F, Shimadzu Co. Ltd., Japan).

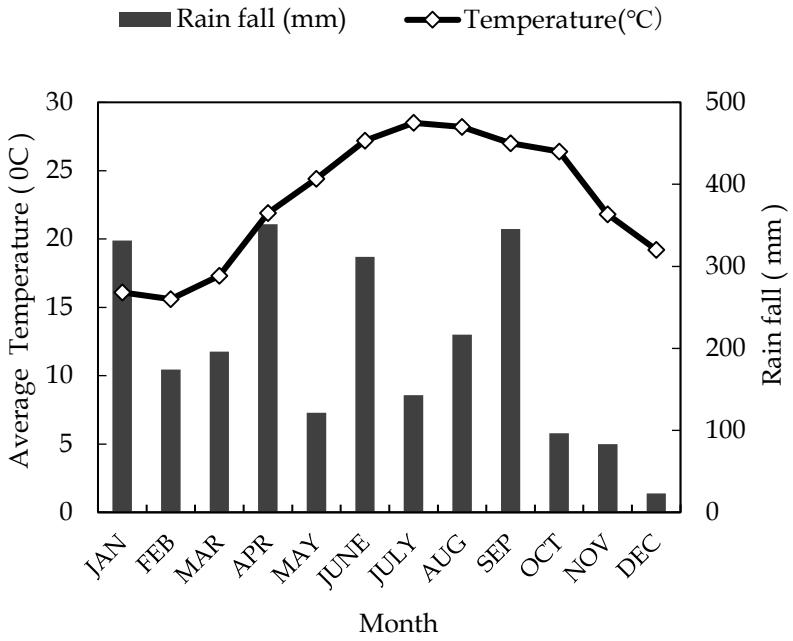


Fig. **I**. 1. Monthly Rain fall (mm) and average temperature (^{0}C) during experiment, 2016 (Data source: Japan Meteorological Agency).

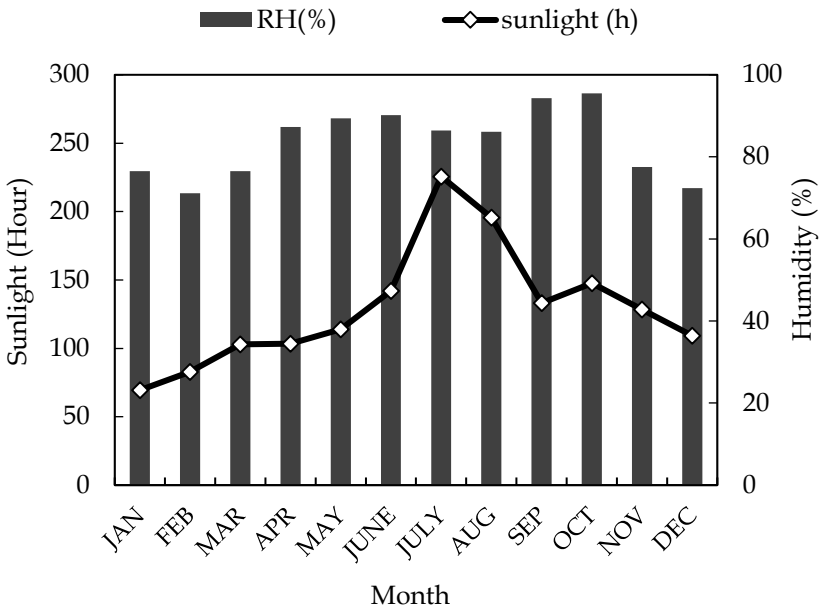


Fig. **I**. 2. Monthly sunlight (h) and average humidity (%) during experiment, 2016 (Data source: Japan Meteorological Agency).

Data analysis

All the data were subjected to analysis of variance. A one-way ANOVA was used for all the parameters. Means were separated by the Tukey's protected least significance difference (LSD) test at $p<0.05$.

RESULTS

In March plantation (photo **I**. 1, A), a significant difference in plant height was observed among the legume species throughout the growth period (Fig **I.** 3, A). Plant height of all legume species, except dhaincha, increased rapidly until 55 DAS, thereafter slowly. While dhaincha plant increased rapidly after 55 DAS. Variation of plant height over the growth period occurred probably due to individual genetic makeup of the legume crops. The number of leaves in chickpea increased rapidly from 55 DAS, whereas gradually in other plants from 40 DAS (Fig **I.** 3, B). At 65 DAS, chickpea produced leaf number 35 times compared to lowest produced in mungbean plant. The highest plant biomass was obtained from dhaincha (41.84g DM pot^{-1}) followed by chickpea (16.45g DM pot^{-1}), grasspea (9.8g DM pot^{-1}), soybean (8.92g DM pot^{-1}), mungbean (8.47g DM pot^{-1}) and Lentil (4.4g DM pot^{-1}) shown in fig **I.** 3, C. In March plantation (photo **I**, B) whereas all legume species examined in red soil, the highest plant biomass was obtained from soybean (4.35 g DM pot^{-1}) followed by sesbania (2.84g DM pot^{-1}), chickpea (2.80g DM pot^{-1}), grasspea (2.75g DM pot^{-1}), mungbean (1.87g DM pot^{-1}) and Lentil (0.54g DM pot^{-1}) shown in fig **I.** 3, D. Total nitrogen (%) was highest in dhaincha (4.73%) followed by grasspea (3.77%), lentil (3.36%), soybean (2.73%), mungbean (2.68%) and chickpea (2.67%), and Total carbon (%) was highest in dhaincha (41.96%) followed by grasspea (38.86%), soybean (38.54%), mungbean (37.54%), lentil (37.24%) and chickpea (35.83%), (Fig **I.** 4, A, B). All the legume plants contain 1.53-2.51 mg Ca, 1.87-3.79 mg K, 0.5-1.47 mg Mg, 0.08-0.11 mg Na, 0.01-0.18 mg P and 0.27-1.41 mg S per gram dry weight (Fig **I.** 4, C-D and Fig 5, A-D). All legume except dhaincha provided grain whereas soybean seed contain highest N (7.04%) followed by grasspea (5.59%) and C content in soyean (47.93%) followed by grasspea (42.15%). The highest N content was found in grasspea pod shell (4.45%) followed by 3.43% whereas C content in chickpea (41.36%) followed by grasspea (39.81%).

1	2	3	4	5	6

(A)

1	2	3	4	5	6

(B)

I. Photo 1. Growth of legume crops in cultured soil (exp. 1) and red soil (exp. 2) at 65 days after seed sowing in March plantation. [cultured soil exp.: 1-dhaincha; 2-lentil; 3-chickpea; 4-grasspea; 5-mungbean, 6-soybean, and red soil exp.: 1-soybean; 2-mungbean; 3-lentil; 4-chickpea; 5-grasspea; 6-dhaincha]

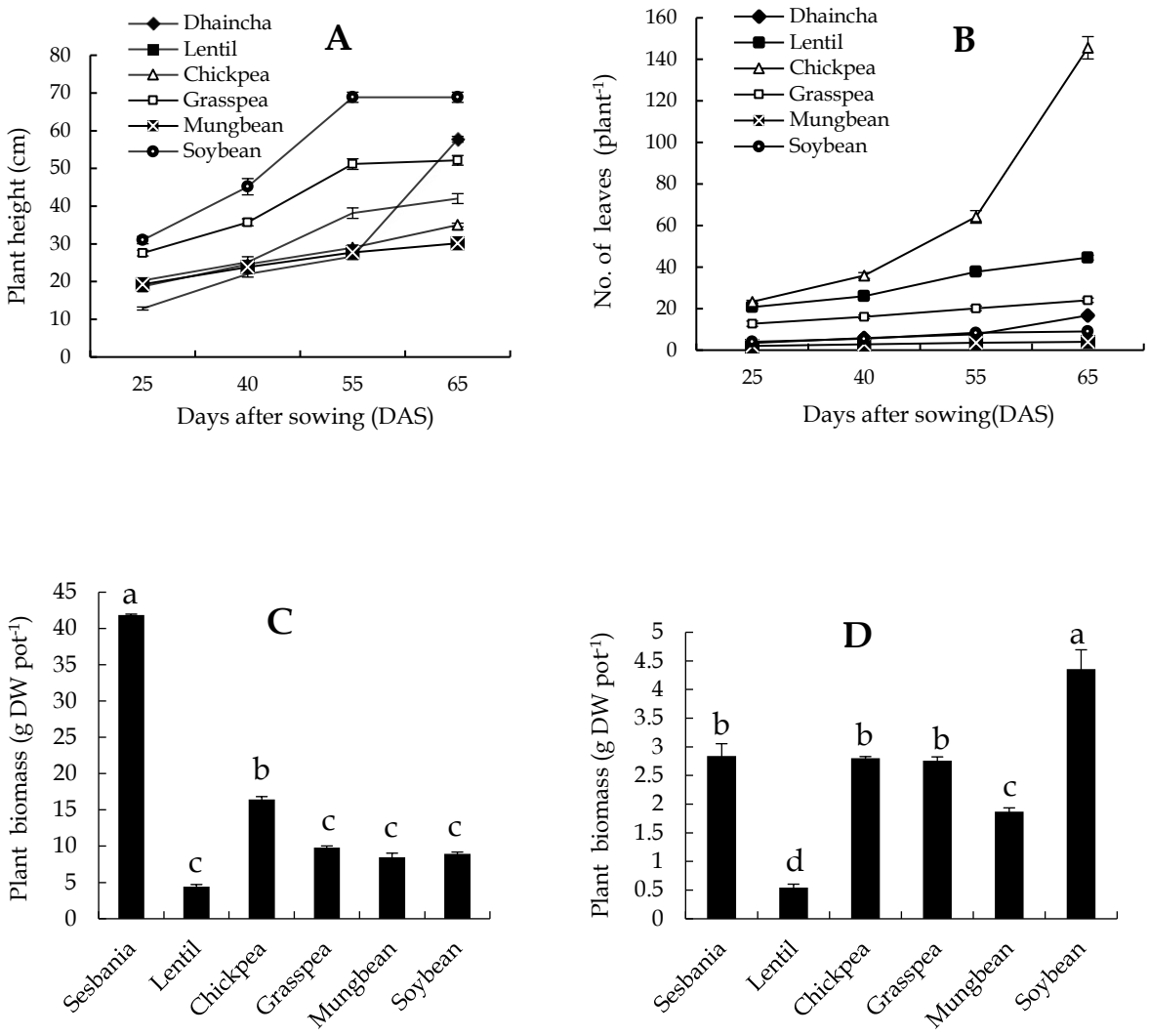


Fig **I.** 3. Plant height(cm), (**A)**; leaf number (plant^{-1}), (**B)**; biomass of different legume crops cultivated in cultured soil, (**C)** and red soil (**D**) at 65 DAS in March plantation. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p<0.05$.

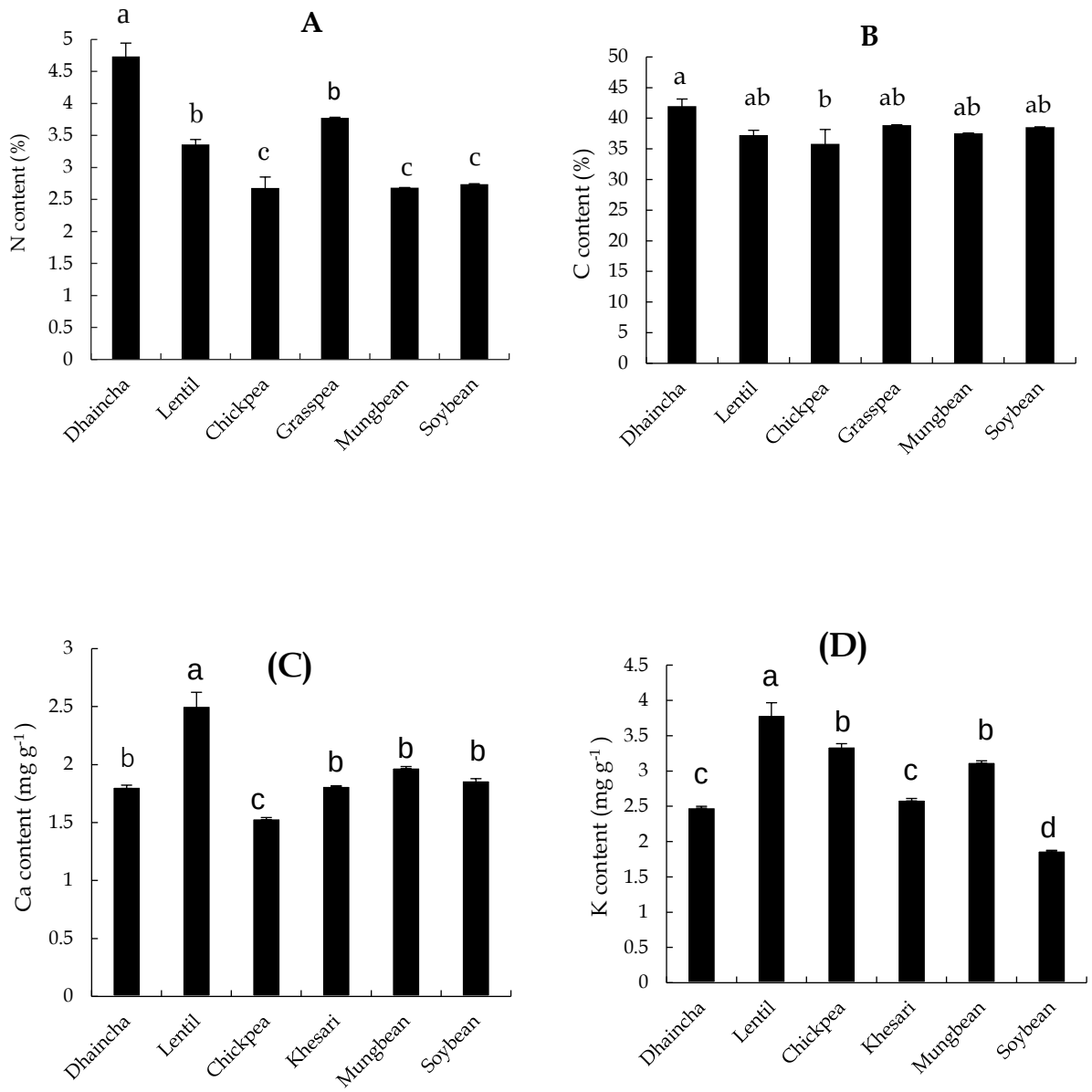


Fig **I**. 4. N (A), C (B), Ca (C) and K (D) content in dry biomass of different legume crops. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p<0.05$.

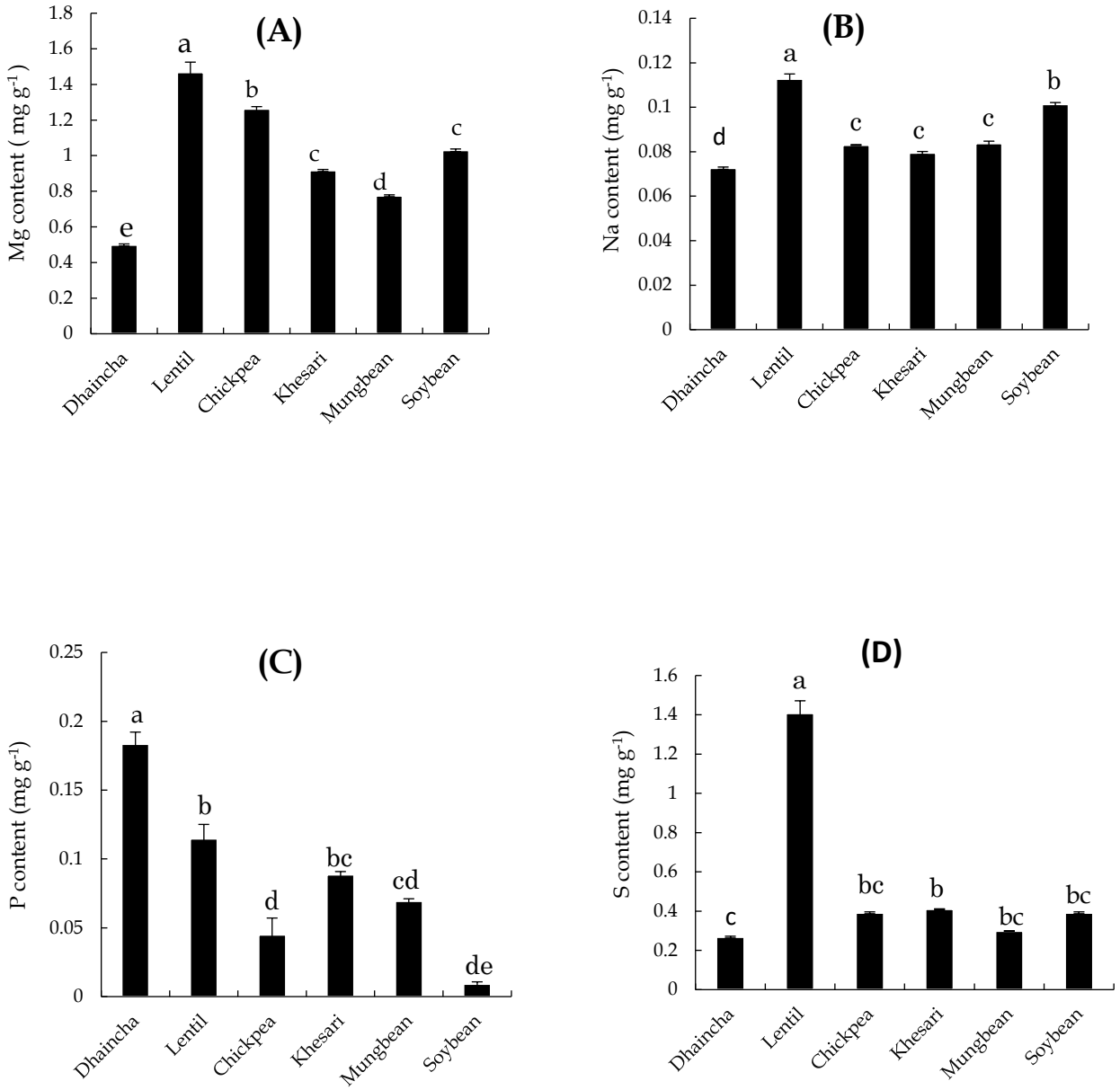


Fig**. I.** 5. Mg (A), Na (B), P (C) and S (D) content in dry biomass of different legume crops. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p<0.05$.

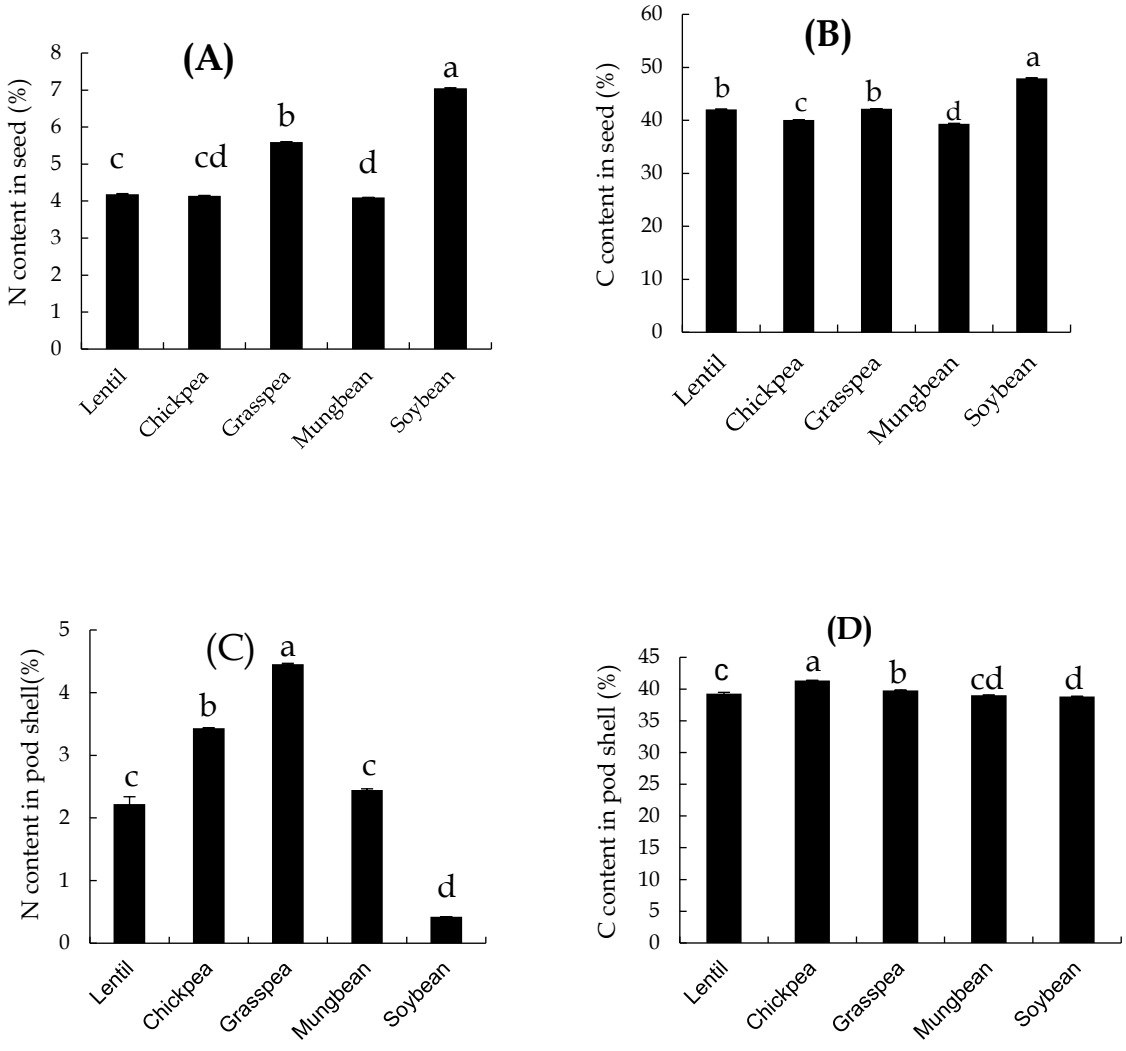


Fig**. I.** 6. N, C content in different seed and pod shell of grain legume crops. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p<0.05$.

In June plantation using cultured soil (Photo **I**. 2), plant height of mungbean and dhaincha was recorded 2.3 and 2.6 times higher than those in March cultivation in same soil and leaf number was 3.5 and 1.8 times higher, respectively, than those in March plantation. Mungbean and dhaincha produced biomass 41.43 g pot^{-1} and 117g pot^{-1}, respectively, which was about 5 and 3 times higher than those in March plantation (Fig **I**. 7A, B and 8 A, B).

In June plantation using red soil (Fig **I**. 9, A), mungbean and dhaincha produced biomass 18.96g DM pot^{-1} and 16.41g DM pot^{-1}, respectively. The mungbean plant biomass was around 11 times higher in June plantation than that in March plantation and dhaincha plant biomass was 6 times higher in June plantation than that in March plantation. In addition, In November plantation using red soil, chickpea and grasspea produced biomass 8.41g DM pot^{-1} and 10.56g DM pot^{-1}, respectively, which were around 3 and 4 times higher, respectively, in November plantation than those in March plantation (Fig **I**. 9, B).

Photo **I**. 2. Growth of Mungbean and Dhaincha crops 65 days after seed sowing in cultured soil on June plantation.

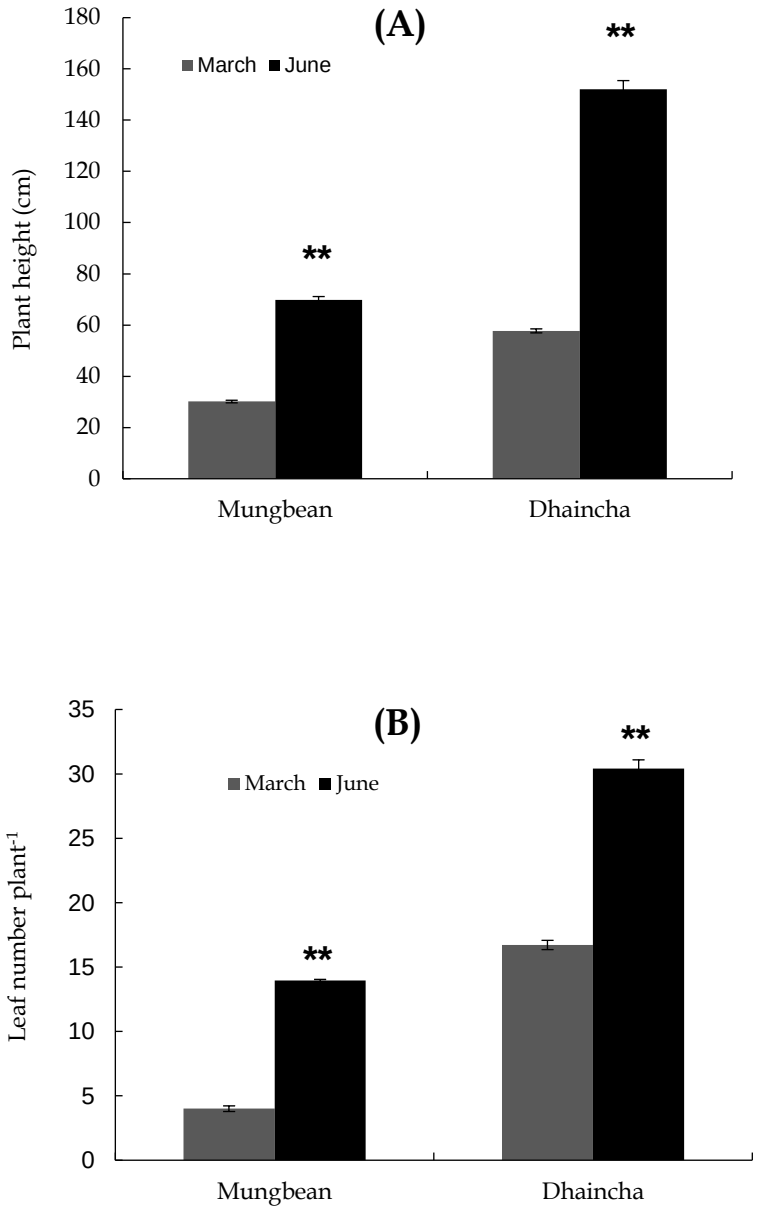


Fig. **I.** 7. Plant height(A), leaf number(B), of Mungbean and Dhaincha cultivated in cultured soil in two season (March and June). The asterisk (**) shows significantly different as determined by Student's T-test Protected LSD (least significance difference) test at $p<0.01$.

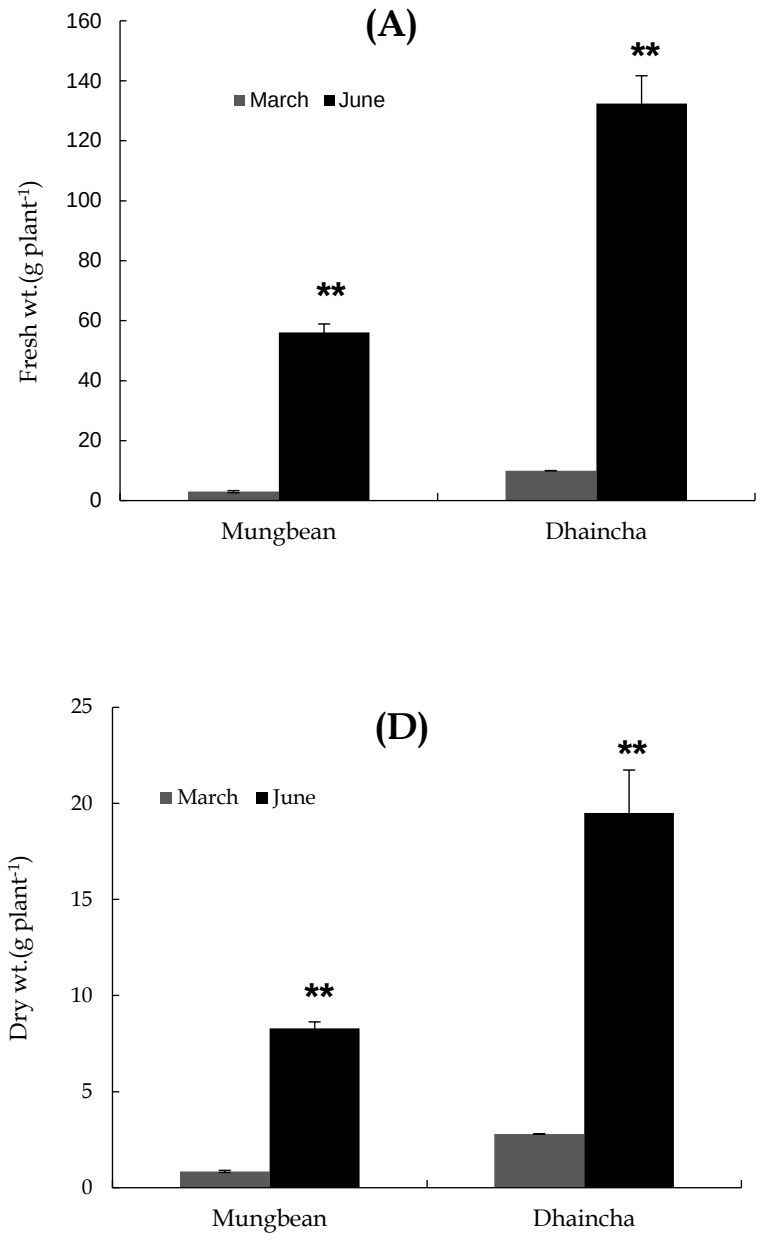


Fig. **I**. 8. Fresh (A) and dry (B) weight of legume plant between two season. The asterisk (**) shows significantly different as determined by Student's T-test Protected LSD (least significance difference) test at $p<0.01$.

June Plantation

Mungbean
(*Vigna radiata*)

Dhaincha
(*Sesbania aculeata*)

(A)

November Plantation

Grasspea
(*Lathyrus sativus*)

Chickpea
(*Cicer arietinum*)

(B)

Photo **I.** 3. Growth of legume crops cultivated in red soil at 65 days after seed sowing in June **(A)** and November Plantation **(B)**.

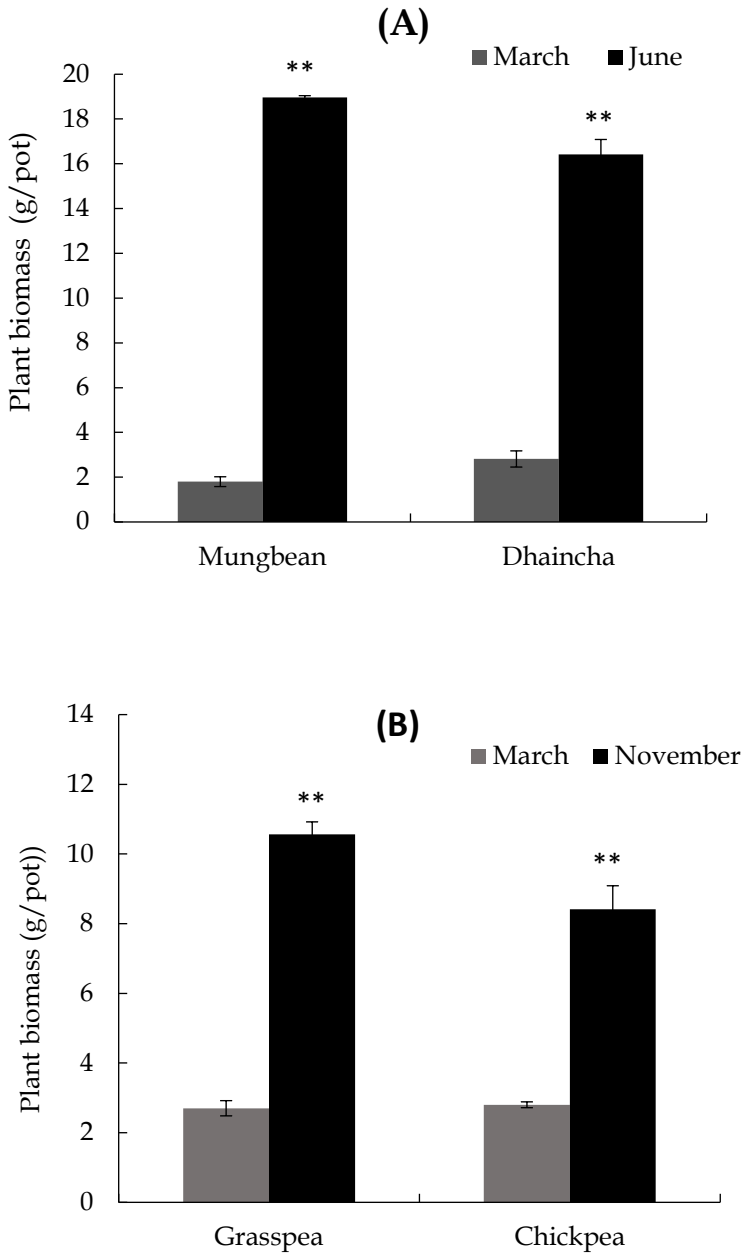


Fig. **I**. 9. Biomass of different legume crops (A and B) grown in red soil at two seasons. The asterisk (**) shows significantly different as determined by Student's T-test Protected LSD (least significance difference) test at $p<0.01$.

DISCUSSIONS

The legume species were different in growth characteristics and biomass production as well as nutrient content. Cultured soil had optimum moisture than other soils, which contributed to higher vegetative growth and shoot biomass as well as nutrient content of the legume plants. On the other hand, waterlogging was observed for a while in red soil after water application and the soil was compact when dried, which probably affected soil aeration, soil microbial activities, and nutrient absorption, and resulted in a lowest legume plant biomass in a same plantation time. In addition, increased length of growing season may or may not increase legume biomass and N content (Sainju and Singh, 2001)

Several studies reported that N is more significant than other nutrients for vegetative growth of plants (Akamine *et al.,* 2007; Chowdhury *et al.,* 2008; Hossain *et al.,* 2012). When climatic features were considered, mungbean and dhaincha plant produced higher biomass in June plantation compared to march plantation. The higher and rapid biomass growth of mungbean and dhaincha in subtropical Okinawa might have been related to the higher average temperature which is more favourable for these legume crops. Chickpea and grasspea produced higher plant biomass in November plantation compared to the March plantation. Time of sowing of legume plants might have a greater influence on plant to grow well coupled with favourable environment condition producing maximum yield contributing characters.

On red soils in subtropical regions, low inputs of chemical fertilizer and high inputs of organic matter derived from legume may be a more sustainable agricultural system than systems based on high chemical fertilizer inputs and clover cover crops. This will better determine the suitability of legume crops as a low-cost green manure for meeting the nutrients demand of subsequent crops.

Chapter II

Influence of Tropical Legumes on Growth, Yield and Quality of Turmeric (*Curcuma longa* L.) in Red Soil in a Subtropical Region

Abstract: Several tropical legumes such as Soybean (*Glycine max*), mungbean (*Vigna radiata*) and dhaincha (*Sesbania aculeata*) can play a significant role in crop production by incorporated to soil as green manure to reduce chemical N fertilizer by increase of minerals N in soil and add organic matter influence the availability of soil minerals. To investigate the effect of soybean, mungbean and dhaincha on red soil physico-chemical properties and growth, yield and quality of turmeric, an experiment was conducted with fresh biomass of soybean, mungbean and dhaincha at the rate equivalent to dry weight of 0, 92.4, 69.1, 81.4 g, respectively, per turmeric plant. Each pot was filled with 10 kg of red soil and one turmeric seed rhizome was planted. Soybean, mungbean and dhaincha provided 3.7-4.16 % N, 0.98-1.45 mgg^{-1} P and 21.07-40.73 mgg^{-1} K. The soil with green manure plant was found to be loose and had significantly higher soil moisture than untreated soil. Soil bulk density decreased from 0.92 to 0.84 (g cm^{-3}) with green manure application. Turmeric plants treated with soybean, mungbean and dhaincha survived longer and resulted in significantly higher growth parameters, shoot biomass and yield as compared to those with the control plants. When turmeric plant was cultivated with the application of soybean, dhaincha and mungbean plants, plant height of turmeric plant increased by 54 to 68 %, 35 to 42%, respectively as compared to control plants. The shoot dry weight of soybean, mungbean and dhaincha treated plants increased by 155-216%, respectively, compared to control plants. Mungbean, dhaincha and soybean green manure increased rhizome yield by 143, 101, 89.4%, respectively, compared to control plants. The highest turmeric yield was obtained with mungbean (213 g $plant^{-1}$) followed by dhaincha (176.9 g $plant^{-1}$) and soybean (166.4 g $plant^{-1}$). The results indicated that green manure crops improved soil nutrients and enhanced plant growth and yield of turmeric.

Key words: ***Tropical legume, green manure plants, growth, yield and quality, turmeric, red soil.***

INTRODUCTION

Legumes are important in agriculture as they form associations with bacteria in their root nodules and fix atmospheric nitrogen (Delfin *et al*., 2008). Legumes increase soil fertility through green manuring. Green manure legumes are fast growing and accumulate high biomass. Green manure legume plants add organic matter influence the availability of soil minerals. They also decrease the soil bulk density, increase soil aeration and water holding capacity and improve the soil texture, acting as a source of nutrients and increasing biological activity in the soil, restricting growth of weeds, helping in soil-borne disease control in suppressive soils (Gallandt *et al.* 1999; Urzua *et al*., 2001, Staver and Brinsfield, 1998; Teasdale, 1998; McGuire, 2003).

Dhaincha (*Sesbaniea aculeata*) is a quick growing succulent green manure crop which improve the soil fertility due to fast growth, high biomass production and ability to conversion of large amount of atmospheric nitrogen into the useable form of plants. Dhaincha produced dry matter 5-5.5 t ha^{-1} y^{-1} and accumulated 101-113 kg N ha^{-1} y^{-1}, all of which was returned to the soil when the above ground residue was incorporated as green manure (Sharma and Prasad, 1999). Soybean has a tremendous value in agriculture for source of high quality plant protein and vegetable oils and also capable to fix nitrogen. It's contains about 40-45% protein, 20-22% oil, 20-26% carbohydrate and a high amount of Ca, P and vitamins. Soybean is the best nitrogen fixer which produced 120 kg N ha^{-1} (Thonnissen *et al*., 2000). Mungbean accumulates large amount of biomass in a short period, so it could be used as a manure or as a cash cum soil improvement crop, by incorporating into the soil. Mungbean produced 0.5-0.6 t ha-1 y^{-1} grain and 3-3.4 t ha-1 y^{-1} residue and accumulated 92-96 kg N ha^{-1}, 20% of which was in the grain. Thus, incorporation of mungbean residue resulted in recycling of about 77 kg N ha^{-1} y^{-1}. (Morris *et al.,* 19860 and Jhon *et al.,* 1989, 1992) reported that a short duration green manure crop accumulated 62-74 Kg N ha^{-1}.

Turmeric (*Curcuma longa* L.) is an important herbaceous perennial spice crop with short stem and tufted leaves, commercial crop of the tropics belonging to the family Zingiberaceae whose origin is considered to be in South Asia. Turmeric is widely used as spice in Bangladesh, India, Myanmar, Pakistan, Sri Lanka, Nepal, Thailand and other Asian countries (Ahmed *et al.,* 1981, Hossain *et al.,* 2005; Singh *et al.,*1992; Umate

et al., 1984; Yamager *et al.*, 2001). The principal ingradient of turmeric called curcumin, whose medicinal values have been accepted worldwide. Curcumins of turmeric have antioxidant, anti-inflammatory, anti-carcinogenic, antibacterial and detox properties (Ammon and Wahl, 1991; Nakamura *et al*., 1998; Sugiyama *et al.*, 1996) And its effects to prevent tumor formation, to improve liver and kidney functions, and could be used to alleviate biliary and hepatic disorders (Herman and Martin, 1991). Chemical fertilizer application in turmeric production is not usually allowed since it is used for keeping good health as a natural medicine.

Turmeric is cultivated in tropical and subtropical regions in the world and also widely cultivated in Asian countries. In Japan, turmeric is commercially produced mainly in Okinawa prefecture where acid soil covers about 80% of land (red-55.1%, dark red-27.4%) which contains low organic matter, nutrients, microorganisms, and has low water holding capacity and high bulk density. Soybean, mungbean and dhaincha legume could be used as green manure to supply nutrients and organic matters, and to improve physical, chemical and biological properties of the soil.

Therefore, present study has been done to evaluate influence of soybean, mungbean and dhaincha green manure on growth, yield and quality of turmeric (*Curcuma longa* L.) in red soil of Okinawa, Japan.

MATERIALS AND METHODS

Experimental Site and Soil Properties

The pot experiment was conducted in a plastic house at the Subtropical Field Science Center, University of the Ryukyus, Okinawa, Japan. The experiment was conducted on red soil containing 0.74% C, 0.04%N with soil pH 7.84 which was collected from northern part of Okinawa at a depth of 50 cm. Red soil is usually acidic in the world, but the red soil in Okinawa contained high Ca which resulted in high pH vale. In addition, farmers have been using lime continuously in red soil from long before for different crop cultivation, therefore, pH in red soil is very high in Okinawa. Okinawa islands are surrounded by ocean and typhoon hits several times in a year which causes heavy saline rainfall and increases pH level in soil. It was reported that red soil pH is 4.4-8.9 in the northern part of Okinawa main land (in Japanese, www.nda.ac.jp/~yamaguch/5.pdf). The contents of K, P, Ca, Mg, Na and S are 12.89, 3.77, 395.32, 28.78, 57.84 and 193.56 mg kg^{-1} soil, respectively.

Plant Materials and Experimental Design

Soybean, mungbean and dhaincha seeds were collected from different research organizations in Bangladesh, and grown in April up to May, 2016 using cultured soil (Hanasaki monogatari) in Wagner pots (size 0.05 m^2) in a plastic house at the Subtropical Field Science Center of the University of the Ryukyus, Okinawa, Japan. After harvesting, the biomass of Soybean, mungbean and dhaincha were cut in 2 to 3 cm with straw cutter and that dry biomass was measured after 48 hours oven dry. One kilogram of fresh soybean, mungbean and dhaincha biomass equivalent to 264.0 g, 197.42 g, 232.57 g dry weight, respectively, and their chemical properties are shown in table **III**.1 and were mixed with 10 kg of red soil in each Wagner pot (25 cm diameter x 30 cm height, 0.05 m^2) according to the experimental treatments. The study was conducted in same house from June to December 2016 whereas four treatments were used, Control (CTRL), Soybean 350 g (S-TP), Mungbean 350 g (M-TP) and Dhaincha 350 g (D-TP). Each treatment consisted of 15 replications with randomized complete block design. One seed-rhizome (30 ± 1g) of turmeric (cv. Ryudai gold) was planted

per pot at 8 cm depth on June 15, 2016. Water was applied to the plants as required and corks were used to prevent water leaching.

Table **II.** 1. Chemical properties of legume biomass used for the experiment.

Legume	**TN** (%)	**TC** (%)	**Ca**	**K**	**Mg**	**Na**	**P**	**S**
			mgg-1					
Soybean	3.7	40.87	15.21	21.07	7.99	0.73	0.98	1.81
Mungbean	3.45	39.57	19.01	28.47	7.45	0.72	1.45	2.01
Dhaincha	4.16	40.93	16.41	40.73	4.21	0.74	1.42	3.07

Photo **II.** 1. Green manure plants used in turmeric production using red soil, 2016.

Data Measurement

Data regarding growth of turmeric plants started to be recorded from after the planting date on. Plant height, leaf number, tiller number and SPAD value were measured at 60, 80,106, 120, 140, 164 and 180 days after planting (DAP). The SPAD value (Chlorophyll-meter reading) of two fully expanded top leaves from each main shoot was measured using a chlorophyll meter (SPAD-502, Minolta Co Ltd.). Dry leaf and stem, fresh and dry rhizome, dry root, no. of mother and daughter rhizome, length of largest mother and daughter rhizome were measured at 220 days after planting (DAP). Soil bulk density and soil pH were determined at 0 (beginning of turmeric planting) and 220 DAP (after harvesting of turmeric planting). Total N and C, and water-soluble P and K, Ca, Mg, Na, S in soil were determined at 0 (beginning of turmeric planting) and 220 DAP (After harvesting of turmeric planting). Contents of N and C, P and K, Ca, Mg, Na, S in shoot (leaf + stem), rhizome and root, and curcuminoids (curcumin, demethoxy curcumin, and bis-demethoxy curcumin) in rhizome were measured at harvesting time. Chopped legume plants and sliced rhizomes of turmeric were dried at 80 ^{0}C for 48 h using the forced convection oven (DRLF23WA, Advantec) for dry weight measurement.

Soil, Turmeric and Legume Plants Sample Preparation for Chemical Analysis

Soil samples were dried at room temperature 25-28 ^{0}C for 5 days, and Shoots and sliced rhizomes of turmeric were dried at 80 °C for 48 h. Soil, shoot and rhizomes of turmeric were ground finely for chemical analysis. Soybean, mungbean and dhaincha samples were similarly prepared.

Determination of Soil pH, Bulk Density and Moisture Content

Soil samples were dried at 110 ^{0}C for 5 h and the soil bulk density was calculated according to the standard methods (Nakano *et al*., 1995). Dried soil powder of 20 g was diluted with 50 ml distilled water, and the solution was shaken for 1 h using an electrical shaker at 170 rpm (Neo- Shaker, NSA-SNH, As One Corp. Ltd.). The solution was filtered using paper No. 2, and then centrifuged for 90 min (Table top centrifuge 4000, Kubota Co. Ltd.) at 3500 rpm followed by filtering with the disposable syringe filter 0.45 µm (Advantec Co. Ltd.). Soil pH was determined with TOA pH meter (HM-

20 S, Toa Electronic Ltd.). Soil samples were dried at 98 ^{0}C for 24 h to determine the moisture content by Cylinder method (Margesin *et al*., 2005).

Determination of Minerals Content in Soil, Legume Plant and Turmeric Plants

Samples were prepared for chemical analysis according to method as described by Ohshiro *et al*., 2016. Soil samples (10 g) and distilled water (50ml) were taken into a beaker, and the solution was shaken with shaker (NEO SHAKER AS ONE CO. Ltd) for 1 h at 170 rpm. The solution was filtered with paper no. 2, and then centrifuged for 90 min at 3500 rpm, followed by filtering with a disposable syringe filter of 0.45 µm (Advantec Co. Ltd.). Plant and rhizome powder of 0.25 g was taken into a 50 ml beaker, and the beaker was filled with 0.5% nitric acid (HNO_3). For extracting elements, beakers were kept into water bath adjusted to 80 ^{0}C for 24 h and the solution was filtered sequentially with paper No. 2 (Advantec Co. Ltd.) and disposable syringe filter (0.45 µm). The soil and plant solution was diluted as necessary by the addition of deionized water for determining the contents of mineral elements. The contents of Ca, K, Mg, Na, P, and S in soil and plant were determined by using a Multiple Inductivity Coupled Plasma Emission Spectrometer (ICPE-9000, Shimadzu Co. Ltd) and the total C and N contents were determined by using gas chromatograph (NC-220F, Shimadzu Co. Ltd., Japan).

Statistical Analysis

All the data were subjected to analysis of variance. A one-way ANOVA was used for all the parameters. Means were separated by the Tukey's protected least significance difference (LSD) test at $p< 0.05$.

RESULTS

The application of soybean, mungbean and dhaincha plants significantly increased plant height, tiller number, leaf number, SPAD value at different days after planting (DAP) as compared to the control. Differences in plant height of turmeric generally started to be notable among the treatments after 60 DAP (Fig. **II**. 1), plant height of mungbean progress on average of 5 replications was from 68.20 cm to 101.80 cm approximately between 60 DAP up to 180 DAP. The mean of plant height progress was from 60.40 cm to 99.60 cm for dhaincha plants and 58.00 cm to 93.60 cm for soybean plants for the same period whereas control plants was from 53.00 cm to 60.60 cm. When turmeric plant was cultivated with the application of soybean, dhaincha and mungbean green manure plant height increased by 54 to 68 % compared to control plants. Leaf number significantly increased at different days after planting as compared to control plant (Fig. **II**. 1). At 180 DAP, mungbean, soybean and dhaincha treated plants increased leaf number 42, 39 and 35%, respectively, compared to control plants. At 180 DAP, Leaf number of mungbean treated plants increased 2.3 % compared to soybean and 4.8% compared to dhaincha treated plant and soybean treated plants increased 2.4% compared to dhaincha treated plant. At 60 and 80 DAP, leaf number of mungbean treated plants significantly increased rather than soybean and dhaincha treated plants but soybean and dhaincha treated plants showed statistically same result at these days. Also, during the whole growth period, significant difference was observed between treatment and control plants.

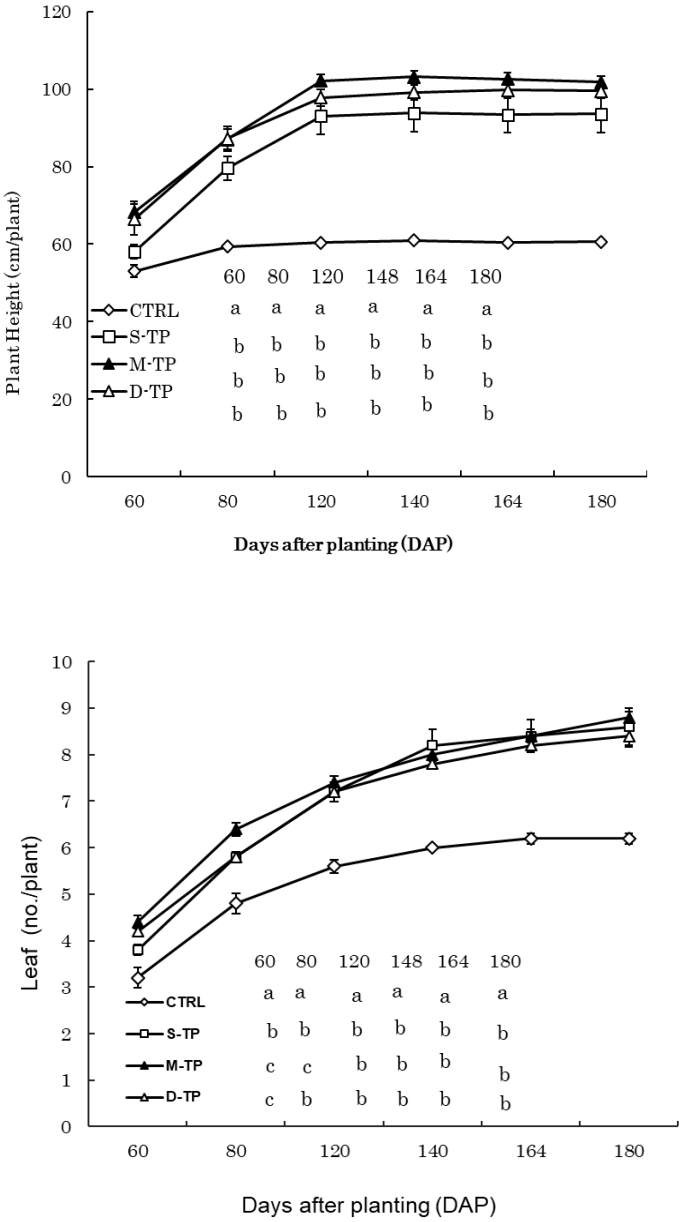


Fig **II.** 1. Effects of soybean, mungbean and dhaincha on plant height and leaf no. of turmeric in pot experiment, 2016. Data are means ±SE of 5 replications. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p<0.05$

In control plants, SPAD value decreased significantly after 60 DAP, while in soybean, mungbean and dhaincha treated plants it decreased significantly after 120 DAP (Fig. **II**. 2). However, SPAD value decreased rapidly in control plants than soybean, mungbean and dhaincha treated plants. Soybean, mungbean and dhaincha treated plants survived longer. Higher SPAD value probably contributed to a higher photosynthesis and resulted in higher growth and yield of turmeric. Similarly, Hossain *et al.* (2015) found that the SPAD value was higher in turmeric leaves due to the additional amount of N supplied by the green manure plants. Shoot dry biomass of turmeric increased significantly with all legume green manure treated plants as shown in Fig **II**. 2. Highest shoot biomass were found in dhaincha (18.16 g plant^{-1}) treated plants followed by mungbean (17.63 g plant^{-1}) and soybean (14.63 g plant^{-1}) treated plants. The shoot dry weight of soybean, mungbean and dhaincha treated plants increased by 155-216%, respectively, compared to control plants whereas Hossain *et al.* (2015) reported that leaf dry weight and shoot dry weight increased by 157-184% and 173-197% respectively, when turmeric was cultivated with the application of 1.66 kg hairy vetch plants.

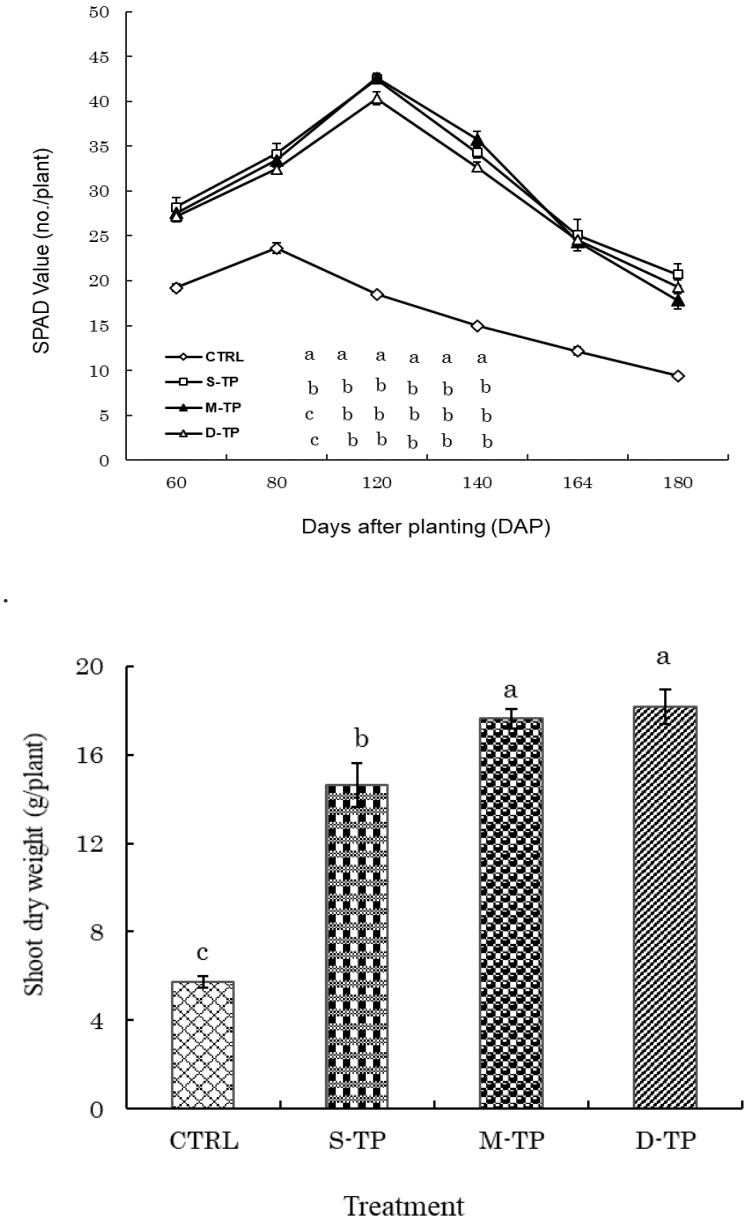


Fig **II**. 2. Effects of soybean, mungbean and dhaincha on SPAD value and shoot dry weight of turmeric in pot experiment, 2016. Data are means ±SE of 5 replications. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p<0.05$.

Fresh weight of rhizome (yield) significantly increased with the application of soybean, mungbean and dhaincha green manure plants compared to control plants as shown in Fig. **II**.3. The highest yield was obtained from mungbean (213 g/plant) followed by dhaincha (176.9 g/plant), soybean (166.4 g/plant) but they showed statistically similar values. Highest yield (213 g/plant) was 2.4 times more than control (87.83 g/plant). Mungbean, dhaincha and soybean green manure increased rhizome yield by 143, 101 and 89.4%, respectively, compared to control plants. Mungbean treated plants increased yield by 20-28% compared to dhaincha and soybean treated plants and dhaincha treated plants increased yield by 6.3% compared to soybean treated plants. Green manures improved the root system of turmeric, so the roots could absorb the minerals and irons from soil solution efficiently, resulting in higher yield.

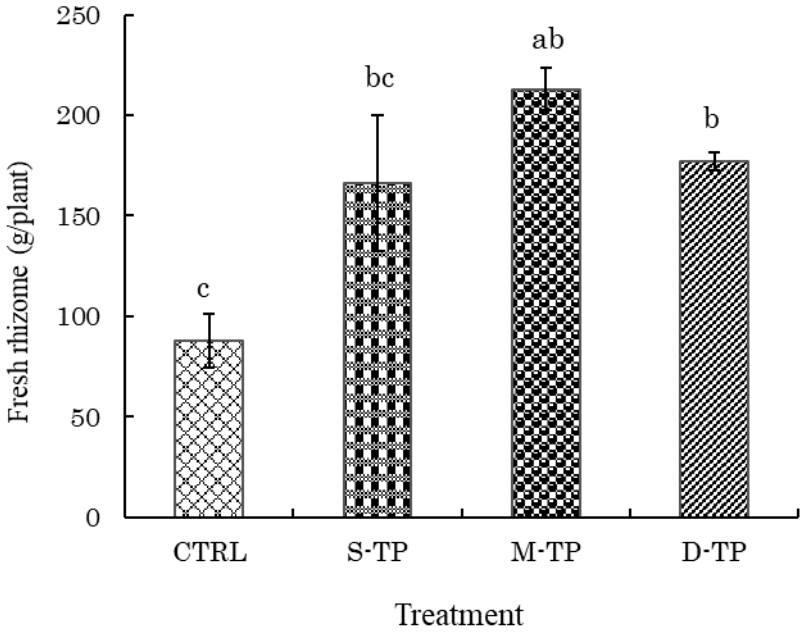


Fig.**II**.3. Effects of soybean, mungbean and dhaincha on fresh yield of turmeric in pot experiment, 2016. Data are means ±SE of 5 replications. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p<0.05$.

Photo II. 2. Effect of soybean, mungbean and dhaincha plants on rhizome yield of turmeric in red soil.

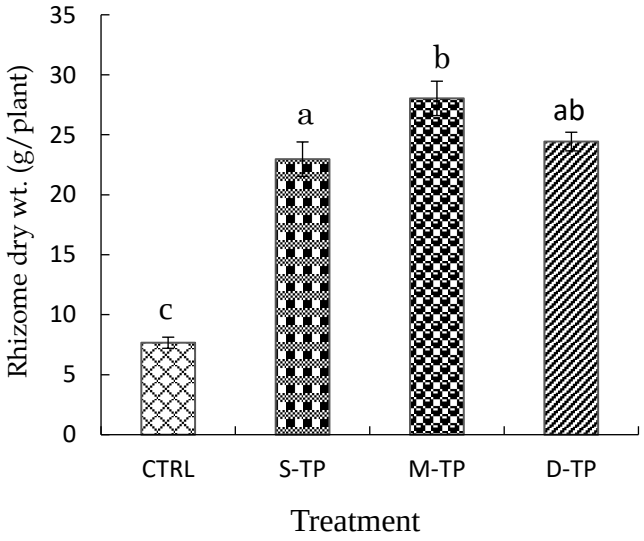


Fig **II**. 4. Effects of soybean, mungbean and dhaincha on dry yield of turmeric in pot experiment, 2016. Data are means ±SE of 5 replications. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p<0.05$.

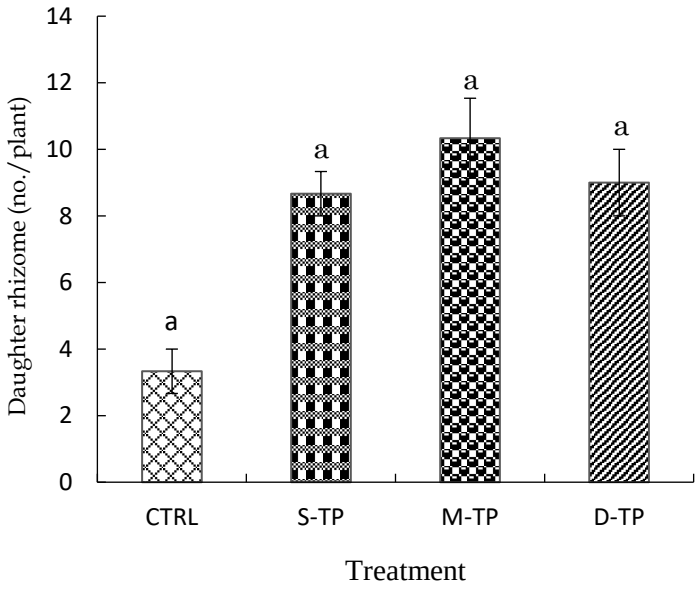


Fig **II**. 5. Effects of soybean, mungbean and dhaincha on daughter rhizome and dry yield of turmeric in pot experiment, 2016. Data are means ±SE of 5 replications. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p<0.05$.

N content is higher in dhaincha treated turmeric rhizome (0.208 g plant^{-1}) followed by mungbean (0.189 g plant^{-1}) and P content is higher in mungbean treated rhizome (48.9 mg plant^{-1}) followed by soybean (40.53 mg plant^{-1}). The K content is higher in mungbean treated turmeric rhizome (1109.30 mg plant^{-1}) followed by dhaincha treated rhizome (1022.31 mg plant^{-1}) and Ca content is higher in mungbean treated rhizome (166.42 mg plant^{-1}) followed by dhaincha treated rhizome(156.5 mg plant^{-1}). The Mg content is higher in mungbean treated rhizome (62.74 mg plant^{-1}) followed by daincha treated rhizome (54.8 mg plant^{-1}). The S content is higher in mungbean treated plant (23.67 mg plant^{-1}) followed by soybean treated turmeric rhizome (Fig. **II.** 6).

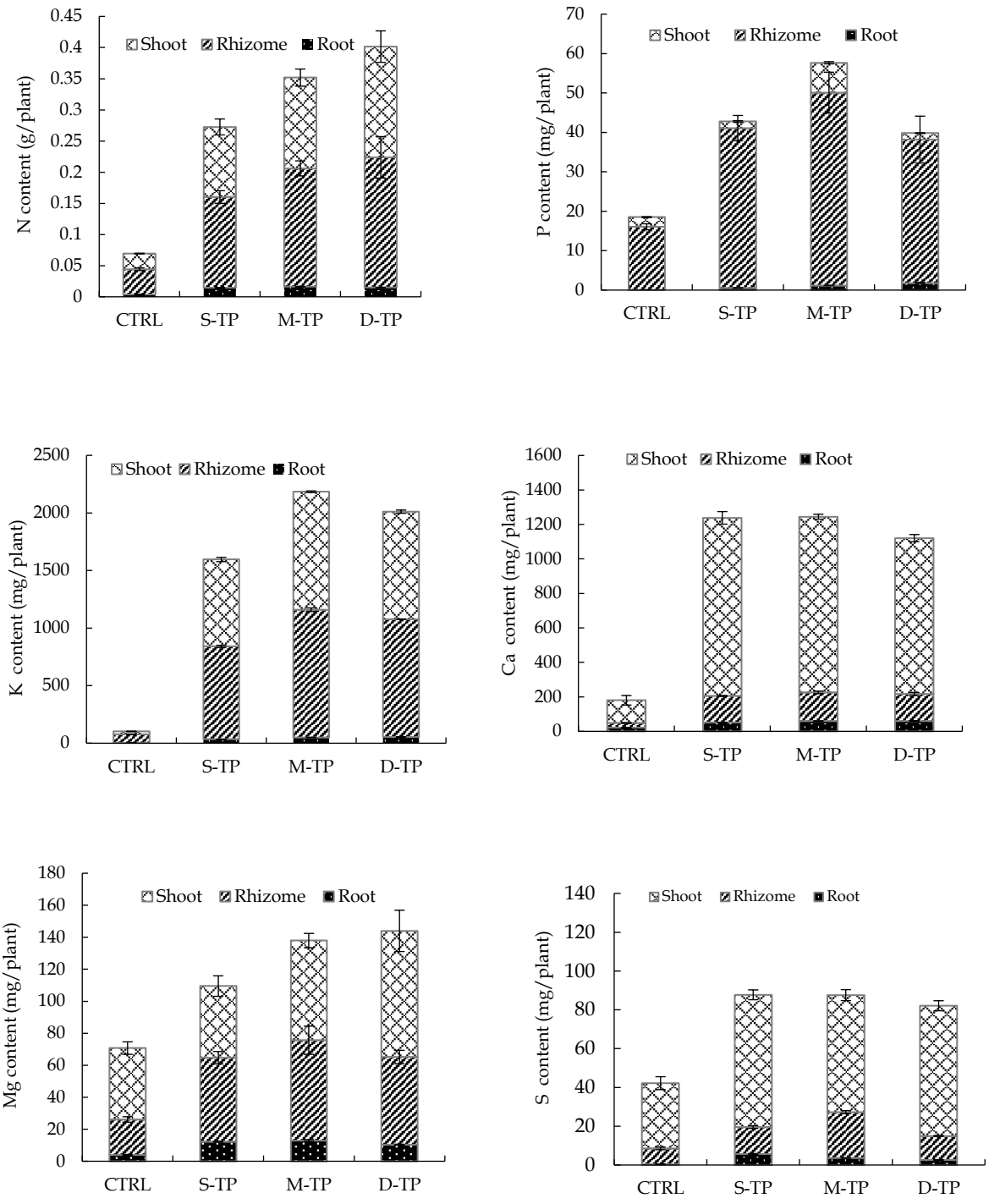


Fig. **II.** 6. Effects of soybean (S-TP), mungbean (M-TP) and dhaincha (D-TP) on total N, P, K, Ca, Mg and S content in turmeric plant. Data are means ± SD of 3 replications and data were checked by Tukey's Protected LSD (least significance difference) test at $p < 0.05$.

Soil bulk density was significantly reduced due to application of legume. The highest bulk density was observed in untreated soil (0.92 gm^{-3}) and the lowest in dhaincha treated soil (D-TP). Soil bulk density decreased by 3-9% (Calculated from Fig. **II**. 5.) with legume incorporation. Due to application of legume biomass, soil pH has been significantly changed. The pH decreased 1.06, 1.33 and 2.42 %, respectively, in soybean (S-TP), mungbean(M-TP), and dhaincha (D-TP) treated soil as compared to untreated soil, and statistical analysis showed that these reductions were significantly different (Fig. **II**. 7). This result indicates that legume plant could be used for adjusting pH level in soil.

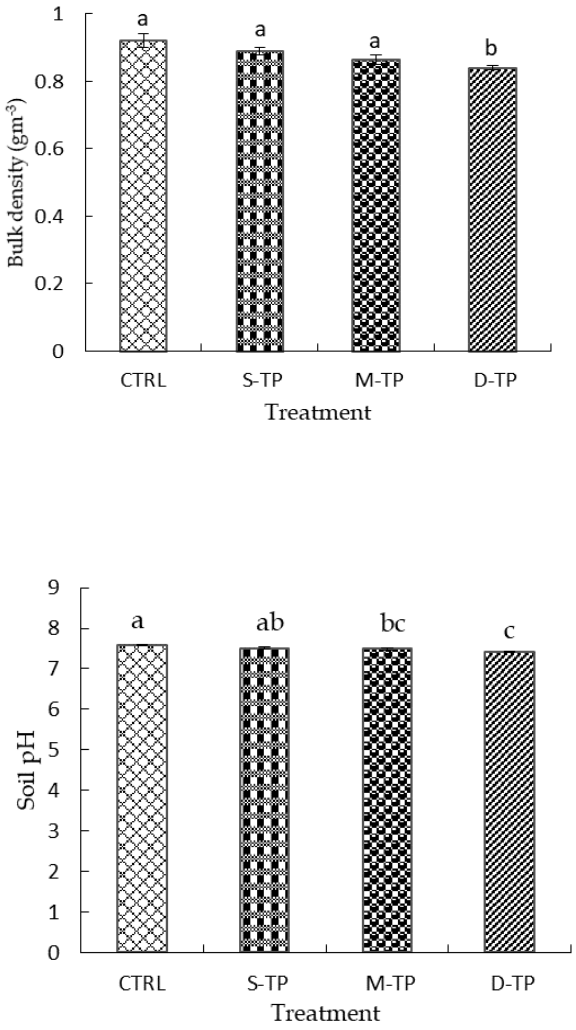


Fig **II**. 7. Effects of soybean, mungbean and dhaincha on soil bulk density and soil pH of turmeric cultivated soil in pot experiment, 2016. Data are means ±SE of 5 replications. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p<0.05$.

DISCUSSION

The application of soybean, mungbean and dhaincha legume plant significantly changed soil physicochemical properties. The soil bulk density was significantly reduced due to application of legume biomass in soil. The availability of legume biomass increased activities of beneficial soil microorganisms in organic matter decomposition which led to enhancement of soil porosity and reduction in soil bulk density. The soil pH was lower in legume treated soil compared with untreated soil which may be attributed to the release of CO_2 and organic acids during decomposition of legume biomass and reduction in exchangeable cations. This result is in line with the findings from other scientists (Hossain *et al*., 2015, Adekiya *et al*., 2017). The increase in plant height is due to the better nutrient availability and higher uptake that promoted plant biomass production. Nitrogen supplied by legume biomass ensured favorable condition for the growth of turmeric with optimum cell division and elongation of cell resulted the tallest plant. This might be due to protein synthesis by the help of nitrogen which enhance vegetative growth of turmeric plant. Hossain *et al*. (2015) reported that turmeric plant height increased by 20% with the application of green manure plants in glasshouse study. Turmeric cultivated with soybean, mungbean and dhaincha treated soil developed maximum number of leaves due to the additional amount of N. This findings was supported by Hossain *et al*. (2015). The SPAD value was higher in turmeric leaves due to the excess amount of N supplied by these legume after incorporation. Higher SPAD value probably contributed to a higher photosynthesis and resulted in higher growth and yield of turmeric. Findings regarding to the SPAD value in turmeric leaves were agreed with the results of Hossain *et al*. (2005). There was an overall delay in senescence in soybean, mungbean and dhaincha treated turmeric plant which was particularly survived longer in compared to untreated plants. It is assumed that longer survival of turmeric plants with higher leaves provided higher photosynthesis which ultimately resulted in higher yield. The application of legume plants increased shoot biomass of turmeric plants. The legume probably improved soil physicochemical properties, water holding capacity, aeration, mineralization and nutrient digestion in soil, which contributed to the increase of shoot weight of turmeric. The legume treated turmeric

plant increased yield as compared with untreated plants. Turmeric plants cultivated with legume showed a higher tiller number with greener leaves and survived longer which generally contributed to higher photosynthesis and resulted in higher yield. Goto *et al*. (2000) and Sullivan *et al*. (2003) reported that application of green manure could improve soil properties, water holding capacity, aeration, mineralization and nutrient releasing in soil that played role to the enhance of shoot biomass and rhizome yield of turmeric. The yield of turmeric significantly increased that generally determined probably due to the supply of higher N and other nutrients by applied legumes which is agreed with other research (Sainju *et al*., 2002, Rochester *et al*., 2005). Hossain *et al*. (2015) showed that when turmeric plant was cultivated with green manure plants, concentration of N in rhizome increased significantly while concentration of K and P decreased. Mappaona *et al*. (1994) reported that P and K concentration decreased in cabbage when green manure was incorporated with soil. The Ca, Mg, Na and S content in rhizome also decreased significantly with soybean, mungbean and dhaincha application. It is considered that the N supplied from applied legumes which accounted as excessive and it did not stimulate the accumulation of P, K, Ca, Mg, and S which resulted in lower values of the elements in rhizome.

Chapter III

Effect of Hairy vetch (*Vicia villosa* R.) Legume on Red Soil Properties, Growth, Yield and Quality of Turmeric (*Curcuma longa* L.) in a Subtropical Region

Abstract: Red soil is widely distributed in tropical and subtropical regions, which contains low organic matter and nutrients. Hairy vetch legume improves low fertile soil by supplying organic matters and crop nutrients. This experiment evaluated the effect of hairy vetch on red soil properties and growth, yield and quality of turmeric. The pot experiment was conducted using fresh hairy vetch at the rate equivalent to dry weight of 0, 94, 186, 280 g per turmeric plant. Each pot was filled with 10 kg of red soil and one turmeric seed rhizome was planted. Hairy vetch provided 5.01-15.03 g N, 0.1-0.3 g P and 1.57-4.71 g K per pot. The soil with hairy vetch was found to be loose and had significantly higher soil moisture than the soil without hairy vetch. Soil bulk density decreased from 0.68 to 0.60 (g cm^{-3}) with the hairy vetch application. All the hairy vetch treated turmeric plants survived longer and had significantly higher plant height, tiller number, leaf number, SPAD value, shoot biomass and yield as compared to control plants. Highest yield was obtained with 280 g hairy vetch treated turmeric plants, which was statistically similar to 186 g hairy vetch treated plants, indicating that about 186 g dry (9 ton ha^{-1}) or equivalent fresh hairy vetch could be incorporated into red soil for higher yield. The overall results indicate that hairy vetch is an important legume in subtropical region which could provide huge organic matter and plant nutrients, and improve soil properties and crop yield.

Key words: Hairy vetch, legume, turmeric growth and yield, rhizome.

INTRODUCTION

The application of leguminous green manure, rather than synthetic fertilizers, is recommended for sustainable agricultural production which is especially environmentally friendly, and it is considered to be an important tool which is effective to reduce the use of synthetic fertilizers and to improve nutrient status, organic matters, soil aeration, water holding capacity, decrease soil bulk density and increase soil microorganism (Sullivan, 2003, Elfstrand *et al*., 2007, Hossain *et al*., 2015, Adekiya *et al*., 2017, Caban *et al*., 2018). The soil having lower organic matter and N with higher soil bulk density can be improved by green manures plants (Sullivan, 2003, Sultani *et al*., 2007, Miyamaru *et al*., 2008). Leguminous plants are more effective than other plants because root nodule bacteria of these plants accumulate N_2 from the atmosphere by fixation. Hairy vetch (*Vicia villosa* R.) is the best green manure plant for nitrogen source that can produce 6-8 ton ha^{-1} dry matter and supplies up to 222 kg N ha^{-1} in a season (Smith *et al*., 1987, Choi *et al*., 2008, Parr *et al*., 2011). Hairy vetch is a fast growing legume which produces shoot biomass ranged from 4-5 ton ha^{-1} in four types of soil (yellow, red, dark red, and gray soil) in Okinawa within 3-4 months. Nitrogen, P, K, Ca and Mg accumulated in hairy vetch shoots were 11.5–15.4, 0.9–1.7, 5.7–8.4, 3.4–6.4 and 0.7–1.2 g m^{-2}, respectively (Fajri *et al*., 2010). Incorporation of hairy vetch residue is rapidly decomposed and supplied much inorganic N in soil due to high plant N concentration (4% N, C/N ratio: 10) and resulted in significantly increased corn yield (Power *et al*., 1991, Varco *et al*., 1989, Sarrantonio *et al*., 1988). Turmeric (*Curcuma longa* L.), belongs to the family Zingiberaceae, is commonly used as medicine, condiment, dye and cosmetic in the most Asian countries (Ahmed *et al*., 1981, Hossain *et al*., 2005, Singh *et al*., 1992, Yamgar *et al*., 2001). It is now very important in medical science because the main bioactive compounds, curcuminoids, of turmeric have antioxidant, anti-inflammatory, anti-malarial, anti-tumor forming, anti-mutagenic, anti-carcinogenic and chemotherapeutic properties (Prasad *et al*., 2014). Calcium, Mg, Fe, protein and fat are also considered to be important quality parameters of turmeric. Various supplements and drinks derived from the turmeric are widely being used for keeping good health (Hossain *et al*., 2005a, Hossain *et al*., 2005b). The application of the chemical fertilizer, herbicide and pesticide pollute

water, air, food, and cause hazard to human health. Therefore, considering the economic and medicinal value of turmeric and environmental issue, it is important to cultivate turmeric using green manure crops. Turmeric is widely cultivated in red soil in the most tropical and subtropical regions throughout the world. Red soils occupy about 40% of the total arable area in the world, most of which are found in tropical and subtropical regions (Haug, 1984). Red soil is generally deficient in most essential nutrients. In Japan, turmeric is commercially cultivated mainly in subtropical region, Okinawa prefecture where red soil covers about 55.1% of land which contains low organic matter, nutrients, microorganisms, and has low water holding capacity and high bulk density. Due to low organic matter and high bulk density water logging condition is found for some days after heavy rainfall in red soil and the soil becomes compact when it is dried (Haug, 1984). This type of soil is not suitable for crop cultivation especially root crop. Attention is increasingly paid to environmental issues and organic farming as well as to the efficient use of natural resources. The interest in organic farming is stimulated not only by the concern for stable and well-balanced development of agricultural production but also increased consumer awareness of food safety. Increasing soil fertility and maintaining it at an optimal level is one of the key factors for sustainable agriculture. Leguminous plants can grow well in poor fertile soil, therefore hairy vetch legume could be grown as green manure in red soil to supply nutrients and organic matters, and to improve physical, chemical and biological properties of the soil. Turmeric yield was improved by using hairy vetch in dark-red soil in Okinawa (Hossain *et al*., 2015). But no study has been done to evaluate hairy vetch effects on red soil covering 51% of land in Okinawa. Therefore, this study aimed to evaluate the effect of hairy vetch legume on physicochemical properties of red soil, and growth, yield and quality of turmeric (*Curcuma longa* L.).

MATERIALS AND METHODS

Experimental Site and Soil Properties

The pot experiment was conducted in a plastic house at the Subtropical Field Science Center, University of the Ryukyus, Okinawa, Japan. The experiment was conducted on red soil containing 0.74% C, 0.04%N with soil pH 7.84 which was collected from northern part of Okinawa at a depth of 50 cm. Red soil is usually acidic in the world, but the red soil in Okinawa contained high Ca which resulted in high pH vale. In addition, farmers have been using lime continuously in red soil from long before for different crop cultivation, therefore, pH in red soil is very high in Okinawa. Okinawa islands are surrounded by ocean and typhoon hits several times in a year which causes heavy saline rainfall and increases pH level in soil. It was reported that red soil pH is 4.4-8.9 in the northern part of Okinawa main land (in Japanese, www.nda.ac.jp/~yamaguch/5.pdf). The contents of K, P, Ca, Mg, Na and S are 12.89, 3.77, 395.32, 28.78, 57.84 and 193.56 mg kg^{-1} soil, respectively.

Plant Materials and Experimental Design

Hairy vetch was grown in a field of the University of the Ryukyus from February to April, 2015 and the required amount of chopped fresh hairy vetch (18.67% dry matter, N 5.37%, C 39.87% and the contents of K, P, Ca, Mg, Na, S are 16.86, 0.97, 12.44, 1.95, 1.36, 2.86 mg g^{-1} dry matter, respectively) was mixed with 10 kg of red soil in each Wagner pot (25 cm diameter x 30 cm height, 0.05 m^2) according to the experimental treatments. The treatments were Control (CTRL), Hairy vetch 0.5 kg (HV-0.5), Hairy vetch 1.0 kg (HV-1.0) and Hairy vetch 1.5 kg (HV-1.5). Each treatment consisted of 15 replications with randomized complete block design. One seed-rhizome (30 ± 1g) of turmeric (cv. Ryudai gold) was planted per pot at 8 cm depth on May 15, 2015. Water was applied to the plants as required and corks were used to prevent water leaching.

Data Measurement

Plant height, leaf number, tiller number and SPAD value were measured at 60, 85, 106, 127, 148, 169 and 190 days after planting (DAP). The SPAD value (Chlorophyll-meter reading) of two fully expanded top leaves from each main shoot was measured using

a spade meter (SPAD-502, Minolta Co Ltd.). Dry leaf and stem, fresh and dry rhizome, dry root, number of mother and daughter rhizome, and length, diameter and weight of largest mother and daughter rhizome were measured at 220 DAP. Soil samples were collected at 0 (beginning of turmeric planting), 70, 100, 130, 160, 190 and 220 DAP by the core sampling method from a 10 cm depth in each pot, and the contents of N, C, P, K, Ca, Mg, Na and S were determined. Soil bulk density and soil pH were determined at 0 and 220 DAP. The contents of N, C, P, K, Ca, Mg, Na and S in shoot, rhizome and root, and curcuminoids (curcumin, demethoxy curcumin, and bis-demethoxy curcumin) in rhizome were measured at harvesting time. Chopped hairy vetch plants and sliced rhizomes of turmeric were dried at 80 ^{0}C for 48 h using the forced convection oven (DRLF23WA, Advantec) for dry weight measurement.

Soil, Turmeric and Green Manure Sample Preparation for Chemical Analysis

Soil, turmeric and hairy vetch plant samples were prepared for chemical analysis according to technique described in previous **Chapter II.**

Determination of Soil pH, Bulk Density and Moisture Content

Soil pH, soil bulk density and soil moisture content were measured according to the method which has been described previous **chapter II**.

Determination of Minerals Content in Soil, Green Manure Plant and Turmeric Plants

Samples prepared for chemical analysis and mineral content in soil, hairy vetch plant and turmeric plant were determined according to method as described in previous **chapter III.**

Determination of Curcumin Content in Turmeric Rhizome

Curcumin content was determined according to the method as described by Hossain *et al*., 2015. Turmeric powder (0.1 g) was taken into a 100 ml beaker and 40ml of ethyl alcohol (99.5%) was added. Extraction was completed by supersonic wave for 10 min and the solution was filtered with paper no. 5A. Residues on filter paper were re-extracted three times following the same procedure and ethyl alcohol was added up to a 250 ml solution. The solution was filtered with disposable syringe filter (0.45 µm)

and then curcumin content was determined by HPLC (Shimudazu Co Ltd.). Column (Intact Cadenza CD-C18 100 x 3.0 mm, 3 µm) was run at 40 ^{0}C, and the acetonitrile and 1% phosphoric acid solvents were used at the ratio of 43: 57. The 5 µL solution was supplied at 0.5 mL min^{-1}, and analyzed for 11min.The contents of curcumin, demethoxy curcumin, and bis-demethoxy curcumin were determined from three replications at the wave length of 424, 420 and 416, respectively.

Statistical Analysis

All the data were subjected to analysis of variance. A one-way ANOVA was used for all the parameters. Means were separated by the Tukey's protected least significance difference (LSD) test at $p< 0.05$.

Photo **III**. 1. Hairy vetch (*Vicia villosa*) legume

Photo **III**. 2. Hairy vetch grown in the university field

RESULTS

The soil bulk density in all hairy vetch treatments was significantly reduced compared with control. The highest bulk density was observed in control treatment (0.68 gm^{-3}) and the lowest in HV-1.5 treatment (0.60 gm^{-3}). Soil bulk density decreased by 12-14% (Calculated from Fig. **III**. 1. A) with hairy vetch application, and the bulk density tented to decrease with the increasing amount of hairy vetch applications. The soil pH is significantly changed due to use of hairy vetch green manure plants. The pH decreased 2.88, 4.11 and 4.25 %, respectively, in HV-0.5, HV-1.00, and HV-1.5 as compared to control, and statistical analysis showed that these reductions were significantly different (Fig. **III**. 1. B). This result indicates that hairy vetch plant could be used for adjusting pH level in soil. The soil moisture content was significantly increased due to hairy vetch application. The moisture content was higher in HV-1.5 followed by HV-1.00 and HV-0.5 (Fig. **III**. C)

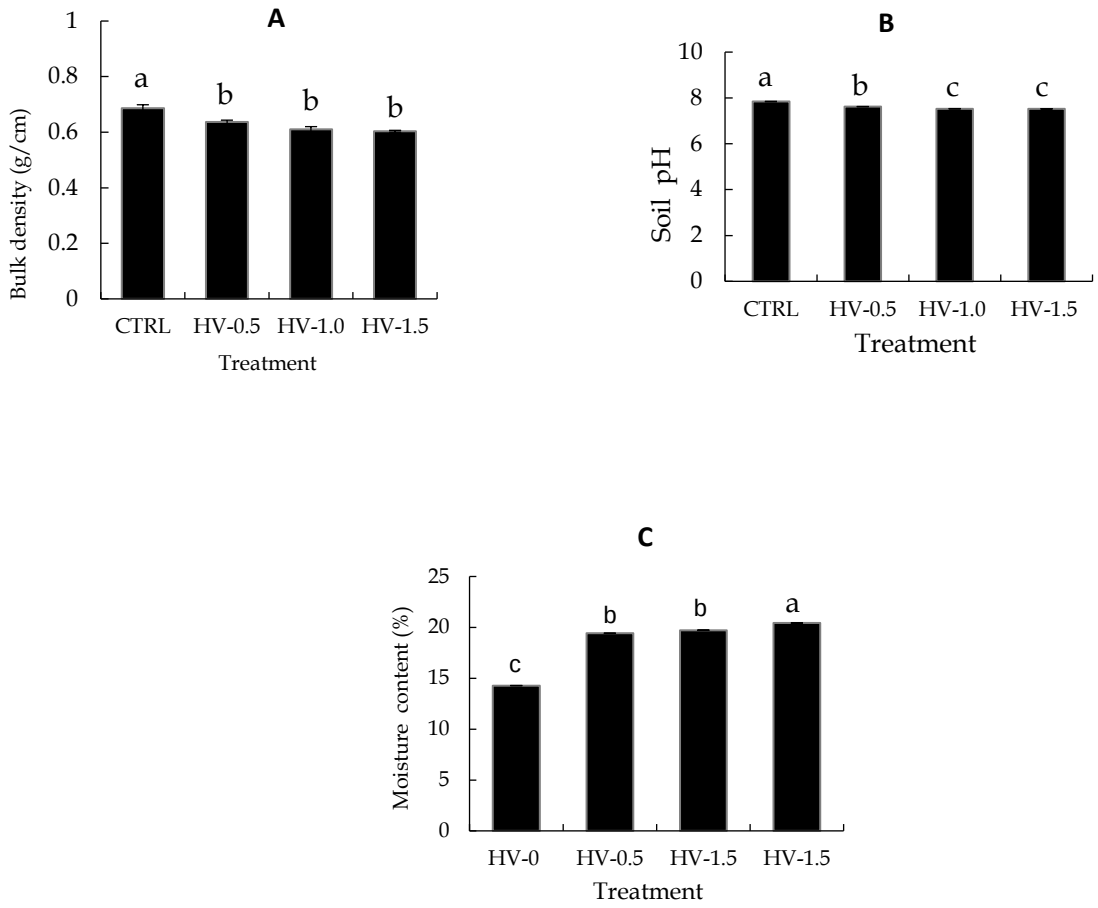


Fig. **III**. 1. Effect of different doses of hairy vetch (HV) on soil Bulk density (A), Soil pH (B) and Moisture content (C) in soil. Data are means ± SD of 3 replications. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p< 0.05$.

Effect of Application of Hairy Vetch Legume on Growth Parameters, SPAD Value and Yield of Turmeric

Different doses of hairy vetch legume significantly increased plant height, tiller number, leaf number and SPAD value at different DAP as compared to control plants. Differences in plant height of turmeric generally started to be notable among the treatments after 60 DAP as shown in fig. **III**. 2 (A). Turmeric cultivated with hairy vetch green manure increased plant height by 100-104% compared to control plants. At 190 DAP, HV-1.5 treated plants increased leaf number 3.4 times (16.13) than control plants (4.73) as shown in fig. **III**. 2 (B). Leaf number of HV-1.5 plants was significantly higher than HV-0.5 and HV-1.0 plants. Significant difference between HV-1.5 and HV-1.0 plants were also found from 148 DAP. Tiller number with the hairy vetch treated plants significantly increased as compared to control (Fig. **III**. 2. C). At 190 DAP, hairy vetch (HV) treated plants increased tiller number up to 2.27 fold as compared to control plant. In control plants, SPAD value decreased significantly after 85 DAP, while in hairy vetch treated plants it decreased after 106 DAP. Hairy vetch treated plants survived longer than control plants (Fig. **III**. 2. D). Leaf and stem dry weight of hairy vetch treated turmeric plants increased by 12-13 and 13-14 times, respectively compared to control plants. Highest leaf and stem dry weight were obtained from HV-1.5 followed by HV-1.0 and HV-0.5 but they were statistically similar (Fig. **III**. 2. E & F). Shoot dry weight of turmeric increased significantly as compared with control plants (Tab. **III**. 1). The shoot dry weight of turmeric treated with the 0.5-1.5 kg fresh hairy vetch increased by 12-13 times. Root dry weight was significantly higher with the HV-1.5 followed by HV-1.0 and HV-0.5 treatment, but HV-1.0 and HV-0.5 were statistically similar (Tab. **III**. 1). Daughter rhizome number significantly increased with hairy vetch treated plants compared to control plants. Daughter rhizome was highest with the HV-1.5 followed by HV-1.0 but these two treatments showed statistically similar result (Fig. **III**. 2. G). The hairy vetch treated turmeric plant increased yield 10-12 times as compared to control plants. The highest dry yield was obtained from HV-1.5 followed by HV-1.0 but HV-1.5 and HV-1.0 treatments showed statistically similar result (Fig. **III**. 2. H).

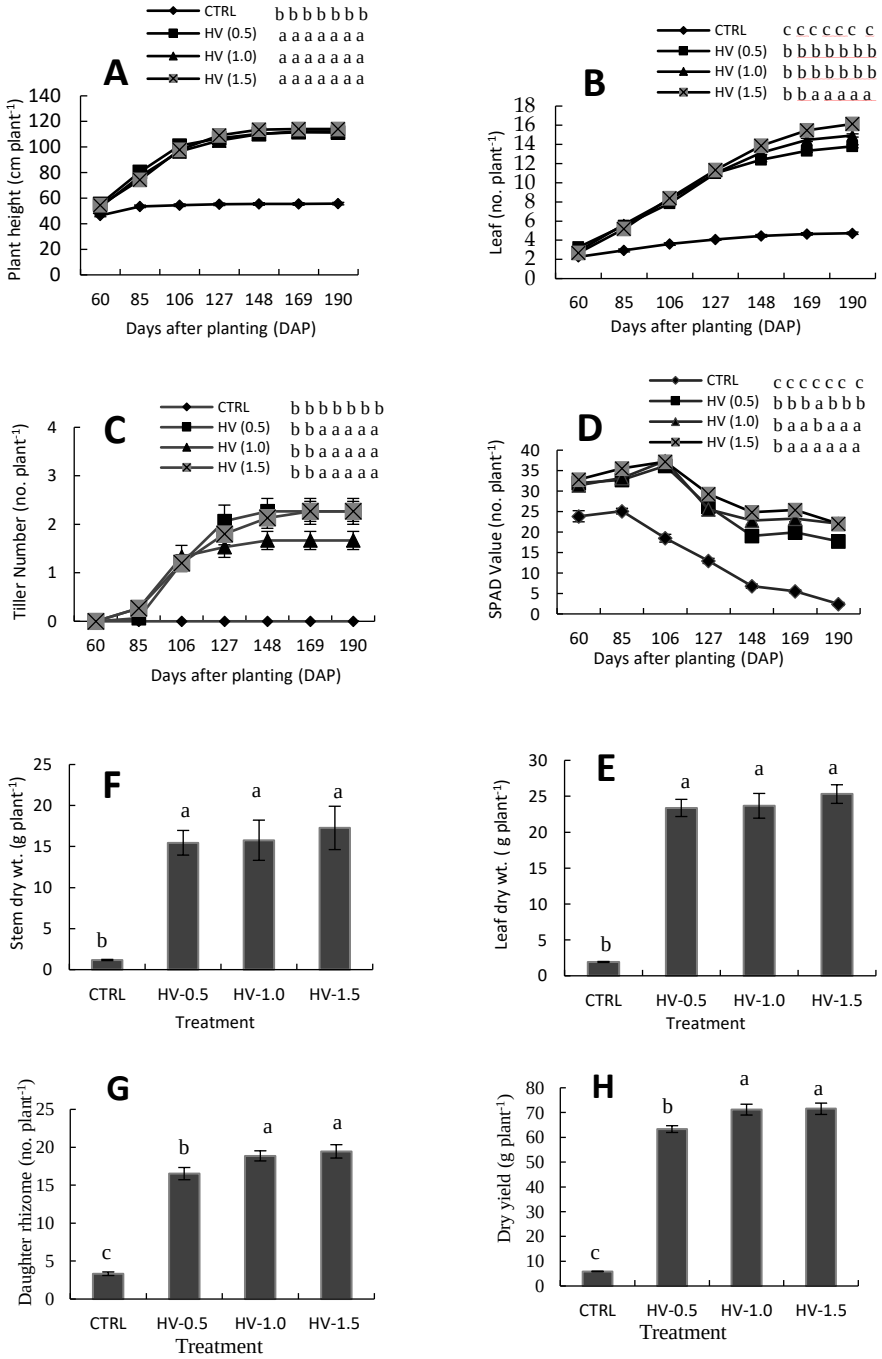


Fig. **III**. 2. Effect of different doses of hairy vetch (HV) on Plant height (A), Leaf no. (B), Tiller no. (C), SPAD value (D), Leaf dry wt.(E), Stem dry wt.(F), Daughter rhizome no.(G) and Dry yield (H) of turmeric plant. Data are means ±SD of 15 replications. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p<0.05$.

CTRL HV-0.5 HV-1.0 HV-1.5

Photo **III**. 3. Yield of turmeric influenced by hairy vetch in pot experiment.

Table **III.** 1. Effects of hairy vetch green manure on growth parameters of turmeric in red soil

Treatment	190 days after planting				At harvest time (220 DAP)	
	Plant Height (cm)	Leaves (no. plant^{-1})	Tiller (no. plant^{-1})	SPAD value	Shoot dwt. (g plant^{-1})	Root dwt. (g plant^{-1})
CTRL	55.73±0.69b	4.73±0.13c	0.00±0.00b	2.43±0.38c	3.10±0.00c	1.70±0.13c
HV-0.5	111.40±1.40a	13.80±0.16b	2.27±0.27a	17.74±0.78b	38.66±0.16b	4.36±0.15b
HV-1.0	112.20±1.45a	14.93±0.17b	1.67±0.19a	22.05±0.76a	39.46±0.21b	4.40±0.20b
HV-1.5	114.07±0.81a	16.13±0.19a	2.27±0.21a	22.05±1.07a	42.53 ±0.03a	5.18±0.22a

Data are means ±SD of 15 replications. Bars with the same letters are not significantly different as Determined by Tukey's Protected LSD (least significance difference) test at $p<0.05$.

Effect of Hairy Vetch on Nutrient of Turmeric Shoot and Rhizome

Total N content in turmeric shoot increased by 18 times due to the application of hairy vetch plants. The highest N uptake in shoot was observed in HV-1.5 (0.9%) followed by HV-1.0 (0.8%) and HV-0.5 (.7%). Nitrogen content in rhizome was highest in HV-1.5 (0.85%) followed by HV-1.0 (0.67%) and HV-0.5 (0.63%), and HV-1.0 and HV-0.5 were statistically similar. The N content in root significantly increased which was highest in HV-1.5 followed by HV-0.5 and HV-1.0 (Fig. **III**. 3, A). Total N and C content per turmeric plant was 19 and 17 times higher, respectively with hairy vetch application, compared to control plant. The highest C uptake in shoot was observed in HV-1.0 (2 g plant^{-1}) followed by HV-1.5 (1.8 g plant^{-1}) and HV-0.5 (1.7 g plant^{-1}). Total C content in rhizome was highest in HV-1.0 (3 g plant^{-1}) followed by HV-1.5 (2.7 g plant^{-1}) and HV-0.5 (2.6 g plant^{-1}) but all treatments were statistically similar. The C content in root significantly increased which was highest in HV-1.5 (0.15 g plant^{-1}) followed by HV-0.5 (0.11 g plant^{-1}) but HV-1.0 and HV-0.5 were statistically similar (Fig. **III**. 3, B). Total Ca content in shoot increased by 7-11 times compared to control plants, which was highest in HV-1.5 (1221.63 mg plant^{-1}) followed by HV-1.0 (1148.53 mg plant^{-1}) and HV-0.5 (742.76 mg plant^{-1}). Total Ca content in rhizome was 5-8 times higher in hairy vetch treated plants, which was highest in HV-1.5 (205.61 mg plant^{-1}) followed by HV-1.0 (182.11 mg plant^{-1}) and HV-0.5 (155.81 mg plant^{-1}). Calcium content in root significantly decreased but total Ca was 2-3 times higher in hairy vetch treated plants. Highest Ca was obtained in HV-1.5 followed by HV-0.5 and HV-1.0 (Fig. **III**. 3, C). The K content in shoot increased significantly in hairy vetch treated plants, which was highest in HV-1.0 (1024.17 mg plant^{-1}) followed by HV-1.5 (934.43 mg plant^{-1}) and HV-0.5 (754.29 mg plant^{-1}). The K concentration in rhizome decreased significantly due to hairy vetch application. Total K content was highest in HV-1.0 (875.38 mg plant^{-1}) followed by HV-1.5 (835.30 mg plant^{-1}) and HV-0.5 (669.11 mg plant^{-1}) but HV-1.0 and HV-1.5 treatments were statistically similar. Whereas, K content in root was highest in HV-1.5 followed by HV-1.0 and HV-0.5 (Fig. **III**. 3, D). Total K content per turmeric plant was 17-28 times higher in hairy vetch treated plants than control plant. Total Magnesium content in shoot was 4-6 times higher in hairy

vetch treatments compared to control plants. The highest Mg content was in HV-1.5 (175.01 mg plant^{-1}) followed by HV-1.0 (166.70 mg plant^{-1}) and HV-0.5 (129.57 mg plant^{-1}). The Mg content in rhizome significantly decreased in hairy vetch treated plants but total Mg content was about 10 times higher compared to control, which was highest in HV-1.0 (158.21 mg plant^{-1}) followed by HV-1.5 (147.86 mg plant^{-1}) and HV-0.5 (145.13 mg plant^{-1}). The Mg content in root was 3-6 times higher in hairy vetch treated plants, which was highest in HV-1.5 followed by HV-0.5 and HV-1.0 (Fig. **III**. 3, E). The Sodium (Na) content in shoot was highest inHV-1.5 (51.23 mg plant^{-1}) followed by HV-1.0 (49.34 mg plant^{-1}) and HV-0.5 (49.08 mg plant^{-1}). The Na content in rhizome was significantly increased and highest in HV-1.0 (53.81 mg plant^{-1}) followed by HV-0.5 (46.20 mg plant^{-1}) and HV-1.5 (44.84 mg plant^{-1}). The Na content in root was significantly higher due to hairy vetch application (Fig. **III**. 3, F). The Phosphorus content in shoot was highest in HV-1.5 (23.51 mg plant^{-1}) followed by HV-1.0 (20.35 mg plant^{-1}) and HV-0.5 (8.72 mg plant^{-1}). The P concentration in rhizome decreased significantly due to hairy vetch application. Total P content in rhizome was highest in HV-1.0 (113.08 mg plant^{-1}) followed by HV-1.5 (107.29 mg plant^{-1}) and HV-0.5 (83.42 mg plant^{-1}) due to hairy vetch application and there were significant difference among the treatments (Fig. **III**. 3, G). Highest S content in shoot was in HV-0.5 (189.61 mg plant^{-1}) followed by HV-1.0 (162.15 mg plant^{-1}) and HV-1.5 (153.19 mg plant^{-1}). The total S content in rhizome was 8-10 times higher in hairy vetch treated plants, which was highest in HV-1.0 (58.05 mg plant^{-1}) followed by HV-1.5 (48.84 mg plant^{-1}) and HV 0.5 (37.98 mg plant^{-1}). The S content in root was 23 times higher with hairy vetch treatments than control (Fig. **III**. 3, H)

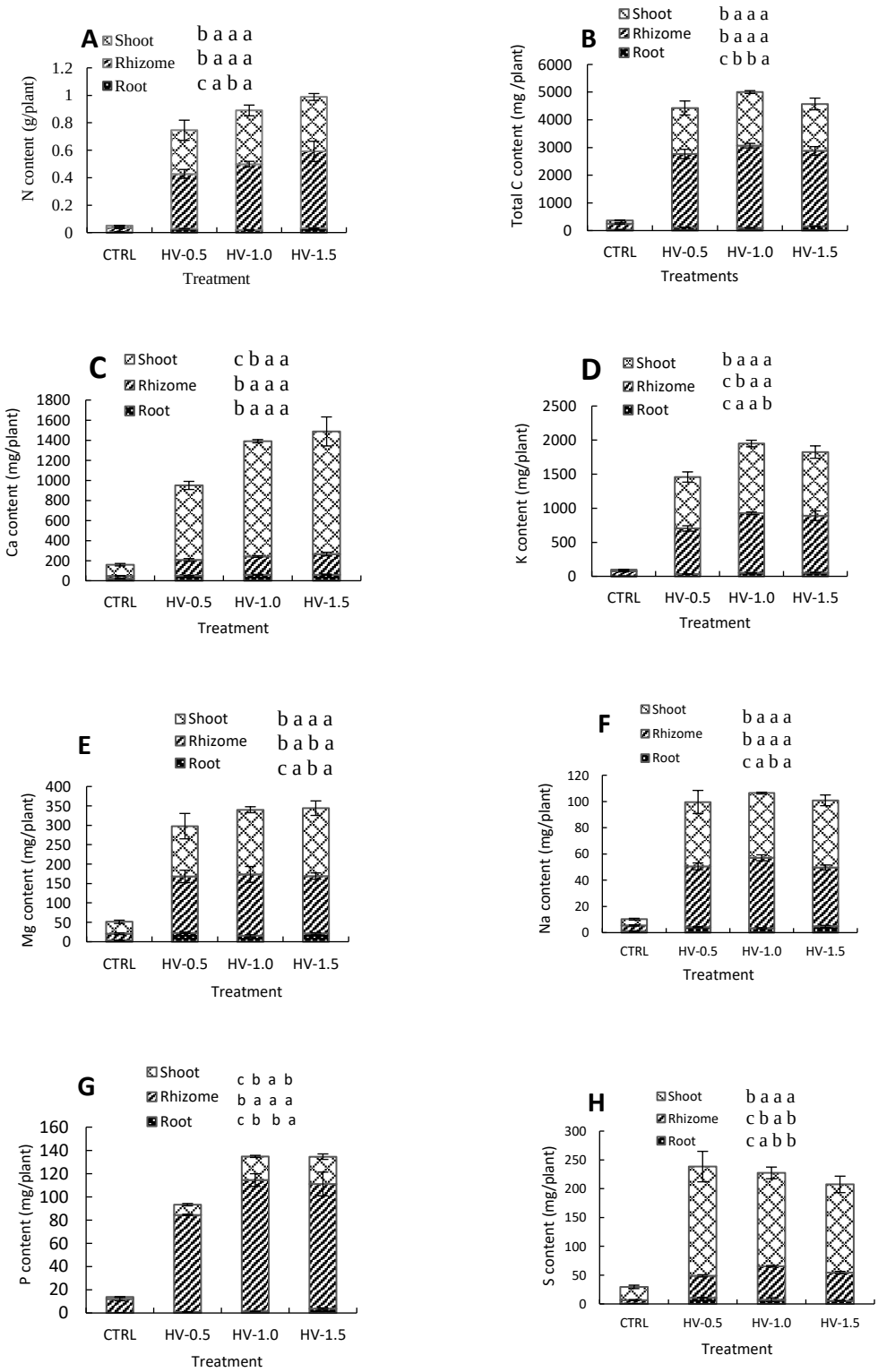


Fig. **III**. 3. Effects of different doses of hairy vetch on total N (A), C (B), Ca (C), K (D), Mg (E), Na (F), P (G) and S (H) content in turmeric plant. Data are means ± SD of 3 replications. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p< 0.05$.

Effect of Hairy Vetch on Curcuminoid Content in Turmeric

Curcuminiods of turmeric decreased significantly in hairy vetch treated plants. Curcumin, bis-methoxy curcumin and methoxy curcumin concentration decreased by 119-128%, 62-77% and 96-113%, respectively when turmeric was grown with hairy vetch (Calculated from Fig. **III**. 4. A, B, and C). The Rhizome showed higher concentration of N, lower concentration of K and P when turmeric was cultivated with hairy vetch green manure.

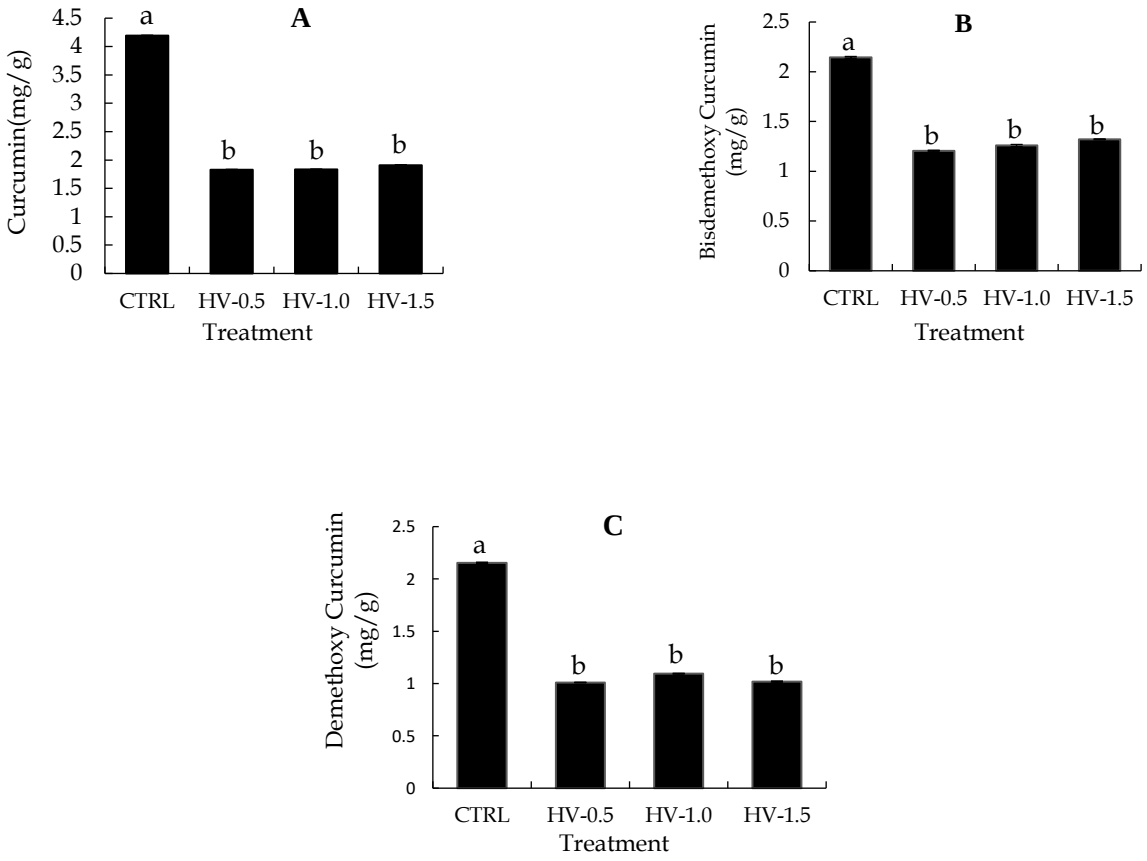


Fig. **III**. 4. Effects of different doses of hairy vetch on Curcumin (A), Bisdemethoxy curcumin (B) and Demethoxy curcumin (C) content in rhizome of turmeric plant. Data are means ± SD of 3 replications. Bars with the same letters are not significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p< 0.05$.

DISCUSSION

The incorporation of green manure significantly changed soil physical and chemical properties. The soil bulk density in all hairy vetch treatments were significantly reduced compared with control. The availability of hairy vetch increased activities of beneficial soil microorganisms in organic matter decomposition which led to enhancement of soil porosity and reduction in soil bulk density. The soil pH was lower in hairy vetch treated soil compared with untreated soil which may be attributed to the release of CO_2 and organic acids during decomposition of hairy vetch biomass and reduction in exchangeable cations. This result is in line with the findings from other research (Salahin *et al*., 2013, Hossain *et al*., 2015, Adekiya *et al*., 2017). Different doses of hairy vetch legume significantly increased plant height, tiller number, leaf number, SPAD value at different DAP as compared to control plants. The increase in plant height is due to the better nutrient availability and higher uptake that promoted plant biomass production. Nitrogen supplied by hairy vetch ensured favourable condition for the growth of turmeric with optimum cell division and elongation of cell resulted the tallest plant. This might be due to protein synthesis by the help of nitrogen which enhance vegetative growth of turmeric plant. Hossain *et al*. (2015) reported that turmeric plant height increased by 20% and tiller number increased by 76-165% with the application of green manure plants in glasshouse. Turmeric cultivated with hairy vetch treated soil developed maximum number of leaves due to the additional amount of N. This result was supported by Hossain *et al*. (2015). The SPAD value was higher in turmeric leaves due to the additional amount of N supplied by hairy vetch. Higher SPAD value probably contributed to a higher photosynthesis and resulted in higher growth and yield of turmeric. Findings regarding to the SPAD value in turmeric leaves were agreed with the results of Hossain *et al*. (2005). There was an overall delay in senescence in hairy vetch treated turmeric plant which was particularly survived longer in compared to untreated plants. It is assumed that longer survival of turmeric plants with higher leaves provided higher photosynthesis which ultimately resulted in higher yield. The application of hairy vetch increased leaf and stem dry weight of turmeric plants. The hairy vetch probably improved soil physical and chemical properties, water holding capacity, aeration, mineralization and nutrient digestion in

soil, which contributed to the increase of shoot (leaf + stem) weight of turmeric. Hossain *et al*. (2015) reported that turmeric shoot dry weight increased by 173-197% with the application of 1.66 kg hairy vetch plants in dark red soil. The hairy vetch treated turmeric plant increased yield as compared with untreated plants. Turmeric plants cultivated with hairy vetch showed a higher tiller number with greener leaves and survived longer which generally contributed to higher photosynthesis and resulted in higher yield. Goto *et al*. (2000) and Sullivan *et al*. (2003) reported that application of green manure could improve soil properties, water holding capacity, aeration, mineralization and nutrient releasing in soil that played role to the enhance of shoot biomass and rhizome yield of turmeric. The yield of turmeric significantly increased that generally determined probably due to the supply of higher N and other nutrients by hairy vetch which is agreed with other research (Kuo *et al*., 2000, Sainju *et al*., 2002, Rochester *et al*., 2005). The turmeric plants have a greener foliage due to more N accumulation from hairy vetch. Hossain *et al*. (2015) showed that when turmeric plant was cultivated with green manure plants, concentration of N in rhizome increased significantly while concentration of K and P decreased. Mappaona *et al*. (1994) reported that P and K concentration decreased in cabbage when green manure was incorporated with soil. The Ca, Mg, Na and S content in rhizome also decreased significantly with hairy vetch application. It is considered that the N supplied from hairy vetch which accounted as excessive and it did not stimulate the accumulation of P, K, Ca, Mg, Na and S which resulted in lower values of the elements in rhizome. Agbede *et al*. (2009) stated N is a major constituent of chlorophyll and carbohydrate synthesis, which enhances root and shoot growth and stimulate uptake and utilization of other nutrient such as K, P and S. The fact that total N, C, P, K, Ca, Mg, Na and S in shoot, rhizome and root of hairy vetch treated turmeric plants increased as the increased dry matter and hairy vetch treated soil provided increased availability of the nutrients as a result of the mineralization of soil. The Rhizome showed higher concentration of N, lower concentration of K and P when turmeric was cultivated with hairy vetch green manure, which probably accounted for lower curcumin concentration. It was reported that curcuminoids concentration in the rhizome was decreased due to higher supply of N by green manure application and

lower K content (Hossain *et al*., 2015). Other scientist reported that curcumin content in rhizome may depend on soil characteristics, nutrients, weather and environment (Kulpapangkorna *et al*., 2011). Therefore, further research should be conducted to clarify the effects of hairy vetch on curcumin content in rhizome of turmeric.

Chapter IV

Effect of Hairy vetch (*Vicia villosa* R.) Legume on Biological Properties of Subtropical Red Soil

Abstract: The use of green manure in soil can provide nutrients, enhance microbial population, and be a more sustainable soil management practice. The activity of soil microbes can affect crop growth, but the extent of this effect on yield remain unclear. The application of hairy vetch enhanced soil biological activity, as measured by microbial biomass and mineralization potential. I investigated soil microbial population, and isolated plant growth promoting fungal strains from hairy vetch amended rhizosphere of turmeric. The investigated result showed that microbial populations in the soils amended with hairy vetch were higher. Two fungal strains, SI-17URAgr and SI-19URAgr, were found in the soils amended hairy vetch, and identified as *Talaromyces pinophilus*, morphological studies and the sequences of ITS region and/or Calmodulin. Subsequently, fungal isolates having excellent phosphate and zinc solubilization efficiency were tested by their potential in solid medium containing insoluble $Ca_3(PO_4)_2$ as P source, and ZnO as Zn source. Both strains had a considerable ability to secrete organic acids and they could survive in acidic environments. The two fungal strains had potential for application as environment-friendly biofertilizer in subtropical agriculture.

Key words: Green manure, biological activity, microbial population, Talaromyces pinophilus.

INTRODUCTION

Soil organic matter (SOM) is a key factor for crop productivity due to its important role in maintaining soil physical, chemical, and biological properties (Sarker *et al.,* 2018). The application of green manure as organic amendment, rather than chemical nutrient sources, is recommended for sustainable soil management and eco-friendly agriculture. Green manures enhance biodiversity of soil micro-organisms, microbial growth and activity, and provides nutrients rich in organic carbon for the microbial biomass which converts unavailable nutrients in plant residues into forms available to crops (Carsky and Suhet, 1990; Elfstrand *et al.,* 2007; Tejada *et al.,* 2008). It is well established that soil microorganisms play a pivotal role in ecosystem functioning through their contribution to SOM decomposition and biogeochemical cycling of nutrients (Bardgett and van der Putten, 2014). Soil microbial biomass is also a precursor and builder of SOM (Miltner *et al.,* 2012). Legume-based green manures supply N to soil. Nitrogen mineralization is more tightly linked to microbial demand for C than phosphate mineralization. In addition, mineralization of phosphate, which is mediated by phosphatases, is also driven by microbial demand for phosphate, independent of C availability (Elfstrand *et al.,* 2007, McGill and Cole, 1981).

However, while phosphorus (P) is an important plant nutrient, large amounts of soluble phosphate applied to soils as fertilizer are fixed in the soil, which limits its availability to plants. Thus, the long-term application of phosphate fertilizers has resulted in an accumulation of total soil phosphate, most of which is poorly soluble (Whitelaw, 1999). Microorganisms capable of solubilizing and mineralizing phosphate in soils are considered vital for promoting phosphate bioavailability (Tao *et al.,* 2008). Green manures enhance soil microbial population and provides nutrients rich in organic carbon for the microbial biomass which converts unavailable nutrients in plant residues into forms available to crops (Carsky and Suhet, 1990). As soil microbial biomass and enzyme activities are considered important components of soil fertility, direct incorporation of a green manure crop can be recommended as a means of promoting this component of soil fertility (Elfstrand *et al.,* 2007). In addition, Acid, calcareous, saline and sodic soils, and coarse-textured soils prone to high weathering, besides soils subjected to intensive cropping and poor drainage exhibit Zn deficiency

(Singh *et al.,* 2005) and application of Zn in the form of chemical fertilizer is inappropriate due to its unavailability to plants. A feasible alternative would be to exploit the innate capacity of certain soil microorganisms, especially, fungi, to solubilize fixed forms of Zn to labile Zn forms for enhanced availability and subsequent uptake by plants. Green manure stimulates the activity and abundance of soil microorganisms, including arbuscular mycorrhizal fungi, which were shown to promote plant Zn uptake (Lehmann *et al.*, 2014).

In the current study, we hypothesized that green manure would enhance the microbial activity and diversity in red soil in response to the result of previous experiment whereas investigation showed that hairy vetch green manure improved soil physical and chemical properties, increased crop growth and yield (*Majumder et al.,* 2018). In subtropical Okinawa, legume-based green manure, notably hairy vetch (*Vicia villosa*) which is an important N source is widely used in sustainable agriculture. Few studies regarding the effects of hairy vetch green manure on soil microbial activity (measured in terms of nutrient solubilizing activity) exist, and Consequently, more research is required in this context. In the above context, this study aims to investigate effect of hairy vetch on soil biological properties, especially isolate fungal strains having role in nutrient solubilization from red soil.

MATERIALS AND METHODS

The microbiological study was carried out in the Mycology Laboratory, Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan during August 2017–November 2018 under a class II biohazard cabinet (BHC-1306IIA/3B, AIRTECH, Tokyo, Japan) followed to the biosafety classification by National Institute of Infectious Disease of Japan, because of possibilities of including toxic fungal species treated as BSL2 during the isolation.

Collection of rhizosphere soil samples

Soil samples were collected from the rhizosphere of turmeric (*Curcuma longa* L.) cultivated in red soil amended with hairy vetch green manure and unamended rhizosphere of turmeric. The turmeric roots were shaken carefully inside plastic bags in order to separate the soil from the roots. The samples were transferred to laboratory in sterile sealed polythene bag under aseptic condition.

Isolation and enumeration of microbial population

Microbial activity was measured by microbial number, which was done by spread plate counting method. Five-gram soil sample was diluted in to 50 ml of sterile water. After making dilution series, from each dilution, 200 µL solutions were spread over petri dish having Nutrient Agar media containing (g L^{-1}): meat extract, 5 g; peptone, 15.0 g; sodium chloride, 5 g; dipotassium phosphate, 5 g; agar, 15 g per 1000 mL distilled water, for bacteria and Potato Dextrose Agar (PDA) media containing (g L^{-1}): potato infusion, 200 g; dextrose, 20 g; agar, 15 g per 1000 mL distilled water with 25 µg/mL chloramphenicol to avoid bacterial growth , for fungi, respectively. The petri dishes were incubated at 25^0C and fungal colonies were counted every day for 4 days for fungi, and 7 days for bacteria. Size of populations were calculated in CFU g^{-1} dry soil. The pure cultures were preserved on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) slants at 4°C for further investigation.

Screening of isolates based on nutrient solubilization

In order to detect the fungi having role in mineralization, each of the isolated rhizosphere fungi were inoculated on petri dish having Pikoveskaya's (PKV) agar medium consisted of 10.0 g glucose, 5.0 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.1 $MgSO_4.7H_2O$, 0.02 g NaCl, 0.02 g KCl, 0.003 g $FeSO_4.7H_2O$, 0.003 g $MnSO_4 \cdot H_2O$ 0.5 g yeast extract, 15.0 g agar dissolved in deionized water to make 1000 mL volume, for phosphate solubilizing fungi (Rao, 1982) where as $Ca_3 (PO_4)_2$ was used as a source of insoluble phosphate. For detecting the fungi having zinc solubilizing ability, isolated fungi were plated on mineral salts medium (g·lit−1) specified by Saravanan et al. 2007, consisted Dextrose: 10.0; $(NH_4)_2SO_4$: 1.0; KCl: 0.2; K_2HPO_4: 0.1; $MgSO_4$: 0.2; pH: 7.0 and insoluble Zn compound (ZnO: 0.1%), Agar: 15.0g and adding deionized water to make 1000 mL volume, and autoclaved at 121∘C for 20 min. The plates were incubated at 28 °C for 7 d and observed for the appearance of a clearing zone around the colonies (caused by the solubilization of inorganic phosphate or zinc by the fungi.

Phosphate or zinc solubilization index was measured using the following formula (Birhanu et al., 2017):

Solubilization index (SI) = [Colony diameter + Halo zone diameter] / colony diameter.

Identification of fungal isolates

The genera of plant growth promoting fungal isolates (SI-17 and SI-19) were identified based on the taxonomic keys based on morphologies (Watanabe, 2010). The keys were the color and tint in colony overs and revers, presence of aerial hyphae, colony surface texture, colony margin and pattern of pigment exudations. Wet mounts prepared from micro culture were mounted in lacto phenol and lacto phenol cotton blue. Microscopic examination and photomicrography were performed with an OLYMPUS BX50 microscopy equipped with image Analysis system (Olympus Corporation, Tokyo, Japan).

DNA was extracted from one piece of fungal mycelia from a culture incubated at 25 °C for 48 h on Sabrouraud medium containing 2% glucose and 1% peptone using a DEXPAT kit (TaKaRa, Japan) to identify the isolates at genetic level (Yamaguchi et al., 2014). Beta- tubulin gene sequences amplified with primers bt2a and bt2b, and the ITS

rRNA gene amplified with ITS 1, 2 and ITS 3, 4 were determined (Samson et al., 2014). Sequences were analysed by the NCBI BLAST (https://blast.ncbi.nlm.nih.gov) tool to classify and identify closely related fungal sequences. We identified the isolates to the certain species if the BLAST results showed similarity values of 98% or higher.

Organic acid production of identified strains under different pH conditions

For conducting this part, fungal cultures were made from the re-slanting of pure culture slants (SI-17 and SI-19) that preserved at 4^0C. Sporulated culture slants were selected for preparation of spore suspension. A total volume of 5 ml sterile water with 0.02% of tween 80 (Polyoxyethylene sorbitan monooleate, Nacalai Tesque, Inc, Kyoto, Japan) was added in culture slants and the fungal colony surface was lightly scraped by a sterile inoculation loop (Thermo Scientific™ , Nunc™ Disposable Loops and Needles, Thermo Scientific™ 251586, Fisher Scientific, Tokyo, Japan). Then cultures were passing through a syringe with a 4 × 4 cm sheet of a sterile absorbant cotton (Kyualet, Kawamoto Sangyo, Osaka,Japan). Spore count was done by a hemocytometer and the suspension was adjusted to approximately10^6 spores mL^{-1}.

The potato dextrose liquid medium was precisely regulated to pH 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 with hydrochloric acid, and sterilized at 121 °C for 20 min. The 1 ml spore suspension of SI-17 and SI-19 were inoculated in 100 ml conical flasks with various acidity. These conical flasks were incubated at 28 °C for 5 days under shaking. Then the culture medium was filtered through 0.22 0.22 µm membrane. All experiments were repeated three times.

For secretion of organic acids under different pH conditions. After five-days incubation, the pH values of the filtered medium were measured. Detection and quantification of organic acids were done by High Performance Liquid Chromatography (Prominence HPLC system, Shimadzu-CBM-20A, Japan) equipped with diode array detector (SPD-M20A), refractive index detector (RID-10A), column ICE-ION-300 (300mm X 7.8mm), auto sampler (LC-20AD) and fraction collector (FRC-10A). The injection volume, temperature and flow rate was 50 µl, 50^oC and 0.5ml/min, respectively. Sulfuric acid of 0.01N was used as solvent of mobile phase. Peaks were

identified against a set of standards from known organic acids (oxalic, citric, tartaric, formic, acetic acid).

RESULTS

As shown in table **IV.** 1, the populations of bacteria and fungi in the soil amended with hairy vetch were significantly higher than populations in the unamended soil. On average, the populations of bacteria and fungi in the soil amended with hairy vetch were 3.7-fold and 4-fold higher, respectively, compared to the untreated soil.

Table **IV.** 1. Effect of hairy vetch on microbial populations of the soils

Treatment	Bacteria	Fungi
	(10^5 c.f.u.g^{-1})	(10^4 c.f.u.g^{-1})
Unamended soil	3.2^b	2^b
Hairy vetch amended soil	12^a	8^a

The different letters (a and b) are significantly different as determined by Tukey's Protected LSD (least significance difference) test at $p<0.05$.

Two fungi, SI-17URAgr and SI-19URAgr were found in the soils amended with hairy vetch which could grow on the medium enriched with 0.5% tricalcium phosphate and 0.1% zinc oxide, and showed a clear zone of dissolved phosphate and zinc in solid medium (Photo **IV.** 1), separately, which indicated that both these plant growth promoting fungi exhibited the desired phosphate and zinc solubilizing ability. The morphological characteristics of hyphae, spores, and conidiophores of the two isolates were examined by optical microscopic (Photo **IV.** 1. A, B; E, F; Fig. **IV.** 2. A). According to the results of ITS and calmodulin gene sequences analysis to identify the selected PGPF, SI-17URAgr and SI-19URAgr were confirmed as *Talaromyces pinophilus*. The sequences of isolates have been submitted to the genetic sequences database at the DNA Data Bank of Japan (DDBJ). The accession number of these isolates in DDBJ are shown in table **IV.** 2.

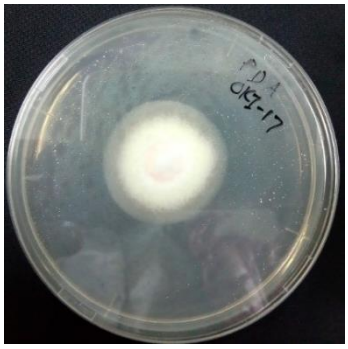
Table **IV**. 2. Strains with their GenBank accession numbers for two genetic markers

Strain in GenBank	**Species**	**GenBank accession no.**	
		ITS region	**Calmodulin**
SI-17URAgr	*Talaromyces pinophilus*	N.D.	LC425341
SI-19URAgr	*Talaromyces pinophilus*	LC425343	N.D.

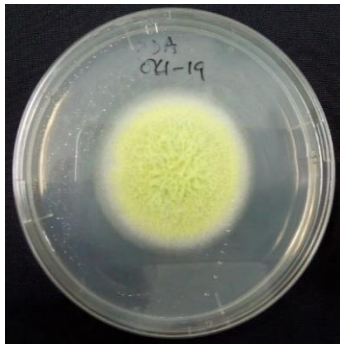
Several organic acids with low molecular weight, e.g., oxalic, citric, tartaric, formic, and acetic acids, were secreted by the two PGPF (Table **IV.** 3). Lactic and malic acids were not detected in culture medium. The major secreted organic acids of SI-17URAgr were oxalic (451.7 mg/mL) and citric acids (398.3 mg/mL), whereas SI-19URAgr has oxalic (427.1 mg/mL) and tartaric acids (366.0 mg/mL). Citric acid was only detected at higher pH environment (Table **IV.** 3). The dominant acids in SI-17URAgr are acetic and oxalic acids at initial pH = 5.5, and 7.5, respectively, and the dominant acids in SI-19URAgr are also oxalic and tartaric acids at initial pH= 7.5 and 5.5, respectively, (Table **IV.** 3). Formic acid in SI-17URAgr was detected at neutral to alkaline environment whereas SI-19URAgr showed same trend. With the increase of the original acidity in the culture medium, the concentration of each single organic acid secreted by the SI-17URAgr and SI-19URAgr varies. Concentration of organic acids secreted by the SI-17URAgr and SI-19URAgr reached the maximum at pH = 5.5 (Table **IV.** 3)

Table **IV.** 3. Types and quantities of the secreted organic acids in the different culture medium (mg/mL).

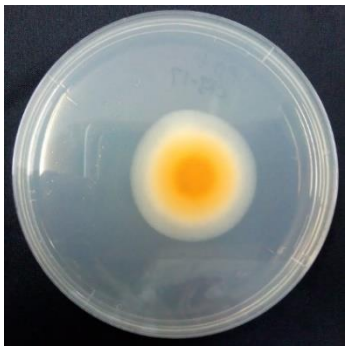
Strains	**Organic acids type**	**pH**					
		8.5	**7.5**	**6.5**	**5.5**	**4.5**	**3.5**
SI-17URAgr	Oxalic	139.0±4.3	245.3±5.1	54.7±4.0	9.0±2.0	3.7±1.5	N.A
	Citric	29.0±3.0	68.0±2.0	37.0±4.5	264.3±13	N.A.	N.A.
	Tartaric	N.A.	N.A.	33.0±4.0	145.0±7.5	164.7±4.0	39.0±2.0
	Formic	48.7±8.0	25.0±2.0	N.A.	N.A.	N.A.	N.A.
	Acetic	28.0±3.0	59.3±6.8	65.0±2.0	80.7±2.5	21.0±2.0	N.A.
	Total	244.7	372.6	214.7	499.0	189.4	39.0
SI-19URAgr	Oxalic	127.7±9.0	134.0±8.8	110.7±1.5	49.7±8.0	5.0±1.0	N.A.
	Citric	83.0±5.5	49.0±2.0	24.3±3.0	160.3±13.6	8.0±2.6	N.A.
	Tartaric	N.A.	12.0±4.2	36.7±4.5	185.0±6.8	104.6±8.5	27.7±2.5
	Formic	50.7±8.3	17.7±3.0	7.7±2.5	N.A.	N.A.	N.A.
	Acetic	78.0±10.0	58.3±5.1	29.3±5.6	59.0±4.3	9.7±2.5	N.A.
	Total	351.4	295.7	172.0	454.0	127.3	27.3



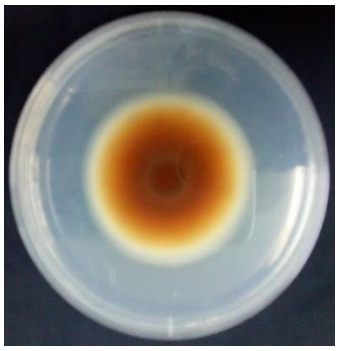
(A)



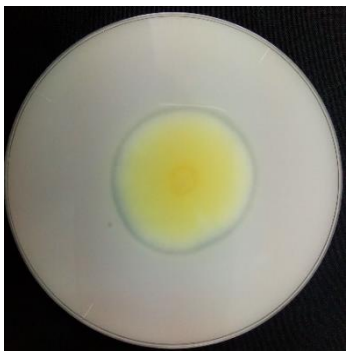
(E)



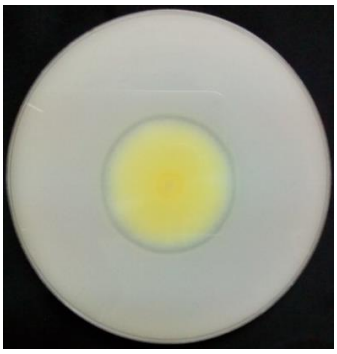
(B)



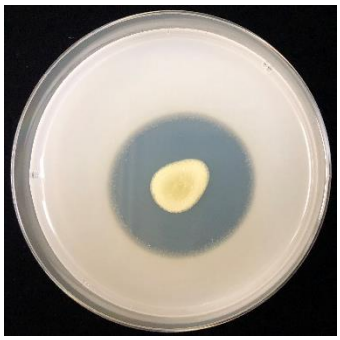
(F)



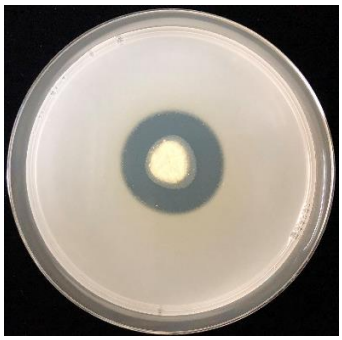
(C)



(G)

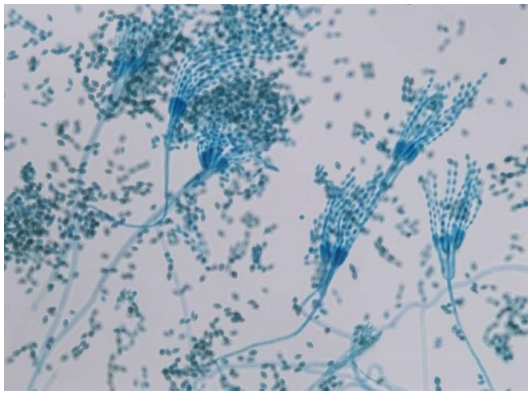


(D)

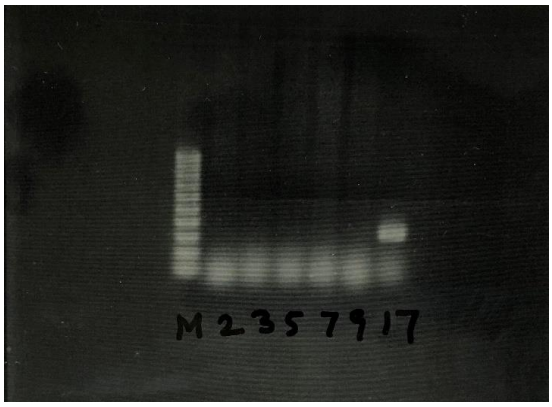


(H)

Photo **IV.**1. Plant growth promoting *Talaromyces pinophilus* isolate SI-17URAgr (A, B) colony morphology on PDA, (C, D); Zone of Phosphate and Zinc solubilization, and SI-19URAgr (E-F); colony morphology on PDA, (G, H) Zone of Phosphate and Zinc solubilization, respectively.

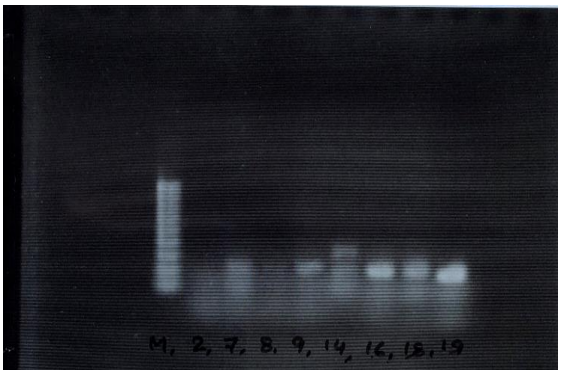


(A)



M 1 2 3 4 5 6

(B)



M 1 2 3 4 5 6 7 8

(C)

Photo **IV.**2. Conidia under microscope (A), DGGE profiles for fungal 18S rDNA gene sequences of soil sample taken from the pots treated with hairy vetch (B, C).

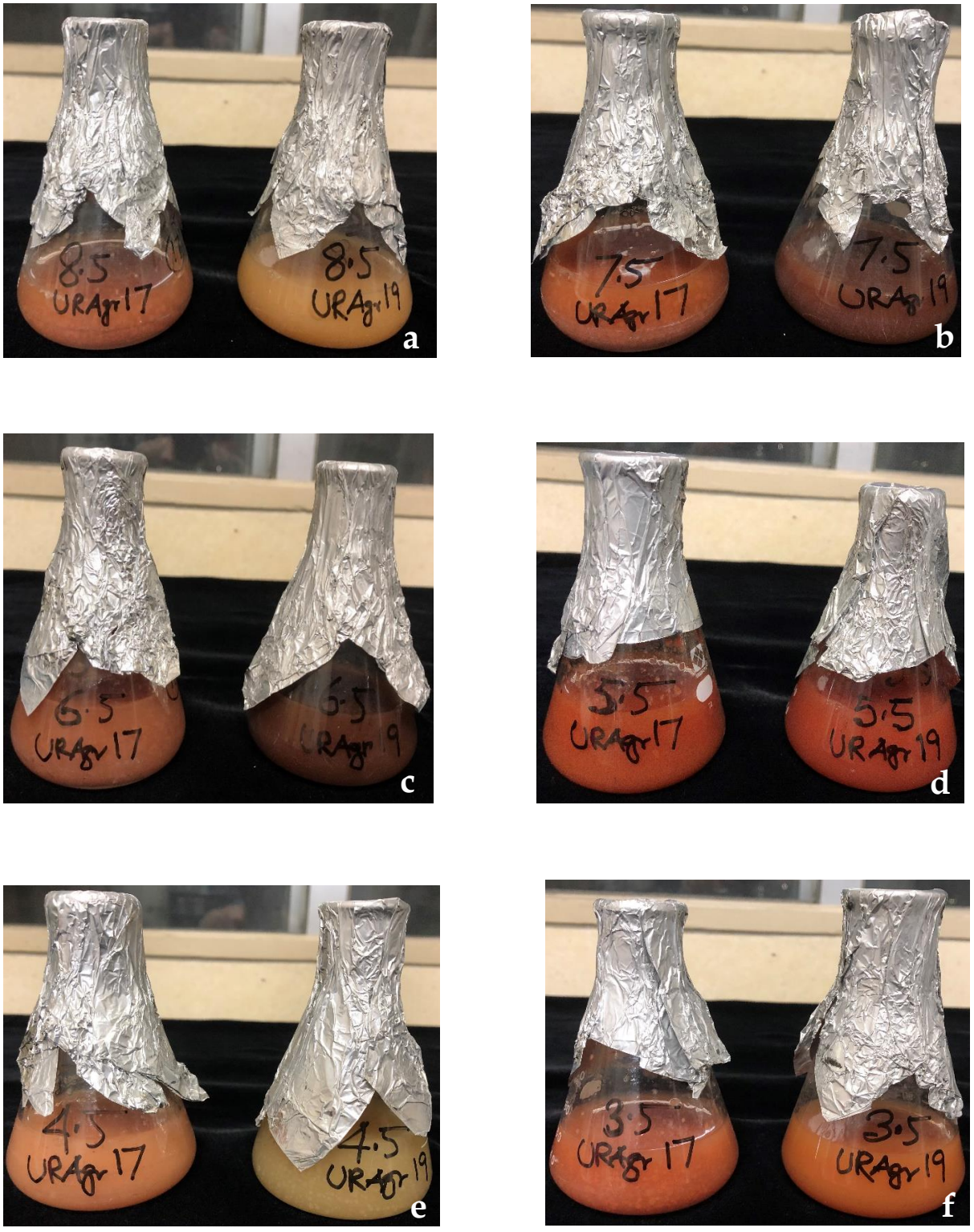


Photo **IV.** 3. Fungal growth and survival at different pH conditions in PDA broth culture (**a.** pH 8.5, **b.** pH 7.5, **c.** pH 6.5, **d.** pH 5.5, **e.** pH 4.5, **f.** pH 3.5).

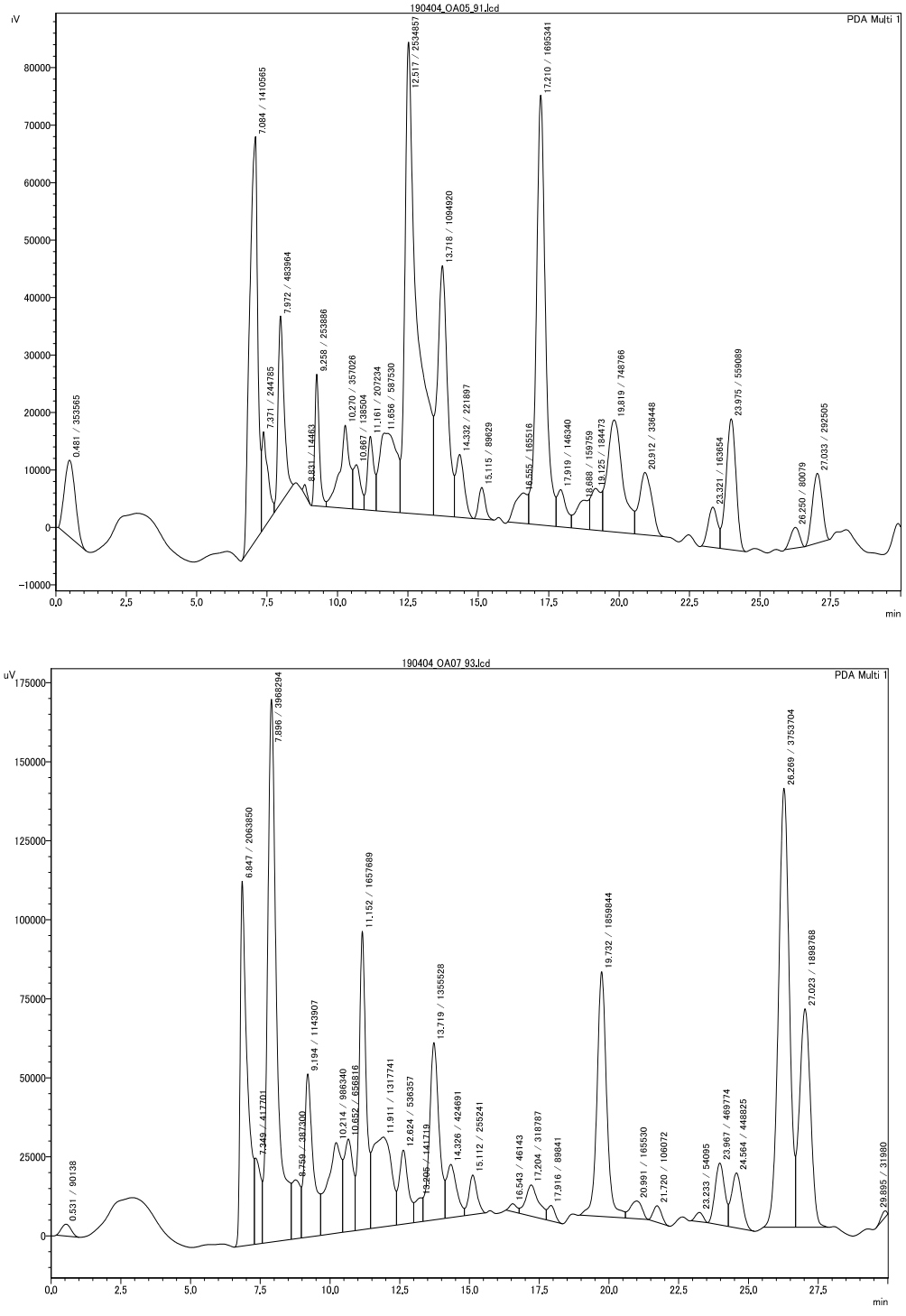


Fig. **IV.** 1. Chromatograms of organic acids analyzed by HPLC.

DISCUSSION

Green manuring is a feasible agricultural practice to mitigate soil degradation and loss of biodiversity caused by long term application of inorganic fertilizer. As the potential replacement of chemical fertilizer, green manure could improve soil nutrients and crop production, but its impacts on crop health haven't been clarified clearly yet. First of all, soil properties were affected by hairy vetch green manure, and the changed soil properties were beneficial for turmeric growth and yield. Previous research showed that hairy vetch green manure greatly increased soil organic C, total N, P and K, available N, P and K content (Majumder et al., 2018). There is no doubt that increased nutrients is beneficial for plant growth and activity of soil microorganisms. Based on the present experiment, we speculated that the increased biodiversity resulted from the increased soil nutrients. Brady and Weil (1999) reported that introduction of organic matter into soil resulted in increasing soil microbial populations and soil biological activity. The five-days culture in this study showed that both SI-17URAgr and SI-19URAgr have impressive potentials in enhancing P and Zn release within such a short time. The organic acids secreted by fungi contribute to their solubilizing ability. To investigate the mechanisms, there are three factors that should be addressed, i.e., pH values, types of organic acids, and feedback to environmental acidity. In the present study, *Talaromyces pinophilus*, SI-17URAgr and SI-19URAgr were isolated from the soils amended with hairy vetch green manure and identified as the fungi with prominent solubilizing ability in phosphate and zinc respectively. The pH reduction can be attributed to diffusion of various organic acids secreted by the SI-17URAgr. In soils, the advantages of organic acids compared to inorganic acids are: 1) few negative effects to soil quality, e.g., salinization; 2) release of more H+ due to their low acidity constants (at the same pH as inorganic acids). A serial of organic acids have various acidity constants, which determine their ability in changing acidity of the environments.

Chapter V

Comparative Study of Phosphate Solubilization Potential of Different *Talaromyces pinophilus* Strains

Abstract: Phosphorus (P) is one of the most essential macronutrients for plant growth and development. Most of the soils in the world contain insoluble P that cannot be utilized by the plants. Phosphate solubilizing fungi (PSF) including *Talaromyces pinophilus* possess more potential for providing available P in soil for plant nutrition. There is no report regarding P solubilization potential among the different strains of *T. pinophilus*. The article aimed to compare the Phosphorous solubilization capabilities of 17 *Talaromyces pinophilus* fungal strains, in order to determine the best options for environmentally friendly fertilizers. The P solubilization efficiency of the fungal strains was investigated in broth containing insoluble $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$ compounds. Result showed that $Ca_3(PO_4)_2$ was solubilized the most followed by $AlPO_4$ and $FePO_4$. The strains SI-17URAgr, NBRC 6345 and NBRC 100533 have strong abilities to solubilize $Ca_3(PO_4)_2$, but SI-19URAgr could solubilize both $AlPO_4$ and $FePO_4$. These results also imply that the strains SI-4URAgr and JCM 22801 have the potential to solubilize $AlPO_4$ and $FePO_4$, respectively. Although the P solubilization by different strains were source dependent, the strains SI-17URAgr, NBRC 6345 and NBRC 100533 were proved to be potential for solubilizing P from three sources tested. Solubilized P was negatively correlated with pH of the medium. This study suggested that these strains have a great potential as eco-friendly biofertilizers for sustainable soil management and crop production.

Key words: : ***Talaromyces pinophilus, insoluble phosphate, solubilization potential, environment friendly, biofertilizer, soil management and plant growth.***

INTRODUCTION

Phosphorus (P) is the second most important limiting element required for plant growth and development (Chai *et al.,* 2011; Ram *et al.,* 2015). The soluble soil P concentration varies from 0.05 to 10 ppm, and more than 80% of the P becomes fixed and unavailable for absorption by plants due to adsorption, precipitation or conversion to organic forms (Holford, 1997). The fixed form of P in alkaline soils is tricalcium phosphate, $Ca_3(PO4)_2$, whereas in acidic soils, it is mainly found as $FePO_4$ and $AlPO_4$ (Subba Rao, 1999).

The application of plant growth promoting microorganisms in agriculture could reduce the use of agrochemicals and support eco-friendly crop production (Herrera *et al.,* 1993; Glick, 1995; Requena *et al.,* 1997). It has been reported that a wide range of microorganisms, especially bacteria, solubilize insoluble phosphate (Badr El-Din *et al.,* 1986; Gaind *et al.,* 1991) although fungi are more efficient than bacteria (Venkateswarlu *et al.,* 1984). A promising biotechnological strategy in the management of P fertilization is the use of phosphate-solubilizing fungi to solubilize rock phosphates and allow the recovery of unavailable P fixed in the soil.

Talaromyces (Trichocomaceae) is an important fungal genus which is derived from the Greek word for 'basket', which capably describes the body in which ascospores are formed. In the past, species producing sexual stages with *Penicillium* anamorphs have been classified in *Eupenicillium* spp. and *Talaromyces* spp. After July 2011, species formally reclassifies as the *Penicillium* subgenus *Biverticillium* were classified in *Talaromyces*.The situation is complicated by the fact that many species now classified in *Talaromyces* will continue to be sought as *Penicillium* species in identifications (Pitt, 2014).

The habitats of *Talaromyces pinophilus* are global in environment, *T. pinophilus* (Samson *et al.,* 2011); anamorph: *Penicillium pinophilum* (Thom, 1910) and ***P.*** *allahabadense* (Mehrotra *et al.,* 1962) currently designated by *T. allahabadensis* **(**Samson *et al.,* 2011) were prevalently isolated from soil, compost (El-Naggar *et al.,* 2015), seeds grains (Ismail *et al.,* 2016), phyllosphere (Lindow *et al.,* 2002), phylloplane (Abdel-Gawad *et al.,* 2017; Abdel-Hafez *et al.,* 2015) and some medicinal plants (Koul *et al.,* 2016; Yao *et al.,* 2017).

T. pinophilus has received increasing attention in mycological research for its ability to act as a fungal antagonist and plant-growth promoter (Nicoletti *et al.,* 2004; Pandey *et al.,* 2008; Wani *et al.,* 2016). A few categorical studies on phosphate solubilizing abilities of *T. pinophilus* were reported previously by others (Sembiring and Fauzi, 2017; Abdul Wahid and Mehana, 2000) but no studies have been made on the in-depth p-solubilization potential and comparative performance of different *T. pinophilus* strains yet. Therefore, the study aimed to compare the Phosphorous solubilization capabilities of 17 *T. pinophilus* fungal strains, in order to determine the best options for environmentally friendly fertilizers.

MATERIALS AND METHODS

The microbiological study was carried out in the Mycology Laboratory, Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan during August 2017–November 2018 under a class II biohazard cabinet (BHC-1306IIA/3B, AIRTECH, Tokyo, Japan) followed to the biosafety classification by National Institute of Infectious Disease of Japan, because of possibilities of including toxic fungal species treated as BSL2 during the isolation.

Fungal Isolates and Culture Preparation

Seventeen *Talaromyces pinophilus* fungal strains were used in this study where 2 strains were isolated from hairy vetch incorporated soil and others collected from different institutions in Japan (Table 1). The isolates were cultured on potato dextrose agar (PDA; Becton, Dickinson and Company, Sparks, MD, USA) slant and kept at 4°C for further study.

Table V. 1. List of the fungal strains used in the study

Isolates	Strain in GenBank	Source	Country of origin	Fungi	Institution
1	SI-4URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
2	SI-15URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
3	SI-17URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
4	SI-19URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
5	IFM 64651	Sputum	Japan	*Penicillium pinophilum*	Chiba University
6	IFM 57309	Sputum	Japan	*Penicillium pinophilum*	Chiba University
7	NBRC 6345	Radio set	Papua New Guinea	*Talaromyces pinophilus*	NITE BRC
8	NBRC 100533	Polyvinyl chloride plastic	France	*Talaromyces pinophilus*	NITE BRC
9	NBRC 33285	Polyvinyl chloride plastic	France	*Talaromyces pinophilus*	NITE BRC
10	NBRC 106907	Soil	Japan	*Penicillium pinophilum*	NITE BRC
11	NBRC 9575	Polyvinyl chloride plastic	France	*Penicillium allahabadense*	NITE BRC
12	JCM 9928	Soil	India	*Penicillium pinophilum*	RIKEN
13	JCM 5593	Radio set	Papua New Guinea	*Penicillium pinophilum*	RIKEN
14	JCM 22801	Wood stakes	Australia	*Penicillium pinophilum*	RIKEN
15	JCM 22802	Barley grain	Australia	*Penicillium pinophilum*	RIKEN
16	JCM 22803	Moldy sorghum grain	Australia	*Penicillium pinophilum*	RIKEN
17	JCM 23043	Radio set	Papua New Guinea	*Penicillium pinophilum*	RIKEN

NITE; National Institute of Technology and Evaluation, Biological Resource Center, NITE (NBRC), Japan. RIKEN; is a large scientific research institute in Japan

Morphological Studies

Morphological studies were done according to the method (Watanabe, 2010). Pigment exudation were investigated on PDA slants. High-temperature resistance and pathogenic potential of the isolates were examined. The isolates were cultured on PDA slants in duplicate and incubated at 25^0C, 35^0C, 37^0C, and 42^0C for 7 days to evaluate the growth of mycelia. Growth of isolates at 25^0C (room temperature) treated as positive control.

Preparation of Spore Suspension

Sporulated pure fungal cultured slants were selected for preparation of spore suspension followed by standard procedure. A total volume of 5 ml sterile water with 0.02% of tween 80 (Polyoxyethylene sorbitan monooleate, Nacalai Tesque, Inc, Kyoto, Japan) was poured on the culture slants and the fungal colony surface was lightly scraped by a sterile inoculation loop (Thermo Scientific™, Nunc™ Disposable Loops and Needles, Thermo Scientific™ 251586, Fisher Scientific, Tokyo, Japan). The cultures were passing through a syringe with a 4 x 4 cm sheet of a sterile absorbent cotton (Kyualet, Kawamoto Sangyo, Osaka, Japan). Spore count was done by a hemocytometer and the suspension was adjusted to approximately 10^6 spores mL^{-1}.

Determination of TCP Solubilization Index on Solid Medium

All the isolates were tested under *in vitro* condition for their phosphate solubilization activity on Pikovskaya's agar medium containing 0.5% tricalcium phosphate (TSP) as an insoluble phosphorus source. A spot inoculation of each fungal isolate was made onto the plates in triplicate under aseptic condition and incubated at 28°C for 7 days in darkness. At 7^{th} day of incubation, phosphate solubilization index was measured using the following formula (Premono et al., 1996):

Solubilization Index (SI) = [Colony diameter + Halo zone diameter]/Colony diameter

Quantitative Estimation of Phosphate Solubilization

It was carried out using Erlenmeyer flask containing 40 ml Pikovskaya's broth medium supplemented with 0.5% tricalcium phosphate [$Ca_3(PO_4)_2$], aluminium phosphate ($AlPO_4$) and iron phosphate ($FePO_4$). After sterilization, the medium of

each flask was inoculated with the 5 % (v/v) spore suspension of a particular fungal isolate containing 10^6 spores mL^{-1}. Sterile distilled water inoculated flaks were treated as control. Three replicates were maintained for each test isolate and mean value was recorded. Incubation was done at 25^0C in an incubator shaker (EYELA Multi Shaker MMS-3010) at 120 rpm for 9 days. The samples were autoclaved and centrifuged (TOMY MX-301) at 5000 rpm for 25 minutes to remove any suspended solids and mycelial parts. Then the cultures were filtered through 0.45 µm pore size syringe filter unit (Advantech, Japan). The filtrates were used for analysis of soluble phosphate and pH value. The pH value of the culture supernatants was determined by a pH meter (HORIBA, Japan) equipped with a glass electrode. The amount of soluble phosphorus in culture supernatants was measured by molybdenum blue method (Murphy and Riley, 1962) and expressed as mg/L. Samples cultured for 3, 6 and 9 days were compared. After calculation of mean phosphate degradation ability from 17 isolates of each day, we selected the adequate period for the comparison depending on the substrate.

Data Analysis

All experiments were conducted in triplicate and data were analyzed using Microsoft Excel program. The mean values were compared by Fisher test and significant differences were detected at $p< 0.05$ level. Correlation between solubilized phosphate and pH of the medium was determined by using Pearson correlation studies.

Photo **V.** 1. Farmented Pikovskaya broth culture inoculated with 17 *Talaromyces pinophilus* strains.

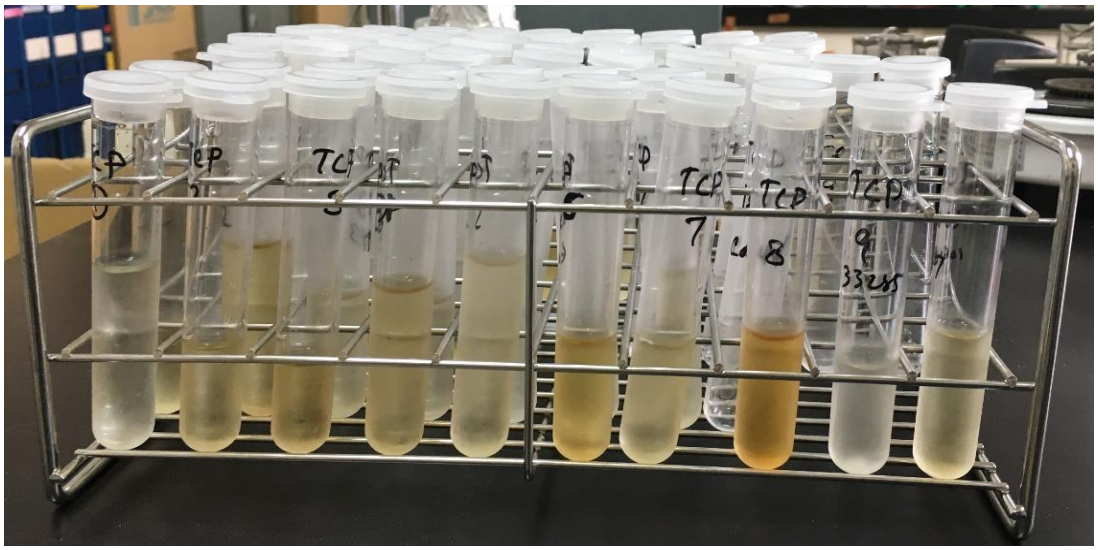
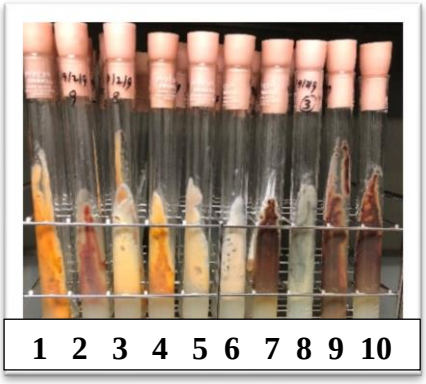
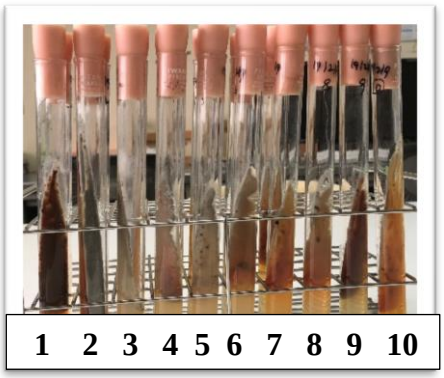


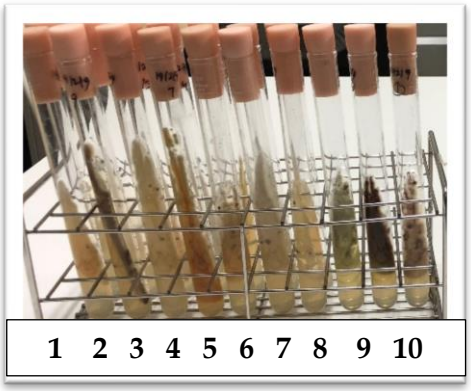
Photo **V**. 2. Farmented Pikovskaya broth culture collected for phosphorus determination by Spectrophotometer



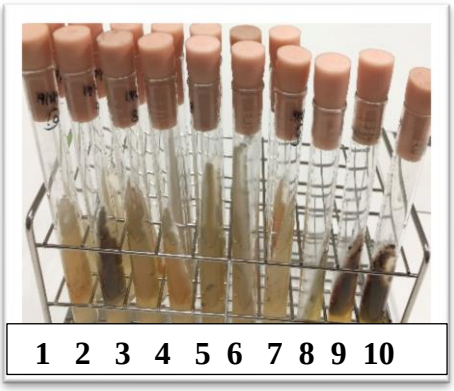
(25^{0}C)



(35^{0}C)



(37^{0}C)



(42^{0}C)

Photo. **V**. 3. The growth and survival of *T pinophilus* strains at different temperature

RESULTS

Pigment exudation

All isolates were reconfirmed based on morphological studies. Morphological studies results are not shown. The isolates produced various color in PDA slant, such as coffee, orange, yellow and cream (Photo **V.** 4). Differential color formation by *T. pinophilus* isolates might be attributed to the presence of bioactive metabolites.

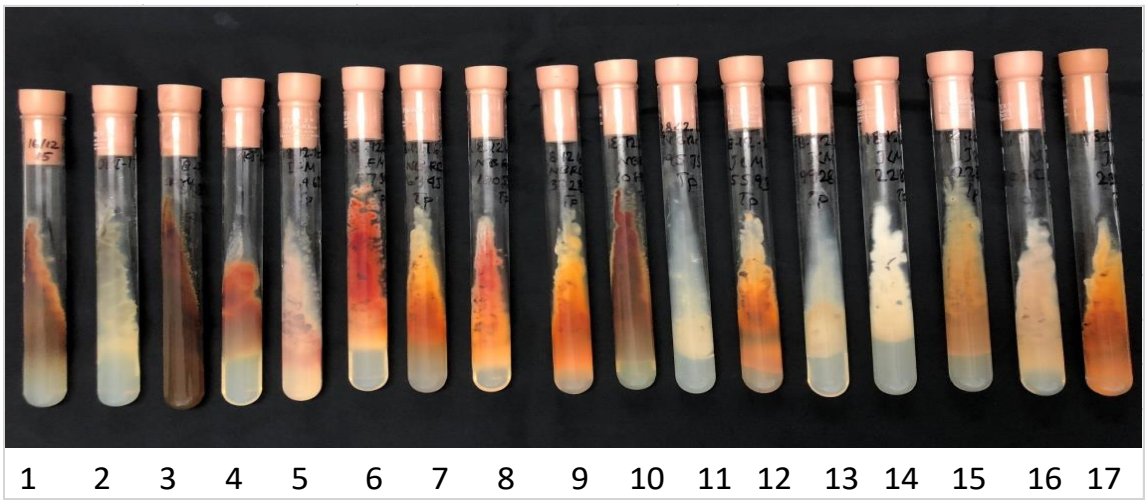


Photo **V.** 4. *Talaromyces pinophilus* isolates grown on Potato Dextrose Agar (PDA) slant for 7 days at 25^0 C [Coffee: 1, 3, 10; Cream: 2, 5, 11, 13, 14, 16; Orange: 4, 6; Yellow=7, 8, 9, 12, 15, 17]. [1, SI-4URAgr; 2, SI-15URAgr; 3, SI-17URAgr; 4, SI-19URAgr; 5, IFM 64651; 6, IFM 57309; 7, NBRC 6345; 8, NBRC 100533; 9, NBRC 33285; 10, NBRC 106907; 11, NBRC 9575; 12, JCM 9928, 13, JCM 5593; 14, JCM 22801; 15, JCM 22802, 16, JCM 22803, 17, JCM 23043].

Qualitative phosphate solubilization

All isolates showed different levels of phosphate solubilization from inconspicuous to a substantial in Pikovskaya's agar medium using tricalcium phosphate as the substrate. Six isolates produced clear zone, 5 developed translucent and the remaining isolates had inconspicuous zones. All isolates also showed significant phosphate solubilization in Pikovskaya's agar medium using tricalcium phosphate as the substrate. The phosphate solubilization index (PSI) ranged from 1.05 to 1.46 (Table **V.** 2).

Table **V. 2.** TCP solubilization in Pikovskaya's agar by 17 fungal strains

Isolate	Fungal strain	Fungi	PSI	Type of zone
1	SI-4URAgr	*Talaromyces pinophilus*	1.31±0.01^{b}	C
2	SI-15URAgr	*Talaromyces pinophilus*	1.46±0.03^{a}	C
3	SI-17URAgr	*Talaromyces pinophilus*	1.09±0.01^{d}	T
4	SI-19URAgr	*Talaromyces pinophilus*	1.12±0.01^{d}	T
5	IFM 64651	*Penicillium pinophilum*	1.07±0.02^{d}	I
6	IFM 57309	*Penicillium pinophilum*	1.09±0.03^{d}	I
7	NBRC 6345	*Talaromyces pinophilus*	1.11±0.02^{d}	T
8	NBRC100533	*Talaromyces pinophilus*	1.21±0.08^{bc}	C
9	NBRC33285	*Talaromyces pinophilus*	1.14±0.08^{cd}	C
10	NBRC 106907	*Penicillium pinophilum*	1.24±0.03^{b}	T
11	NBRC 9575	*Penicillium allahabadense*	1.07±0.03^{d}	I
12	JCM 9928	*Penicillium pinophilum*	1.09±0.06^{d}	T
13	JCM 5593	*Penicillium pinophilum*	1.19±0.09^{bc}	C
14	JCM 22801	*Penicillium pinophilum*	1.05±0.05^{d}	I
15	JCM 22802	*Penicillium pinophilum*	1.08±0.05^{d}	I
16	JCM 22803	*Penicillium pinophilum*	1.09±0.05^{d}	T
17	JCM 23043	*Penicillium pinophilum*	1.06±0.05^{d}	I

PSI: Phosphate solubilization index, Values given are the mean ± standard deviation of three independent replicates, Same letter in the column are not significantly different at $p<0.05$ by Fisher's test, C = clear, T = translucent, I= inconspicuous.

Quantitative phosphate solubilization

Phosphate solubilization by all isolates were tested in Pikovskaya's broth medium using three substrates of recalcitrant phosphate compounds: tricalcium phosphate [$Ca_3(PO_4)_2$], aluminium phosphate ($AlPO_4$) and iron phosphate ($FePO_4$). The phosphate solubilizing ability of fungal isolates varied with incubation period and substrates. The best period of observation was 6 days considering their mean P solubilization for the three substrates (Table **V.** 3).

Table **V.** 3**.** Selection for the best period of phosphate solubilization by 17 isolates

Solubilized phosphate (mg/L)								
TCP			**Al-P**			**Fe-P**		
3 days	**6 days**	**9 days**	**3 days**	**6 days**	**9 days**	**3 days**	**6 days**	**9 days**
196.2±93.0	354.3±136.1*	343.2±125.1	80.6±29.8	111.6±40.7*	63.6±22.2	55.3±16.3	95.8±28.1*	69.4±20.1

TCP: tricalcium phosphate, Al-P: aluminium phosphate, Fe-P: iron phosphate, Values given are the mean ± standard deviation of P solubilized by 17 fungal isolates, An asterisk (*) indicated the best period of phosphate solubilization.

All *T. pinophilus* strains were tested for their ability to solubilize hardly soluble phosphate sources [$Ca_3(PO_4)_2$, $AlPO_4$, and FePO4]. All of the *T. pinophilus* strains showed potential P solubilizing in the medium containing $Ca_3(PO_4)_2$ followed by $AlPO_4$ and $FePO_4$ (Table **V** 4). The solubilized P ranged between 83.7-574.8 mg/L, 50.5-192.4 mg/L and 50.5-192.4 mg/L from $Ca_3(PO_4)_2$, $AlPO_4$, and $FePO_4$, respectively. Among 17 isolates, 6 isolates (SI-4URAgr, SI-17URAgr, SI-19URAgr, NBRC 6345, NBRC 100533 and JCM 22801) were considered as outstanding isolates because solubilized P was higher than the sum of the mean and standard deviation of P solubilized by 17 isolates. The highest amount of P solubilization from $Ca_3(PO_4)_2$ was shown in NBRC6345 (574.8 mg/L) followed by NBRC100533(540.4 mg/L) and SI-17URAgr (537.2 mg/L). The highest amount of P solubilization from $AlPO_4$, was shown in SI-4URAgr (192.4 mg/L) followed by SI-19(153.7 mg/L). The highest amount of P solubilization from $FePO_4$ was shown in SI-19URAgr (145.2 mg/L) followed by JCM 22801(126.1 mg/L). The strain SI-19URAgr showed outstanding performance in both $AlPO_4$, and $FePO_4$ solubilization (Table **V.** 4). In case of total

solubilized phosphate (mgl^{-1}) from three substrates (TCP, AL-P and Fe-P), the highest amount of P solubilization was shown in NBRC 6345(829.6 mg/L) followed by SI-17URAgr (789.2 mg/L) and NBRC100533(770.4 mg/L). The tested isolates produced various colors in broth medium using three P substrates during incubation period (data not shown).

Table **V.** 4. Phosphate solubilization from different substrates by *T. pinophilus* fungal strains

Sl. No	Strain	Fungi	Solubilized phosphate (mg/L)			Total P (mg/L)
			TCP	AL-P	Fe-P	
1	SI-4URAgr	*Talaromyces pinophilus*	268.4	192.4*	46.5	507.3
2	SI-15URAgr	*Talaromyces pinophilus*	363.7	132.9	34.8	531.4
3	SI-17URAgr	*Talaromyces pinophilus*	537.2*	135.7	116.3	789.2*
4	SI-19URAgr	*Talaromyces pinophilus*	314.0	153.7*	145.2*	612.8
5	IFM 64651	*Penicillium pinophilum*	324.3	50.5	85.6	460.5
6	IFM 57309	*Penicillium pinophilum*	228.5	51.4	65.6	345.5
7	NBRC 6345	*Talaromyces pinophilus*	574.8*	148.6	106.2	829.6*
8	NBRC100533	*Talaromyces pinophilus*	540.4*	134.7	95.3	770.4*
9	NBRC 33285	*Talaromyces pinophilus*	263.5	135.8	95.4	494.7
10	NBRC106907	*Penicillium pinophilum*	482.5	107.2	106.5	696.3
11	NBRC 9575	*Penicillium allahabadense*	83.7	96.0	116.5	296.2
12	JCM 9928	*Penicillium pinophilum*	135.2	85.1	105.3	325.6
13	JCM 5593	*Penicillium pinophilum*	387.7	137.2	105.3	630.2
14	JCM 22801	*Penicillium pinophilum*	405.7	54.8	126.1*	586.6
15	JCM 22802	*Penicillium pinophilum*	392.2	83.9	85.9	562.1
16	JCM 22803	*Penicillium pinophilum*	397.1	125.2	116.1	638.4
17	JCM 23043	*Penicillium pinophilum*	325.2	71.7	76.6	473.5
		Mean±SD	354.3±136.1*	111.6±40.7	95.8±28.1	561.8±157.8

TCP: tricalcium phosphate, Al-P: aluminium phosphate, Fe-P: iron phosphate, Values given are the mean ± standard deviation of P solubilized by 17 fungal isolates, An asterisk (*) indicated outstanding values of solubilized phosphate, It was higher than sum of mean and standard deviation of P solubilized by 17 fungal isolates.

Correlation between pH and soluble P

The pH of the culture medium exhibited the opposite changes. It decreased with the increased amount of soluble P in the medium. Correlation studies showed a significant inverse relationship between soluble P and pH of the culture medium (Fig. **V.** 2). The negative correlation was observed in all fermented broth culture and correlation coefficient (r) was -0.88, -0.51 and -0.60 in TCP, Al-P and Fe -P respectively. The strong negative correlation was observed in TCP containing medium.

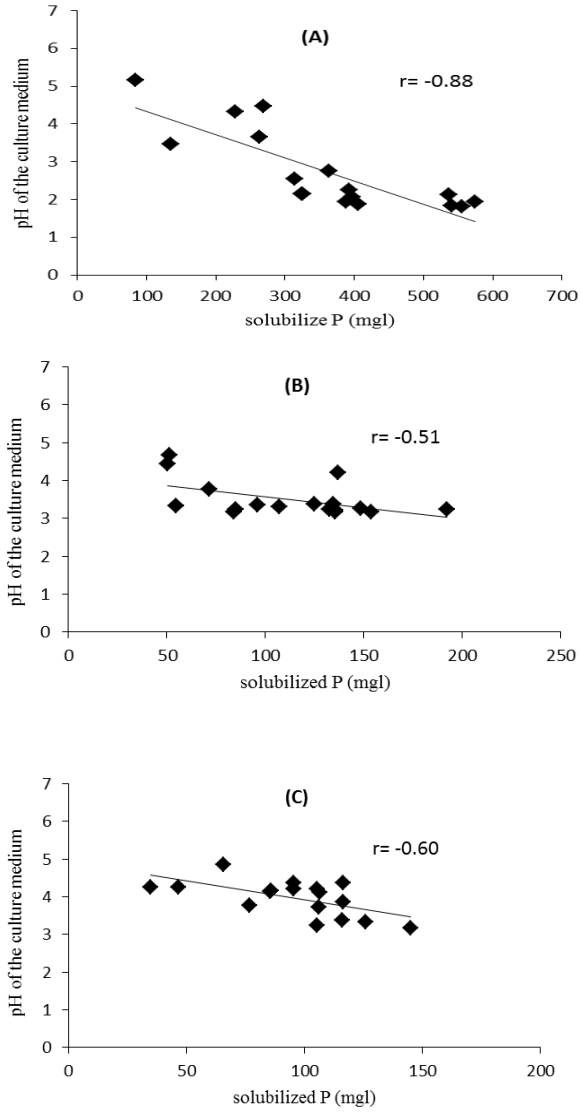


Fig. V. 2. Pearson's correlation between soluble phosphate and pH of the culture medium supplemented with TCP (A), $AlPO_4$ (B) and $FePO_4$ (C); inoculated by 17 fungal strains. [SI-4URAgr, SI-15URAgr, SI-17URAgr, SI-19URAgr, IFM 64651, IFM 57309, NBRC 6345, NBRC 100533, NBRC 33285, NBRC 106907, NBRC 9575, JCM 9928, JCM 5593, JCM 22801, JCM 22802, JCM 22803, JCM 23043].

Temperature effects on strains

Survival of the strains at different temperature was investigated (Table **V.** 5). Most of the strains could grow and survive at 25°C-37°C. Strain SI-4URAgr, SI-15URAgr, SI-17URAgr, SI-19URAgr and NBRC 106907 could grow at 25°C-42°C. These strains are summer tolerant and they could survive both acidic (low pH) and alkaline environment (high pH).

Table **V**. 5. The growth and survival of *T. pinophilus* strains at different temperature

SL No.	Strain	Fungi	Growth temperature			
			25^{0}C	**35^{0}C**	**37^{0}C**	**42^{0}C**
1	SI-4URAgr	*Talaromyces pinophilus*	+	+	+	+
2	SI-15URAgr	*Talaromyces pinophilus*	+	+	+	+
3	SI-17URAgr	*Talaromyces pinophilus*	+	+	+	+
4	SI-19URAgr	*Talaromyces pinophilus*	+	+	+	+
5	IFM 64651	*Penicillium pinophilum*	+	+	+	-
6	IFM 57309	*Penicillium pinophilum*	+	+	+	-
7	NBRC 6345	*Talaromyces pinophilus*	+	+	+	-
8	NBRC100533	*Talaromyces pinophilus*	+	+	+	-
9	NBRC 33285	*Talaromyces pinophilus*	+	+	+	+
10	NBRC106907	*Penicillium pinophilum*	+	+	+	-
11	NBRC 9575	*Penicillium allahabadense*	+	+	+	-
12	JCM 9928	*Penicillium pinophilum*	+	+	+	-
13	JCM 5593	*Penicillium pinophilum*	+	+	+	-
14	JCM 22801	*Penicillium pinophilum*	+	+	+	-
15	JCM 22802	*Penicillium pinophilum*	+	+	+	-
16	JCM 22803	*Penicillium pinophilum*	+	+	+	-
17	JCM 23043	*Penicillium pinophilum*	+	+	+	-

(+) indicated growth of fungi, (-) indicated no growth.

DISCUSSION

Morphological studies reconfirmed that all the 17 strains used in this study belong to *Talaromyces pinophilus.* All fungal isolates produced different colours in the medium regardless of the P substrates, but the colour seemed not to have any relation with P solubilization.

T. pinophilus has various applications based on the production of enzymes (Hansen *et al.,* 2015; Li *et al.,* 2017) as well as a renewable source of pigments, colorants (Caro *et al.,* 2017) and bioactive compounds (Wang *et al.,* 2013; Nicoletti *et al.,* 2016) and degrading ability of agricultural waste (El-Naggar *et al.,* 2015). *T. pinophilus* is able to produce a variety of bioactive metabolites, including alkaloids, peptides, lactones, polyketides and miscellaneous structure type compounds, with different chemical and biological activities. *T. pinophilus* produces biomass-degrading enzymes such as α-amylase (Xian *et al.,* 2015), cellulase (Visser *et al.,* 2013), endoglucanase (Pol *et al.,* 2012), xylanase (Visser *et al.,* 2013), laccase (Dhakar *et al.,* 2014) and α-galactosidase (Visser *et al.,* 2013). *T. pinophilus* produces a variety of medically useful metabolites such as 3-O- methylfunicone, which is used to inhibit mesothelioma cell motility, and talaromycolides 1-3,5 and 11 which inhibit the growth of the human pathogen methicillin resistant *Staphylococcus aureus* (Buommino *et al.,* 2012).

In the present study, all isolates showed better P solubilizing activity in liquid medium compared with solid medium. The possible reason for these anomalous behaviors of isolates on liquid and solid media could be attributed to nutrient availability, varying diffusion rate of different organic acids secreted by fungi, and growth requirement of fungi (Jain *et al.,* 2014). Alam *et al.* (2002) reported that some isolates having little clear zone on solid agar medium exhibited higher phosphate solubilization efficiency in liquid medium. Some fungal isolates showed larger clear zones on solid agar medium but low phosphate solubilization in liquid medium. This shows that higher PSI on solid agar medium does not necessarily show solubilization efficiency in liquid medium. Thus, the plate technique is insufficient to screen the best P solubilizers and to detect all phosphate solubilizers mentioned by Nautiyal C. S. (1999).

The strongest P solubilization was found in $Ca_3(PO4)_2$ followed by $AlPO_4$ and $FePO_4$ because $AlPO_4$ and $FePO_4$ have complex structure than $Ca_3(PO4)_2$ while fungi exhibited low P solubilizing ability in media containing $AlPO_4$ and $FePO_4$ (Son *et al.,* 2006; Zhang *et al.,* 2018). The mechanisms of phosphate solubilization by microorganisms are very complex and are not completely known yet. The very common mechanisms are acidification, chelation and exchange reactions (Chai *et al.,* 2011). Organic acids play an important role in phosphate solubilization processes, which can help the release of P by providing protons and complexing anions, or ligand exchange reactions or complexion of metal ions release to solution. Organic acids production depends on the interaction of P source and fungi (Zhang *et al.,* 2018; Scervino *et al.,* 2013).

In the present study, the isolates (SI-4URAgr, SI-17URAgr, SI-19URAgr, NBRC 6345, NBRC 100533, and JCM 22801) showed the highest efficiency in P solubilization by decreasing pH of the culture medium, which indicated higher amount of organic acid production. Solubilization of the different P sources mostly depended on the amount of organic acids production by fungi (Zhang *et al.,* 2018). Tricarboxylic acids and other lower molecular weight organic acids are considered to be the main contributors to phosphate solubilization and a decrease in pH of the medium (Chai *et al.,* 2011).

It was observed that phosphate solubilization was negatively correlated with pH of the medium. There are several reports where such correlation was documented (Pandey *et al.,* 2008; Jain *et al.,* 2012; Wani *et al.,* 2016;). The activities in lower pH indicated that the increase of organic acids in the medium (Pradhan and Sukla, 2005; Saxena *et al.,* 2013). However, soluble P was increased without changing pH in some occasion because of other mechanism (Jain *et al.,* 2012, 2017) such as; chelation and exchange reactions (Chai *et al.,* 2011).

Chapter VI

Organic Acid Production Efficiency of Different Phosphate Solubilizing *Talaromyces pinophilus* Strains

Abstract: Soils are generally low in phosphorous (P) readily available for plants growth and yield. Microorganisms play an important role to improve available P status in soil by solubilization process. Although the mechanism of phosphate solubilization is still now not well documented but the main mechanism recognized to be responsible for the solubilization of phosphorus is the production of different types of organic acids. Previously we studied the P solubilization potentiality of 17 *Talaromyces pinophilus* strains. Therefore, the present study aimed to evaluate the organic acid production efficiency of these fungal strains in broth containing insoluble tricalcium phosphate [$Ca_3(PO_4)_2$], aluminum phosphate ($AlPO_4$) and iron phosphate ($FePO_4$). Results showed that both type and the quantity of organic acid production depended on the P source and fungal strains. The test fungal strains produced highest amount of acetic, formic and lactic acids in the medium supplemented with $Ca_3(PO_4)_2$, citric and oxalic acids were produced in the medium supplemented with $AlPO_4$ and tartaric and malic acids were produced in the medium supplemented with $FePO_4$. Finally, the strain NBRC106907 has the strongest ability to produce organic acids considering the whole study followed by SI-4URAgr and JCM5593. These strains may become a potential bioresource for agricultural and industrial purposes.

Key words: Phosphorous, Talaromyces pinophilus, organic acid, solubilization mechanism, insoluble phosphate, plant growth and yield.

INTRODUCTION

Phosphorus (P) is a key nutrient required for plant growth and development which plays an influential role in biochemical and physiological activities of plants (Chai *et al.*, 2011; Mittal *et al.,* 2008). Most of the soils in the world having insoluble phosphate that can't be utilized by the plants unless solubilization (Singh *et al.,* 2011). Acidic environment can enhance the solubility of P minerals significantly which is a possible major pathway to increase soluble P release from phosphate minerals (Zhen *et al.,* 2016). Soil microbes solubilize insoluble inorganic phosphates and convert them available to the plants (Pradhan and Sukla, 2005). Although the precise mechanism of phosphate solubilization utilized by different phosphate solubilizing microorganisms (PSM) still now not clear, the production of organic acid is recognized as main mechanism responsible for P solubilization (Nahas, 1996; Alam *et al.*, 2002, Siddique and Robinson, 2003). There are many significant roles of organic acids in agriculture. Soil organic acids have been reported to have both direct and indirect roles in crop production (Barea *et al.,* 2005) and their presence in the soil improves the physico-chemical properties of the soil and may help facilitate uptake of deficient/unavailable/ insoluble nutrients (Morgan *et al.,* 2005). Organic acids also have huge industrial applications as food supplement, pharmaceutical and cosmetic diluents (Sauer *et al.*, 2008). They are fully degradable molecules and can be used as chemical intermediates or as for the production of biodegradable polymers replacing synthetic chemicals (Sauer *et al.,* 2008).

A large number of phosphate solubilizing microorganisms including bacteria, fungi and actinomyces have the ability to secrete organic acids (Kavanagh, 2011) and they help to dissolving insoluble P through the process of acidification, chelation and exchange reaction, which promote plant growth (Gerresten, 1948; Singh *et al.*, 2011). The ability of organic acids secretion by fungi is 10 times higher than bacteria (Kavanagh, 2011). Among the microorganisms, fungal species belonging to *Aspergillus, Penicillium, Talaromyces* and *Eupenicilium* have shown potential for solubilization of insoluble P compounds and they are considered as "key organisms" in the P cycle (Whitelaw, 2000; Achal *et al.*, 2007; Jain *et al.*, 2017).

The organic acid producing capabilities of phosphate solubilizing microorganisms are primarily determined by gene, but can also be affected by environmental conditions. For example, carbon and nitrogen could affect the types of organic acids and phosphate solubilizing (Narsian and Patel, 2000).

In the previous study, 17 *Talaromyces pinophilus* (anamorph: *Penicillium pinophilum;* Thom, 1910*)* strains solubilized different insoluble phosphate compounds such as $Ca_3(PO_4)_2$; TCP, $AlPO_4$; Al-P and $FePO_4$; Fe-P. However, the organic acid production abilities of these strains in different P substrates are unidentified. In this study, We evaluated the organic acid production ability of phosphate solubilizing *T. pinophilus* in the medium supplemented with different insoluble phosphate compounds.

MATERIALS AND METHODS

Fungal strains

Seventeen *Talaromyces pinophilus* fungal strains were used in this study where 4 strains isolated from subtropical soils in Okinawa, Japan and 13 strains collected from different institutions in Japan (Table 1). The isolates were cultured on potato dextrose agar (PDA; Becton, Dickinson and Company, Sparks, MD, USA) slant and kept at 4^{o}C for further study.

Table VI. 1. List of the fungal strain used in the study

Isolates	Strain in GenBank	Source	Country of origin	Fungi	Institution
1	SI-4URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
2	SI-15URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
3	SI-17URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
4	SI-19URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
5	IFM 64651	Sputum	Japan	*Penicillium pinophilum*	Chiba University
6	IFM 57309	Sputum	Japan	*Penicillium pinophilum*	Chiba University
7	NBRC 6345	Radio set	Papua New Guinea	*Talaromyces pinophilus*	NITE BRC
8	NBRC 100533	Polyvinyl chloride plastic	France	*Talaromyces pinophilus*	NITE BRC
9	NBRC 33285	Polyvinyl chloride plastic	France	*Talaromyces pinophilus*	NITE BRC
10	NBRC 106907	Soil	Japan	*Penicillium pinophilum*	NITE BRC
11	NBRC 9575	Polyvinyl chloride plastic	France	*Penicillium allahabadense*	NITE BRC
12	JCM 9928	Soil	India	*Penicillium pinophilum*	RIKEN
13	JCM 5593	Radio set	Papua New Guinea	*Penicillium pinophilum*	RIKEN
14	JCM 22801	Wood stakes	Australia	*Penicillium pinophilum*	RIKEN
15	JCM 22802	Barley grain	Australia	*Penicillium pinophilum*	RIKEN
16	JCM 22803	Moldy sorghum grain	Australia	*Penicillium pinophilum*	RIKEN
17	JCM 23043	Radio set	Papua New Guinea	*Penicillium pinophilum*	RIKEN

NITE; National Institute of Technology and Evaluation, Biological Resource Center, NITE (NBRC), Japan.
RIKEN; is a large scientific research institute in Japan

Culture medium

PKV broth medium was used for this study (Rao, 1982). In this medium $Ca_3(PO_4)_2$ was used as source of insoluble phosphate compound that was replaced by insoluble $FePO_4$ and $AlPO_4$. The medium was autoclaved at 121^{0}C for 15 minutes. Chloramphenicol (Wako Pure Chemical Corporation, Osaka, Japan) was also used to avoid bacterial growth.

Spore suspension preparation

For conducting organic acid production experiment, fungal cultures were made from the re-slanting of pure culture slants that preserved at 4^0C according to previous experiments. Spore suspension was prepared according to the method mentioned in previous **chapter, IV and V.**

Incubation

The experiments were carried out using Erlenmeyer flask containing 40 ml Pikoveskaya's (PKV) broth medium supplemented with 0.5% tricalcium phosphate (Ca_3PO_4; TCP), aluminium phosphate ($AlPO_4$; AL-P) and iron phosphate ($FePO_4$; Fe-P). After sterilization, the medium of each flask was inoculated with the 5 % (v/v) spore suspension of a particular fungal strain containing 10^6 spore mL-1. Sterile distilled water inoculated flaks was treated as control. Three replicates were maintained for each test isolate. Incubation was done at 25^0C in an incubator shaker at 120 rpm up to 7 days (Photo **VI.** 2). The samples were autoclaved and centrifuged at 5000 rpm for 25 minutes to remove any suspended solids and mycelial parts. The culture supernatants were filtered through 0.22 µm pore size syringe filter unit (Merck KGaA, Darmstadt, Germany).

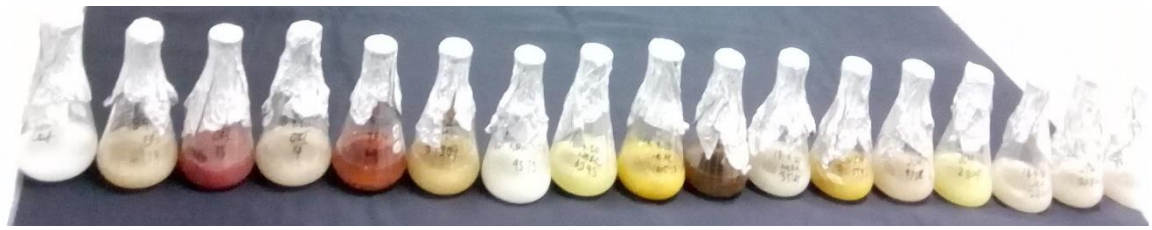


Photo **VI.** 1. Farmented Pikovskaya broth culture for organic acid determination by HPLC inoculated with 17 phosphate solubilizing fungal strains.

Organic acids analysis

Detection and quantification of organic acids were done by High Performance Liquid Chromatography (Prominence HPLC system, Shimadzu-CBM-20A, Japan) equipped with diode array detector (SPD-M20A), refractive index detector (RID-10A), column ICE-ION-300 (300mmX7.8mm), auto sampler (LC-20AD) and fraction collector (FRC-10A). The injection volume, temperature and flow rate was 50 µl, 50^oC and 0.5ml/min,

respectively. Sulfuric acid (0.01N) (Merck, Germany) was used as solvent of mobile phase. Peaks were identified against a set of standards from known organic acids (oxalic, citric, tartaric, malic, lactic, formic and acetic acids; Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis

All experiments were conducted in triplicate and data were analyzed using Microsoft Excel program (version 2016). The mean values were compared by Fisher test and significant differences were detected at $p< 0.05$ level.

RESULTS

I detected and quantified seven different organic acids, oxalic, citric, tartaric, malic, lactic, formic and acetic from medium containing TCP, Al-P and Fe-P.

Organic acid production by fungal strains in the medium supplemented with insoluble P compounds [$Ca_3(PO_4)_2$; TCP, $AlPO_4$; AL-P and $FePO_4$; Fe-P]

In the medium supplemented with TCP, all the strains produced oxalic, citric, tartaric, lactic, formic and acetic acids except malic acid in the medium. The amount ranged from 2.3-26.7, 3.0-404.3, 7.3-77.0, 8.0-285.0, 17.3-484.0, 9.0-142.0 µg/mL, respectively. Excepting SI-15URAgr and JCM 9928, other strains produced malic acid ranged from 17.0-266.0 µg/mL. The highest amount of oxalic (26.7 µg/mL), citric (404.3 µg/mL), tartaric (77.0 µg/mL), malic (266.0 µg/mL) and formic (484.0 µg/mL) were produced by the strain JCM 22803, NBRC 9575, NBRC 6345, NBRC 106907 and SI-17URAgr, respectively whereas the strain SI-19URAgr produced higher both lactic (285.3 µg/mL) and acetic acids(142.3 µg/mL) (table **VI.** 2).

Table **VI.** 2. Types and quantities of produced organic acids in the Pikoveskaya's medium supplemented with $Ca_3(PO_4)_2$, (TCP) compound by phosphate solubilizing *T. pinophilus* strains

		Organic acid (µg/mL)						
Strains	**Fungi**	**Oxalic**	**Citric**	**Tartaric**	**Malic**	**Lactic**	**Formic**	**Acetic**
SI-4URAgr	*T. pinophilus*	22.0±2.5	7.3±0.5	42.7±9.0	115.3±2.5	126.3±6.5	107.0±8.0	24.0±4.0
SI-15URAgr	*T. pinophilus*	15.3±3.0	19.7±3.0	42.7±4.5	N. D.	21.0±4.0	167.0±11.0	37.0±4.0
SI-17URAgr	*T. pinophilus*	9.7±3.0	24.3±6.1	50.7±3.5	17.0±1.0	104.3±13.5	484.0±13	46.0±5.5
SI-19URAgr	*T. pinophilus*	5.7±1.5	6.7±1.5	25.0±4.6	121.0±14.1	285.3±19.5	140.0±17.5	142.3±19.2
IFM 64651	*P. pinophiilum*	9.0±2.0	6.0±1.0	25.7±3.1	45.7±1.5	162.7±4.0	138.0±22.6	45.0±8.2
IFM 57309	*P. pinophiilum*	2.3±0.6	10.0±1.0	21.0±4.6	88.7±9.5	62.3±5.0	73.3±5.5	117.0±11.0
NBRC 6345	*T. pinophilus*	12.0±2.6	6.0±1.0	77.0±9.5	250.0±19.6	25.7±5.5	120.7±7.0	50.7±7.6
NBRC100533	*T. pinophilus*	11.0±2.0	20.3±2.5	52.0±5.5	81.7±8.7	94.0±4.6	116.3±10.5	54.3±7.5
NBRC 33285	*T. pinophilus*	8.0±2.6	144.7±20.6	7.3±2.5	67.3±14.0	9.0±2.0	70.3±16.3	21.3±2.5
NBRC106907	*P. pinophilum*	9.7±3.0	254.3±34.5	36.7±1.5	266.0±19.0	8.0±1.0	145.0±11.0	33.0±4.0
NBRC 9575	*P. allahabadense*	7.0±2.0	404.3±17.2	36.7±9.5	154.7±18.0	13.0±3.0	17.3±4.0	82.0±7.0
JCM 9928	*P. pinophiilum*	4.3±1.5	5.7±1.5	12.0±3.0	N. D.	14.0±2.6	101.7±11.3	66.3±5.0
JCM 5593	*P. pinophiilum*	10.3±3.5	14.5±2.1	49.0±2.0	156.7±4.5	162.7±26.0	131.3±10.5	42.0±9.0
JCM 22801	*P. pinophiilum*	13.0±2.0	12.0±1.0	18.7±3.5	126.3±7.5	109.3±10.7	216.7±12.0	28.3±2.5
JCM 22802	*P.pinophiilum*	12.0±2.6	11.0±2.0	10.7±3.1	94.0±3.0	98.3±10.7	168.3±5.5	14.0±3.0
JCM 22803	*P. pinophiilum*	26.7±4.0	3.0±1.0	23.0±2.0	173.0±10.0	211.3±22.5	81.3±3.5	9.0±2.0
JCM 23043	*P. pinophiilum*	11.0±2.0	9.7±2.5	10.7±1.5	129.0±11.5	143.7±17.0	125.3±8.5	36.7±9.0

Values given are the mean of three replicates ± standard deviation of the mean, N.D.: Not detected, Organic acid calculated as micrograms per milliliter.

In the medium supplemented with AL-P, most of the strains produced oxalic acid, citric, tartaric, malic, lactic, formic and acetic acids ranged from 1.3-46.0, 2.0-857.7, 4.7-218.0, 31.0-132.3, 3.3-169.7, 4.3-112.7 and 10.7-122.3 µg/mL, respectively. Exception was that the strain SI-4URAgr, SI-15URAgr, SI-17URAgr and NBRC33285 could not produce malic acid, and JCM 22803 could not produce formic acids. The highest amount of citric (857.7 µg/mL), tartaric (218.7 µg/mL), malic (132.3 µg/mL), formic (112.7 µg/mL) and acetic (122.3 µg/mL) acids were produced from Al-P containing broth by the strain SI-4URAgr, NBRC 9575, JCM5593, SI-19URagr and NBRC100533, respectively whereas NBRC 6345 produced highest amount both oxalic acid (46.0 µg/mL) and lactic acid (169.7 µg/mL) (Table **VI.** 3).

Table **VI. 3.** Types and quantities of produced organic acids in the Pikoveskaya's medium supplemented with $AlPO_4$ (Al-P) compound by phosphate solubilizing *T. pinophilus* strains

		Organic acid (µg/mL)						
Strains	**Fungi**	**Oxalic**	**Citric**	**Tartaric**	**Malic**	**Lactic**	**Formic**	**Acetic**
SI-4URAgr	*T. pinophilus*	22.7±4.0	857.7±77.0	27.3±2.1	N.D	8.6±3.0	38.3±10.5	10.7±2.1
SI-15URAgr	*T. pinophilus*	2.3±0.5	3.3±1.5	30.0±4.0	N.D.	17.0±4.0	59.0±12.0	21.7±3.5
SI-17URAgr	*T. pinophilus*	2.0±1.0	5.3±1.5	44.0±2.0	N.D	17.0±4.0	60.3±17.0	19.7±2.5
SI-19URAgr	*T. pinophilus*	6.3±1.5	2.0±1.0	25.0±6.0	46.0±5.0	38.0±11.0	112.7±14.2	29.0±5.1
IFM 64651	*P. pinophiilum*	17.0±4.0	7.0±2.0	5.0±2.0	52.3±1.5	10.0±1.0	56.0±14.5	33.7±7.3
IFM 57309	*P. pinophiilum*	5.3±1.5	5.0 ±1.0	16.7 ±5.5	86.0 ±2.0	151.0 ±27.0	19.0±8.0	21.3 ±3.5
NBRC 6345	*T. pinophilus*	46.0±13.4	6.0 ±2.0	12.7±3.2	88.0±2.0	169.7±10.5	72.0±9.0	86.3±10.5
NBRC100533	*T. pinophilus*	44.7±12.0	5.0 ±1.0	35.7±5.5	62.7±17.0	24.7±6.0	24.0±5.5	122.33±19.1
NBRC 33285	*T. pinophilus*	7.3±2.1	7.3±1.5	5.3±1.5	N.D	12.7±2.5	4.3±1.5	22.7 ±4.5
NBRC106907	*P. pinophilum*	1.3±0.5	513.7±73.5	24.7±6.5	68.3±17.0	33.7±7.0	7.0±1.0	60.3±5.0
NBRC 9575	*P. allahabadense*	9.7±2.5	58.7±12.0	218.7±26.5	31.0±5.5	20.0±2.0	29.3±6.0	26.7±6.0
JCM 9928	*P. pinophiilum*	1.3±0.5	140.3±19.2	22.7±2.5	N. D.	34.0±9.5	37.0±6.0	52.7±5.5
JCM 5593	*P. pinophiilum*	23.0±4.0	6.3±1.5	6.3±1.5	132.3±6.5	120.3±16.5	52.7±11.5	69.3±11.4
JCM 22801	*P. pinophiilum*	24.0±3.0	9.7±1.5	13.0±1.0	51.0±3.0	158.0±7.0	75.7±12.0	42.3±6.5
JCM 22802	*P. pinophiilum*	26.0±5.0	8.3±2.5	15.7±2.5	56.0±9.1	3.3±1.5	110.3±6.5	32.3±8.3
JCM 22803	*P. pinophiilum*	20.0±4.0	6.7±1.5	30.0±5.0	61.3±14.6	9.0±2.0	N.D	17.7±3.0
JCM 23043	*P. pinophiilum*	24.0±4.0	7.0±2.0	4.7±1.5	79.7±4.5	88.7±4.5	44.7±11.0	24.3±5.0

Values given are the mean of three replicates ± standard deviation of the mean, N.D.: Not detected, Organic acid calculated as micrograms per milliliter.

In the medium supplemented with Fe-P, all strains produced oxalic, citric, tartaric and lactic acids ranged from 1.3-54.7, 2.0-238.7, 4.0-288.0 and 8.0-140.0 µg/mL, respectively. The malic acid was produced ranged 9.0-727.0 µg/mL by the strain SI-4URAgr, SI-17URAgr, SI-19URAgr, IFM64651, JCM5593, JCM22802, JCM22803 and JCM23043. Most of the strains produced formic acid ranged from 6.3-119.7 µg/mL excepting IFM64651, IFM57309, NBRC33285, JCM5593, JCM22802, JCM22803 and JCM23043. All the strains except NBRC 6345 and JCM 22803 produced acetic acid

ranged from 16.3-104.3 µg/mL. The highest amount of formic (119.7 µg/mL), lactic (140.0 µg/mL) and malic acids (727.0 µg/mL), whereas, acetic acid (104.0 µg/mL) and citric acid (238.7 µg/mL) were produced by the strain NBRC106907 and the strain SI-19URAgr produced highest in both oxalic (54.7 µg/mL) and tartaric acids (288.0 µg/mL) respectively (Table **VI.** 4).

Table **VI. 4**. Types and quantities of produced organic acids in the Pikoveskaya's medium supplemented with $FePO_4$ (Fe-P) compound by phosphate solubilizing *T. pinophilus* strains.

		Organic acid (µg/mL)						
Strains	**Fungi**	**Oxalic**	**Citric**	**Tartaric**	**Malic**	**Lactic**	**Formic**	**Acetic**
SI-4URAgr	*T. pinophilus*	3.0±1.0	21.0±4.0	4.0±1.0	371.0±55.0	107.0±7.2	8.0±2.0	82.0±6.0
SI-15URAgr	*T. pinophilus*	4.3±1.5	8.7±2.1	31.0±6.0	N.D	24.0±6.0	15.7±3.0	23.0±4.0
SI-17URAgr	*T. pinophilus*	35.0±2.0	2.0±1.0	245.7±6.0	9.0±5.0	54.0±6.5	119.0±21.5	71.3±24.5
SI-19URAgr	*T. pinophilus*	54.7±1.5	7.0±2.0	288.0±5.0	71.0±4.0	43.0±6.0	103.7±16.2	64.3±8.5
IFM 64651	*P. pinophiilum*	14.0±3.6	25.3±2.5	8.0±2.0	294.7±30.8	140.0±8.5	N.D	60.0±13.0
IFM 57309	*P. pinophiilum*	1.3±0.5	7.3±2.5	28.0±1.0	N.D	23.3±5.6	N.D	26.3 ±9.5
NBRC 6345	*T. pinophilus*	6.0±1.0	8.0±3.0	40.0±3.0	N.D	19.0±4.0	19.33±3.5	N.D
NBRC100533	*T. pinophilus*	3.0±1.0	216.0±26.7	41.0±2.0	N.D	19.0±2.0	83±5.0	23.0±2.0
NBRC 33285	*T. pinophilus*	3.3±1.5	8.0±2.0	7.0±2.0	N.D	8.0±1.0	N.D	22.0±2.0
NBRC106907	*P. pinophilum*	3.0±2.0	238.7±39.3	29.7±0.5	N.D	15.33±4.5	6.3±1.5	104.0±15.5
NBRC 9575	*P. allahabadense*	4.7±2.0	6.3±1.5	66.7±6.5	N.D	35.0±16.0	45.3±2.5	23.0±2.0
JCM 9928	*P. pinophiilum*	2.3±0.5	21.3±4.5	20.0±1.0	N.D.	14.7±3.2	45.3±3.0	31.3±5.5
JCM 5593	*P. pinophiilum*	2.0±1.0	19.0±2.0	37.7±6.0	727.0±67.1	110.0±19.0	N.D	23.0±2.0
JCM 22801	*P. pinophiilum*	2.0±1.0	7.0±1.0	33.0±2.0	N.D	37.0±4.0	119.7±6.0	21.3±2.5
JCM 22802	*P. pinophiilum*	2.0±1.0	20.0±2.6	33.3±3.5	446.0±39.5	19.0±7.0	N.D	16.3±4.0
JCM 22803	*P. pinophiilum*	2.7±2.1	9.3±1.5	31.7±3.2	534.0±54.8	94.3±15.5	N.D	N.D
JCM 23043	*P. pinophiilum*	2.3±1.1	20.7±5.7	29.7±1.5	571.7±48.2	87.0±21.0	N.D	19.3±1.5

Values given are the mean of three replicates ± standard deviation of the mean, N.D.: Not detected, Organic acid calculated as micrograms per milliliter.

Effect of phosphate compounds in medium on the quantities of different organic acids

Insoluble phosphate compounds strongly affect the quantities of different organic acids produced by *T. pinophilus* strains. The highest amount of acetic acid, formic acid and lactic acid were produced in the medium supplemented with TCP followed by Al-P and Fe-P. On the other hand oxalic acid and citric acid were produced in the medium containing Al-P followed by TCP and Fe-P. Tartaric acid and malic acid were produced in the medium containing Fe-P followed by TCP and AL-P (Table **VI.** 5).

Table **VI. 5**. Phosphate sources effect on the quantities of different organic acids produced by *T. pinophilus* strains

Insoluble P sources	Organic acid (µg/mL)						
	Oxalic	**Citric**	**Tartaric**	**Malic**	**Lactic**	**Formic**	**Acetic**
TCP	179.3	959.5	541.3	1886.3	1651.0*	2403.7*	849.0*
AL-P	283.0*	1649.3*	537.3	814.0	915.7	802.3	693.3
FE-P	145.7	645.7	974.3*	3024.3*	850.7	565.3	610.3
Mean±SD	202.7±71.6	1145.9±501.9	684.3±251.2	1917.8±1105.2*	1139.3±444.5	1256.0±1000.0	717.5±121.2

An asterisk (*) indicated outstanding values of produced organic acids, It was higher than sum of the mean and standard deviation of organic acids produced by 17 fungal strains, Values given are the mean ± standard deviation of organic acids produced by 17 fungal strains.

Comparison of produced organic acids from different P substrate

The strongest organic acid production ability of *T. pinophilus* strains was found in medium containing TCP followed by Fe-P and Al-P. The produced organic acid ranged between 204.0-752.7 µg/mL, 59.7-965.3 µg/mL and 48.3-918.7 µg/mL in TCP, Al-P and Fe-P medium, respectively. Among the isolates, the highest amount of organic acid was produced by strain SI-4URAgr (965.3 µg/mL) followed by JCM 5593 (918.7 µg/mL) and NBRC 106907 (752.7 µg/mL). The strain SI-4URAgr, SI-17URAgr, SI-19URAgr, NBRC 106907, NBRC 9575, JCM 5593, JCM 22803 and JCM 23043 were considered as outstanding because their produced organic acid was higher than sum of the mean and standard deviation of organic acid produced by 17 *T. pinophilus* strains (Table **VI.** 6). Investigated result showed that the stain NBRC106907 had outstanding performance in both TCP and AL-P broth medium.

Table **VI.** 6. Comparison between different P substrate (TCP, Al-P and Fe-P) in Pikoveskaya's broth based the quantity of produced organic acids by *T. pinophilus* strains

Strain	**Fungi**	Organic acid (µg/mL) released in medium		
		TCP	**AL-P**	**Fe-P**
SI-4URAgr	*Talaromyces pinophilus*	435.0	965.3*	596.0
SI-15URAgr	*Talaromyces pinophilus*	302.7	133.3	106.7
SI-17URAgr	*Talaromyces pinophilus*	736.0*	148.3	536.3
SI-19URAgr	*Talaromyces pinophilus*	726.0*	259.3	631.7
IFM 64651	*Penicillium pinophiilum*	432.0	181.3	542.7
IFM 57309	*Penicillium pinophiilum*	374.7	304.3	86.3
NBRC 6345	*Talaromyces pinophilus*	542.0	480.7	92.3
NBRC100533	*Talaromyces pinophilus*	429.7	318.3	385.0
NBRC 33285	*Talaromyces pinophilus*	328.0	59.7	48.3
NBRC106907	*Penicillium pinophilum*	752.7*	709.0*	397.0
NBRC 9575	*Penicillium allahabadense*	715.0*	394.0	181.0.
JCM 9928	*Penicillium pinophiilum*	204.0	288.0	135.0
JCM 5593	*Penicillium pinophiilum*	566.5	410.0	918.7*
JCM 22801	*Penicillium pinophiilum*	524.3	373.7	220.0
JCM 22802	*Penicillium pinophiilum*	408.3	252.0	536.7
JCM 22803	*Penicillium pinophiilum*	527.3	144.7	672.0*
JCM 23043	*Penicillium pinophiilum*	466.0	273.0	730.7*
	Mean±SD	498.2±162.0*	335.0±223.6	401.0±268.7

TCP: tricalcium phosphate; Al-P: aluminium phosphate and Fe-P: iron phosphate, An asterisk (*) indicated outstanding values of produced organic acids, It was higher than sum of the mean and standard deviation of organic acids produced by 17 fungal strains, It also indicated the best substrate for organic acid production, Values given are the mean ± standard deviation of organic acids produced by 17 fungal strains.

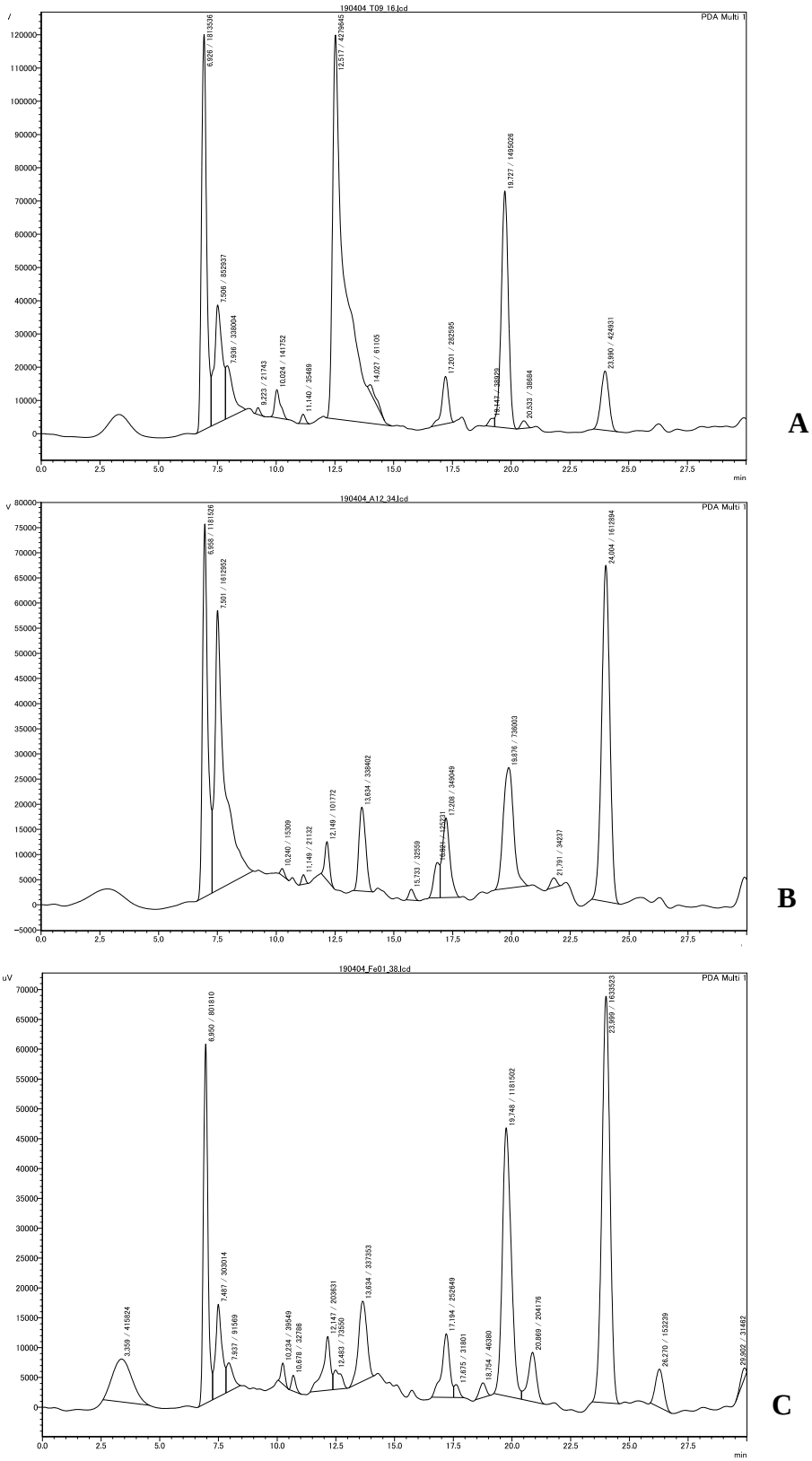


Fig.**VI.** 1. Chromatograms of organic acids analyzed by HPLC.

DISCUSSION

In the present study, results showed that the type and the amount of organic acids produced by *T. pinophillus* strains were varied on both fungal strains and phosphate substrate. TCP supplemented medium enhanced acetic acid, lactic acid and formic acid production but Fe-P medium enhanced the production of malic and tartaric acid. The Al-P medium enhanced the production of oxalic and citric acid. Based on the total quantity of organic acids, TCP medium is the best followed by Fe-P and Al-P. It might be the result of interaction between fungal strains and the P sources (Zang *et al.*, 2018 and Scervino *et al.*, 2013).

In our study, eight strains of *T. pinophilus* (SI-4URAgr, SI-17URAgr, SI-19URAgr, NBRC106907, NBRC9575, JCM5593, JCM22803 and JCM23043) could produce higher amount of organic acids in the medium supplemented with TCP, Al-P and Fe-P. Among the isolates, the strain SI-17URAgr and NBRC 9575 could produce higher amount of organic acids in the medium supplemented with TCP whereas NBRC106907 produced higher organic acids in both TCP and AL-P medium. The strain SI-4URAgr also could produce higher organic acids in the medium supplemented with AL-P. The strain JCM5593, JCM22803 and JCM23043 were capable to produce higher amount of organic acids in the medium supplemented with Fe-P. It suggested that the type and the quantity of organic acids produced by each fungal species varied according to the P source (Jose *et al.*, 2010). Protiva Vyas and Arvind Gulati (2009) have reported that organic acid production by microorganism is independent of their genetic relatedness and each strain has its own ability of producing organic acid during the P solubilization. Jose *et al.* (2010) suggested that the production of organic acids depends both on the microorganism and source of P in which the microorganisms grows. Cunningham and Kuiack (1992) reported that total amount and type of acids produced in the medium affect the solubilization of the different sources of P. Phosphorus is fixed as insoluble iron and aluminium phosphates in acidic soil or as calcium phosphates in alkaline soils (Zhang *et al.*, 2001). Soil microbes use organic acids for various purposes such as making available relatively insoluble elements in the soil for themselves and plants (Morgan *et al.*, 2005, Balogh-Brunstad *et al.*, 2008). These organic acids decrease the pH of the medium that

is responsible for solubilization of the insoluble phosphates (Chen *et al.,* 2006). Some of the phosphorus-solubilizing microbial strains produce phosphorus-hydrolyzing enzymes known as phosphatases and phytases, in addition to organic acids (Pawar *et al.,* 2009, Rodriguez *et al.,* 1999). These enzymes convert insoluble phosphates to the soluble phosphorus. Bacterial cells and fungal mycelia require phosphorus for their own growth; thus, they produce phosphorus-hydrolyzing enzymes. Large quantities of phos-phates are available in soil, and when the microbial culture is added in the form of biofertilizer, they solubilize the phos-phates. The soluble phosphorus is then easily available for plant.

T. pinophilus has received increasing attention in mycological research for its ability to act as a fungal antagonist and plant-growth promoter (Nicoletti et al., 2004; Pandey *et al.,* 2008; Wani *et al.,* 2016). They are capable of dissolving insoluble phosphate compounds in soil. Out of P solubilization activity, *T. phinophilus* are considered as the significant source of enzymes, pigments and secondary metabolites that are essential for sustainable crop production and industrial utilization (*Koul et al.,* 2016; Yao *et al.,* 2017; Caro *et al.,* 2017; Zhai *et al.,* 2015). They also play an important role to biomass degradation and act as bio control agent against some plant pathogen (Fujii *et al.,* 2014; Ismail and Kamal, 2018).

Chapter VII

Comparative study of Zinc Solubilization Potential of *Talaromyces pinophilus* Strains

Abstract: Zinc(Zn) is one of the essential micro nutrients required for optimum plant growth. Zinc deficiency is well known problem occuring in plants which retards photosynthesis and nitrogen metabolism. Most of the soils in the world contain insoluble Zn that cannot be utilized by the plants and substantial quantity of applied inorganic zinc in soil also is converted into unavailable form. Zinc solubilising microorganisms are potential alternates for zinc supplement. Zinc solubilizing fungi (PSF) including *Talaromyces pinophilus* possess more potential for providing available Zn in soil for plant nutrition. The present study aimed to assess the zinc solubilization potential of 17 *T. pinophilus* strains in qualitatively and quantitatively under invitro conditions, in order to determine the best options for eco-friendly fertilizers. The Zn solubilization efficiency of *T. pinophilus* strains was investigated in broth containing insoluble ZnO and $ZnCO_3$ compounds. Result showed that ZnO was solubilized higher in comparison to $ZnCO_3$ whereas the strain JCM 9928 and SI-17URAgr have marked zinc solubilization ability in broth amended with ZnO and $ZnCO_3$, respectively. In addition, there was inverse proportion between the pH and zinc solubilizaing capacities. Findings indicated that these fungal strains can be potential bio-inoculants for sustainable soil management and crop production.

Key words: Zn solubilization, Talaromyces pinophilus, insoluble zinc compound, eco-friendly fertilizer.

INTRODUCTION

Zinc (Zn) is an essential micronutrient required by plants for better growth and nutrition. Its deficiency in plants retards photosynthesis and nitrogen metabolism, causes the reduction in flowering and fruit development, decreases the synthesis of carbohydrates and phytohormones, delays crop maturity leading to decrease in crop yield and nutritional quality of grains. The worldwide prevalence of Zn deficiency in crop is due to low solubility of Zn, rather than low Zn availability in soil (Iqbal *et al.*, 2010). However, Zn deficiency in millions of hectares of agricultural soils has not only reduced crop yields but also severely hampered the nutritional quality of the crop produce causing critical nutritional and health problem in one-third of the world's human population (Hotz and Brown, 2004; Myers *et al.*, 2015). Acid, calcareous, saline and sodic soils and coarse-textured soils prone to high weathering, besides soils subjected to intensive cropping and poor drainage exhibit Zn deficiency (Singh *et al.*, 2005).

Apparently, enhancing the available Zn pool in the soil by application of Zn containing synthetic fertilizers or organic manures becomes imperative. Unfortunately, exogenous application of chemical fertilizers alone cannot help in combating soil Zn deficiency in the long term since 96.0–99.0% of the applied Zn is once again converted to unavailable Zn pools by precipitation to carbonates or oxides or phosphates etc (Ma and Uren, 1997; Zhang *et al.*, 2017). Hence, decreased use efficiency of chemical Zn fertilizers, remains an issue, especially in the long-term.

Nevertheless, a redeeming feature is that the worldwide occurrence of Zn scarcity issues in crops is not due to low levels of total Zn but is due to low solubility of Zn in soils (Cakmak, 2008). In fact, the total Zn content in soils is substantially high and exists in fixed forms such as smithsonite ($ZnCO_3$), sphalerite (ZnS), zincite (ZnO), franklinite ($ZnFe_2O_4$), wellemite (Zn_2SiO_4), and hopeite ($Zn_3(PO_4)_2.4H_2O$), which are only sparingly soluble. Values in the literature indicate that available Zn level in soils is very low (4.0–270.0 μgL^{-1}) in relation to the mean total Zn level of 64.0 $mgkg^{-1}$ (Alloway, 2009). Reports suggest that Zn deficiency due to low amounts of bioavailable Zn is rampant in at least one-third of the cultivated soils globally (Sillanpää and Vlek, 1985). Apparently, low bioavailability not only hampers crop

productivity but also markedly lowers Zn density in the harvested produce (seeds, grains, rhizomes etc.) thereby impairing nutritional quality (Cakmak and Hoffland, 2012).

Hence, a feasible alternative would be to exploit the innate capacity of certain soil microorganisms, especially, fungi, to solubilize these fixed forms of Zn to labile Zn forms for enhanced availability and subsequent uptake by plants. However, the ability to solubilize immobilized Zn (ZnO, $ZnCO_3$ or $ZnPO_4$) is not a common characteristic of cultivable fungi in soils, though in the recent past, there are in vitro studies on a few genera of fungi like *Beauveria caledonica* (Fomina *et al.,* 2004), *Lecanicillium psalliotae* (Senthil *et al.,* 2018) etc, capable of solubilizing Zn that have exhibited terrific ability to improve zinc availability in root zone and enhance zinc in plants. Compared with the use bacteria, the use of fungi as zinc solubilizer has not been extensively investigated. *T. pinophilus* has received increasing attention in mycological research for its ability to act as a fungal antagonist and plant-growth promoter (Nicoletti *et al.,* 2004; Pandey *et al.,* 2008; Wani *et al.,* 2016). There are no studies have been made on Zn-solubilization potential and comparative performance of different *T. pinophilus* strains yet. Therefore, the study aimed to compare the Zinc solubilization capabilities of 17 *T. pinophilus* fungal strains, in order to determine the best options for eco-friendly fertilizers.

MATERIALS AND METHODS

Fungal isolates

Seventeen *T. pinophilus* fungal strains were used in this study where 2 strains were isolated from hairy vetch incorporated soil and others collected from different institutions in Japan (Table VII. 1). The isolates were cultured on potato dextrose agar (PDA; Becton, Dickinson and Company, Sparks, MD, USA) slant and kept at 25^0C (room temperature) for further study.

Culture medium

Mineral salts medium was used for this study (Saravanan *et al.*, 2007). In this medium $ZnCO_3$ was used as source of insoluble Zn compound that was replaced by insoluble ZnO. The medium was autoclaved at 121^0C for 15 minutes. Chloramphenicol (Wako Pure Chemical Corporation, Osaka, Japan) was also used to avoid bacterial growth.

Spore suspension preparation

For conducting this experiment, spore suspension of pure fungal cultures were made from the re-slanting of pure culture slants that preserved at 25^0C (room temperature) according to the method mentioned in previous experiment **Chapter, V**.

Table VII. 1. List of the *T. pinophilus* strains used in the study

Isolates	Strain in GenBank	Source	Country of origin	Fungi	Institution
1	SI-4URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
2	SI-15URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
3	SI-17URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
4	SI-19URAgr	Soil	Japan	*Talaromyces pinophilus*	Univ. Ryukyus
5	IFM 64651	Sputum	Japan	*Penicillium pinophilum*	Chiba University
6	IFM 57309	Sputum	Japan	*Penicillium pinophilum*	Chiba University
7	NBRC 6345	Radio set	Papua New Guinea	*Talaromyces pinophilus*	NITE BRC
8	NBRC 100533	Polyvinyl chloride plastic	France	*Talaromyces pinophilus*	NITE BRC
9	NBRC 33285	Polyvinyl chloride plastic	France	*Talaromyces pinophilus*	NITE BRC
10	NBRC 106907	Soil	Japan	*Penicillium pinophilum*	NITE BRC
11	NBRC 9575	Polyvinyl chloride plastic	France	*Penicillium allahabadense*	NITE BRC
12	JCM 9928	Soil	India	*Penicillium pinophilum*	RIKEN
13	JCM 5593	Radio set	Papua New Guinea	*Penicillium pinophilum*	RIKEN
14	JCM 22801	Wood stakes	Australia	*Penicillium pinophilum*	RIKEN
15	JCM 22802	Barley grain	Australia	*Penicillium pinophilum*	RIKEN
16	JCM 22803	Moldy sorghum grain	Australia	*Penicillium pinophilum*	RIKEN
17	JCM 23043	Radio set	Papua New Guinea	*Penicillium pinophilum*	RIKEN

NITE; National Institute of Technology and Evaluation, Biological Resource Center, NITE (NBRC), Japan. RIKEN; is a large scientific research institute in Japan

Determination of zinc solubilization index on solid medium

All the isolates were tested under *in vitro* condition for their zinc solubilization activity on mineral salts medium (g·lit−1) specified by Saravanan *et al.* (2007), consisted Dextrose: 10.0; $(NH_4)_2SO_4$: 1.0; KCl: 0.2; K_2HPO_4: 0.1; $MgSO_4$: 0.2; pH: 7.0 and insoluble Zn compound (ZnO), Agar: 15.0g and adding deionized water to make 1000 mL volume, and autoclaved at 121◦C for 20 min. A spot inoculation of each fungal isolate was made onto the plates in triplicate under aseptic condition and incubated at 28°C for 7 days in darkness. At 7^{th} day of incubation phosphate solubilization index was determined by using the following formula: ratio of the total diameter (colony + halo zone) and the colony diameter (Premono *et al.,* 1996).

Solubilization Index (SI) = [Colony diameter + Halo zone diameter]/Colony diameter

Quantitative estimation of zinc solubilization

The quantitative assessment of zinc solubilization was done in 100 ml conical flasks containing 40 ml of liquid mineral salts medium (g·lit−1) specified by Saravanan *et al.*, (2007), consisted Dextrose: 10.0; $(NH_4)_2SO_4$: 1.0; KCl: 0.2; K_2HPO_4: 0.1; $MgSO_4$: 0.2; pH: 7.0 and insoluble Zn compound (ZnO and $ZnCO_3$-0.1%, separately), Agar: 15.0g and adding deionized water to make 1000 mL volume, and autoclaved at 121◦C for 20 min. After sterilization, the medium of each flask was inoculated with the 5 % (v/v) spore suspension of a particular fungal isolate containing 10^6 spores mL^{-1}. Sterile distilled water inoculated flaks were treated as control. Three replicates were maintained for each test isolate and mean value was recorded. Incubation was done at 25^0C in an incubator shaker (EYELA Multi Shaker MMS-3010) at 120 rpm for 6 days. The samples were autoclaved and centrifuged (TOMY MX-301) at 5000 rpm for 25 minutes to remove any suspended solids and mycelial parts. Then the cultures were filtered through 0.45 µm pore size syringe filter unit (Advantech, Japan). The filtrates were used for analysis of soluble phosphate and pH value. The pH value of the culture supernatants was determined by a pH meter (HORIBA, Japan) equipped with a glass electrode. One ml of this supernatant was directly used to estimate the soluble zinc content using a Multiple Inductivity Coupled Plasma Emission Spectrometer (ICPE-9000, Shimadzu Co. Ltd) and expressed as mg/L. When sample were taken for

organic acids analysis, the culture supernatants were filtered through 0.22 μm pore size syringe filter unit (Merck KGaA, Darmstadt, Germany).

Organic acids analysis

Detection and quantification of organic acids were done by High Performance Liquid chromatography (Prominence HPLC system, Shimadzu-CBM-20A, Japan) equipped with diode array detector (SPD-M20A), refractive index detector (RID-10A), column ICE-ION-300 (300mmX7.8mm), auto sampler (LC-20AD) and fraction collector (FRC-10A). The injection volume, temperature and flow rate was 50 μl, 50^{o}C and 0.5ml/min, respectively. Sulfuric acid (0.01N) (Merck, Germany) was used as solvent of mobile phase. Peaks were identified against a set of standards from known organic acids (oxalic, citric, tartaric, malic, lactic, formic and acetic acids; Wako Pure Chemical Industries, Osaka, Japan).

Data analysis

All experiments were conducted in triplicate and data were analyzed using Microsoft Excel program (version, 2016). The mean values were compared by Tukeys's test and significant differences were detected at $p< 0.05$ level. Correlation between solubilized Zinc and pH of the medium was determined by using pearson's correlation studies.

RESULTS

Qualitative zinc solubilization

All isolates showed different levels of Zinc solubilization in solid medium enriched Zinc Oxide (ZnO) as the substrate. All isolates also showed significant zinc solubilization in mineral salts medium ($g \cdot lit^{-1}$) whereas ZnO as the substrate. The zinc solubilization index (SI) ranged from 1.19 to 1.59 (Table **VII.** 2).

Table **VII.** 2. ZnO solubilization on mineral salts medium by 17 *T. pinophilus* strains

Isolate	Fungal strain	Fungi	SI
1	SI-4URAgr	*Talaromyces pinophilus*	1.37±0.01^{b}
2	SI-15URAgr	*Talaromyces pinophilus*	1.33±0.03^{b}
3	SI-17URAgr	*Talaromyces pinophilus*	1.19±0.01^{d}
4	SI-19URAgr	*Talaromyces pinophilus*	1.34±0.01^{b}
5	IFM 64651	*Penicillium pinophilum*	1.39±0.02^{b}
6	IFM 57309	*Penicillium pinophilum*	1.25±0.03^{d}
7	NBRC 6345	*Talaromyces pinophilus*	1.38±0.02^{b}
8	NBRC100533	*Talaromyces pinophilus*	1.28±0.08^{c}
9	NBRC33285	*Talaromyces pinophilus*	1.38±0.08^{b}
10	NBRC 106907	*Penicillium pinophilum*	1.45±0.03^{a}
11	NBRC 9575	*Penicillium allahabadense*	1.50±0.03^{a}
12	JCM 9928	*Penicillium pinophilum*	1.36±0.06^{b}
13	JCM 5593	*Penicillium pinophilum*	1.27±0.09^{c}
14	JCM 22801	*Penicillium pinophilum*	1.50±0.05^{a}
15	JCM 22802	*Penicillium pinophilum*	1.59±0.05^{a}
16	JCM 22803	*Penicillium pinophilum*	1.27±0.05^{c}
17	JCM 23043	*Penicillium pinophilum*	1.42±0.05^{b}

SI:Solubilization index, Values given are the mean ± standard deviation of three independent replicates, Same letter in the column are not significantly different at $p<0.05$ by Fisher's test.

Quantitative zinc solubilization

All the strains of *Talaromyces pinophilus* used could effectively solubilize the insoluble Zn compounds used, namely, ZnO and ZnCO3, under the assay conditions. Zinc solubilization by all isolates were tested in broth mineral salts medium using two substrates of recalcitrant zinc compounds: ZnO and $ZnCO_3$. The zinc solubilizing ability of fungal isolates varied with incubation period and substrates. All *T. pinophilus* strains were tested for their ability to solubilize hardly soluble zinc sources [ZnO and

$ZnCO_3$]. All of the *T. pinophilus* strains showed potential Zn solubilizing in the medium containing ZnO followed by $ZnCO_3$ (Table **VII.** 2). The solubilized Zn ranged between 8.47-69.5 ppm and 3.23—28.5 ppm from ZnO and $ZnCO_3$, respectively. Out of 17 isolates, 2 isolates (JCM 9928 and SI-17URAgr) were considered as outstanding isolates because solubilized Zn was higher than the sum of the mean and standard deviation of Zn solubilized by 17 isolates. The highest amount of Zn solubilization from ZnO was shown in JCM 9928 (69.5 ppm) followed by SI-15URAgr (28.9 ppm) and JCM 22802 (24.5ppm). The highest amount of Zn solubilization from ZnCO3, was shown in SI-17URAgr (28.5 ppm) followed by JCM 22802 (15.3 ppm) and JCM 23043 (14.5 ppm).

Table **VII.** 3. Comparison of zinc solubilization from two insoluble substrates by zinc solubilizing *T. pinophilus* strains

Sl. No	Strains	Fungi	Solubilized Zinc (ppm)	
			ZnO	**$ZnCO_3$**
1	SI-4URAgr	*Talaromyces pinophilus*	21.31	8.34
2	SI-15URAgr	*Talaromyces pinophilus*	28.90	8.90
3	SI-17URAgr	*Talaromyces pinophilus*	8.51	28.51*
4	SI-19URAgr	*Talaromyces pinophilus*	15.34	3.23
5	IFM 64651	*Penicillium pinophiilum*	16.0	7.86
6	IFM 57309	*Penicillium pinophiilum*	11.67	13.0
7	NBRC 6345	*Talaromyces pinophilus*	14.43	9.34
8	NBRC100533	*Talaromyces pinophilus*	13.0	8.47
9	NBRC 33285	*Penicillium pinophiilum*	8.47	3.67
10	NBRC106907	*Talaromyces pinophilus*	19.34	11.86
11	NBRC 9575	*Penicillium allahabadense*	11.77	6.14
12	JCM 9928	*Penicillium pinophiilum*	69.5*	8.45
13	JCM 5593	*Penicillium pinophiilum*	14.23	7.89
14	JCM 22801	*Penicillium pinophiilum*	19.12	12.78
15	JCM 22802	*Penicillium pinophiilum*	24.56	15.34
16	JCM 22803	*Penicillium pinophiilum*	20.45	11.89
17	JCM 23043	*Penicillium pinophiilum*	21.56	14.56
		MEAN±SD	19.89±13.93*	10.60±5.74

Correlation between pH and soluble Zn

The pH of the culture medium exhibited the opposite changes. It decreased with the increased amount of soluble Zn in the medium. Correlation studies showed a significant inverse relationship between soluble Zn and pH of the culture medium (Fig. **VII**. 1). The negative correlation was observed in both fermented broth culture and correlation coefficient (r) was -0.526 and -0.422 in ZnO, and $ZnCO_3$, respectively.

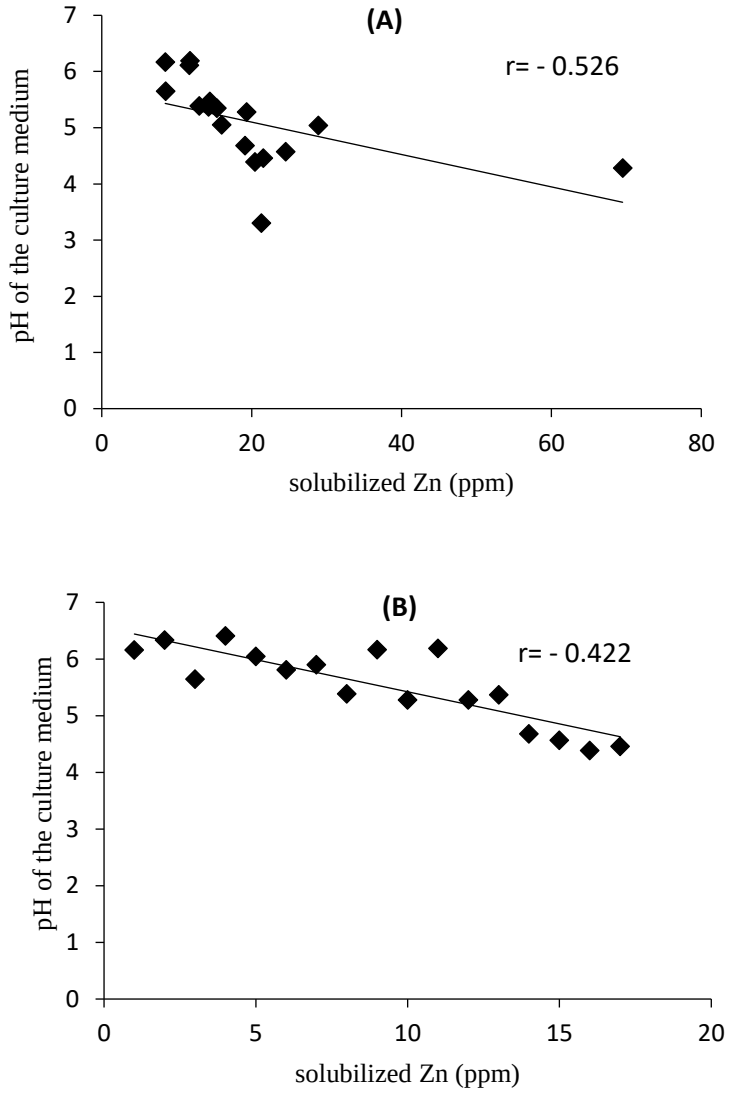


Fig. **VII.** 1. Pearson's correlation between soluble Zn and pH of the culture medium supplemented with ZnO (A) and $ZnCO_3$ (B); inoculated by 17 fungal strains. [SI-4URAgr, SI-15URAgr, SI-17URAgr, SI-19URAgr, IFM 64651, IFM 57309, NBRC 6345, NBRC 100533, NBRC 33285, NBRC 106907, NBRC 9575, JCM 9928, JCM 5593, JCM 22801, JCM 22802, JCM 22803, JCM 2304.

Organic acid production by fungal strains in the medium supplemented with insoluble Zn compounds [ZnO and $ZnCO_3$]

In the medium supplemented with ZnO, most of the strains produced oxalic acids, citric acids, tartaric acids and formic acids in the medium, ranged from 1.00-17.00, 2.0-22.0, 3.67-27.67 and 6.33-44.33, µg/mL, respectively, whereas malic, lactic and acetic acids were not detected. Excepting IFM57309, NBRC6345, NBRC100533, JCM5593 and JCM22802, and NBRC9575, JCM5593, JCM22801 and JCM23043, other strains produced citric, tartaric and formic acids ranged from 2.0-22.00, 3.67-27.67 and 6.33-44.33, µg/mL, respectively. The highest amount of citric acids (22.33 µg/mL) and tartaric acids (27.67 µg/mL) were produced by the strain JCM 22802 and SI-15URAgr, resepectively, whereas the strain JCM9928 produced higher both oxalic acids (17.00 µg/mL) and formic acids (44.33 µg/mL) (Table **VII.** 4).

Table **VII.** 4. Types and quantities of produced organic acids in the mineral salts medium supplemented with ZnO compound by zinc solubilizing *T. pinophilus*

Strains	Fungi	Organic acid (µg/mL)						
		Oxalic	Citric	Tartaric	Malic	Lactic	Formic	Acetic
SI-4URAgr	*Talaromyces pinophilus*	2.33gh	11.00b	15.00b	N.D	N.D	N.D	N.D
SI-15URAgr	*Talaromyces pinophilus*	11.33b	8.67bc	27.67a	N.D	N.D	13.00b	N.D
SI-17URAgr	*Talaromyces pinophilus*	1.00h	2.00e	3.67de	N.D	N.D	10.67bc	N.D
SI-19URAgr	*Talaromyces pinophilus*	1.00h	7.00bcde	5.00cde	N.D	N.D	11.00bc	N.D
IFM 64651	*Penicillium pinophiilum*	5.00efg	4.33cde	8.00cd	N.D	N.D	11.00bc	N.D
IFM 57309	*Penicillium pinophiilum*	1.00h	N.D	N.D	N.D	N.D	9.33bc	N.D
NBRC 6345	*Talaromyces pinophilus*	1.00h	N.D	N.D	N.D	N.D	7.67c	N.D
NBRC100533	*Talaromyces pinophilus*	1.00h	N.D	N.D	N.D	N.D	7.67c	N.D
NBRC 33285	*Talaromyces pinophilus*	3.00fgh	8.00bcd	7.00cd	N.D	N.D	10.67bc	N.D
NBRC106907	*Penicillium pinophilum*	9.00bcd	3.33de	7.00cd	N.D	N.D	6.33c	N.D
NBRC 9575	*Penicillium allahabadense*	2.0gh	6.33bcde	N.D	N.D	N.D	41.00a	N.D
JCM 9928	*Penicillium pinophiilum*	17.00a	21.33a	9.00c	N.D	N.D	44.33a	N.D
JCM 5593	*Penicillium pinophiilum*	7.00cde	N.D	N.D	N.D	N.D	N.D	N.D
JCM 22801	*Penicillium pinophiilum*	2.33gh	7.00bcd	N.D	N.D	N.D	N.D	N.D
JCM 22802	*Penicillium pinophiilum*	10.00bc	22.33a	6.67cd	N.D	N.D	40.67a	N.D
JCM 22803	*Penicillium pinophiilum*	5.33efg	N.D	25.33a	N.D	N.D	9.33bc	N.D
JCM 23043	*Penicillium pinophiilum*	6.33def	21.00a	N.D	N.D	N.D	12.67b	N.D

Organic acid calculated as micrograms per milliliter, Values given are the mean of three independent replicates, Same letter in the column are not significantly different at $p<0.05$ by Fisher's test, N.D.: Not detected.

In the medium supplemented with $ZnCO_3$, most of the strains produced oxalic acid, citric, tartaric and formic acids ranged from 1.00-4.00, 3.33-52.00, 5.33-27.67 and 9.0-32.67, µg/mL, respectively, whereas malic acids (28.33 µg/mL) and lactic acids (27.45 µg/mL) were produced only by strain SI-17URAgr, whereas acetic acids were not detected. The highest amount of citric acids (52.0 µg/mL) and formic acids (32.67 µg/mL) were produced by the strain JCM23043 and NBRC100533, respectively, whereas the strain SI-15URAgr produced higher both oxalic acids (4.0 µg/mL) and tartaric acids (27.67 µg/mL) (Table **VII.** 5).

Table **VII.** 5. Types and quantities of produced organic acids in the mineral salts medium supplemented with $ZnCO_3$ compound by zinc solubilizing *T. pinophilus*

Strains	**Fungi**	**Organic acid (µg/mL)**						
		Oxalic	Citric	Tartaric	Malic	Lactic	Formic	Acetic
SI-4URAgr	*Talaromyces pinophilus*	2.33bc	N.D.	23.00b	N.D.	N.D.	9.00c	N.D.
SI-15URAgr	*Talaromyces pinophilus*	4.00ab	N.D.	27.67a	N.D.	N.D.	N.D.	N.D.
SI-17URAgr	*Talaromyces pinophilus*	2.00bc	3.33e	N.D.	28.33a	27.45a	N.D.	N.D.
SI-19URAgr	*Talaromyces pinophilus*	5.33a	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
IFM 64651	*Penicillium pinophiilum*	3.33abc	N.D.	N.D.	N.D.	N.D.	9.33c	N.D.
IFM 57309	*Penicillium pinophiilum*	3.00abc	N.D.	N.D.	N.D.	N.D.	20.67b	N.D.
NBRC 6345	*Talaromyces pinophilus*	1.33c	14.67b	N.D.	N.D.	N.D.	N.D.	N.D.
NBRC100533	*Talaromyces pinophilus*	2.00bc	7.67cd	22.00b	N.D.	N.D.	32.67a	N.D.
NBRC 33285	*Talaromyces pinophilus*	2.67bc	8.00cd	N.D.	N.D.	N.D.	N.D.	N.D.
NBRC106907	*Penicillium pinophilum*	1.33c	N.D.	N.D.	N.D.	N.D.	21.00b	N.D.
NBRC 9575	*Penicillium allahabadense*	2.00bc	6.33de	N.D.	N.D.	N.D.	N.D.	N.D.
JCM 9928	*Penicillium pinophiilum*	2.00bc	N.D.	22.67b	N.D.	N.D.	10.33c	N.D.
JCM 5593	*Penicillium pinophiilum*	3.00abc	9.33cd	N.D.	N.D.	N.D.	N.D.	N.D.
JCM 22801	*Penicillium pinophiilum*	2.33bc	7.00cde	5.33d	N.D.	N.D.	N.D.	N.D.
JCM 22802	*Penicillium pinophiilum*	1.00c	5.33de	15.00c	N.D.	N.D.	9.00c	N.D.
JCM 22803	*Penicillium pinophiilum*	2.00bc	11.00bc	N.D.	N.D.	N.D.	N.D.	N.D.
JCM 23043	*Penicillium pinophiilum*	2.33bc	52.00a	14.00c	N.D.	N.D.	N.D.	N.D.

Organic acid calculated as micrograms per milliliter (µg/mL), Values given are the mean of three independent replicates, Same letter in the column are not significantly different at $p<0.05$ by Fisher's test, N.D.: Not detected.

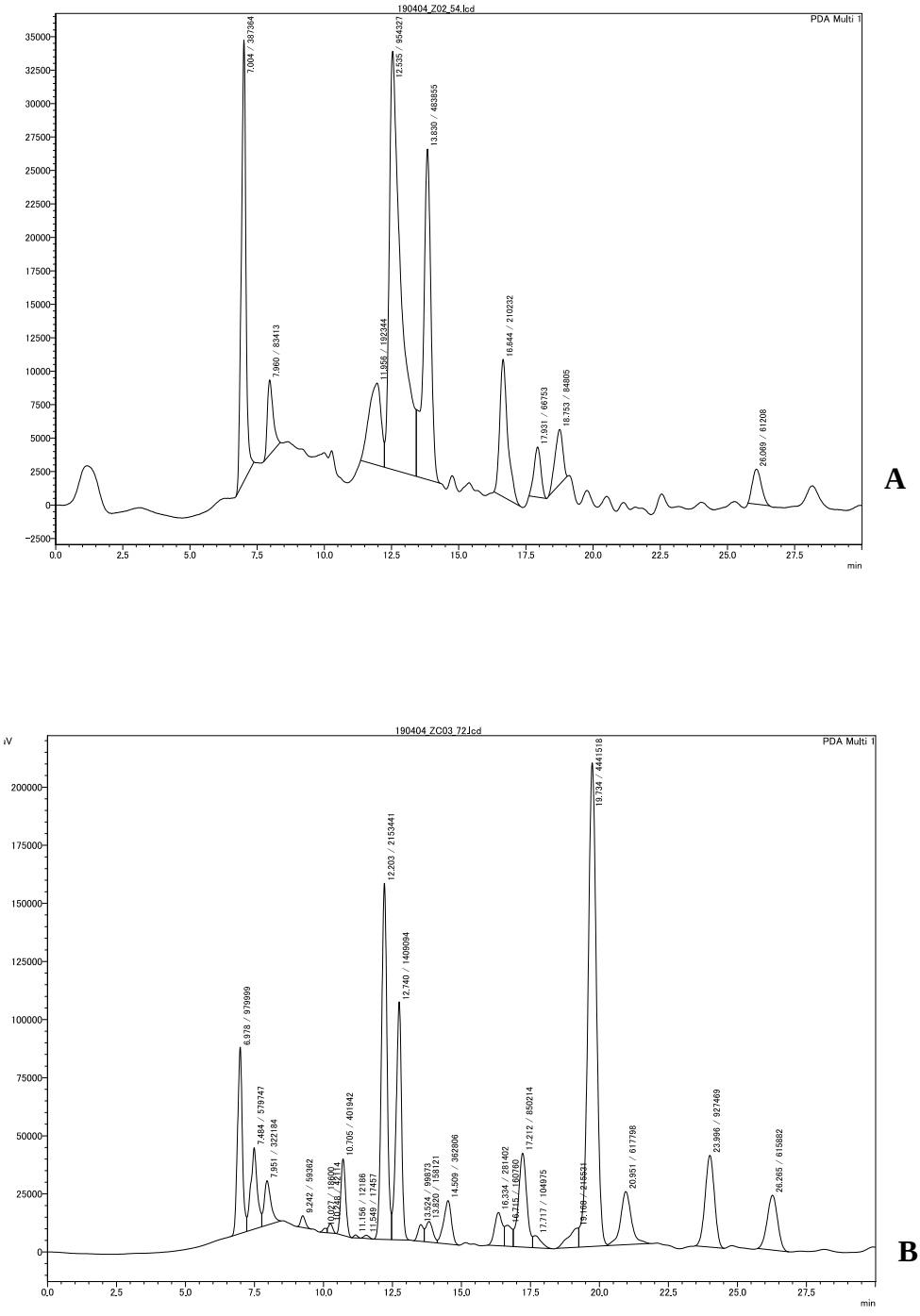


Fig. **VII.** 2. Chromatograms of organic acids analyzed by HPLC.

DISCUSSION

The study demonstrates that all *T. pinophilus* isolates could solubilize Zn in mineral salts medium amended with Zn substrates, ZnO and $ZnCO_3$. Zinc is an important micronutrient required in little amount by plants for growth and reproduction (Wissuwa *et al.,* 2006). Solubilization of inorganic mineral nutrients such as insoluble form zinc into available form for plant absorption is a well-known plant growth promoting traits exhibited by fungal species. Although huge works have been done on zinc solubilization by bacterial isolates. Fungi are used largely in industrial fermentation and bioremediation and they can grow on medium amended with sufficiently high levels of Zn (Akhtar and Mohan, 1995; Price *et al.*, 2001). Sayer *et al.* (1995) reported that fungal species could make a colony and solubilize Zn in the medium amended with zinc substrates.

In the study, all isolates showed Zn solubilizing activity in both solid and liquid medium. Some isolates showed larger clear zones on solid medium but low in liquid medium. This shows that higher zinc solubilization index (SI) on solid agar medium does not requisite to show solubilization efficiency in liquid medium. It is assumed that this anomalous behaviors could be occurred in liquid and solid media due to nutrient availability and varying diffusion rate of organic acids released by isolates which is supported with others (Jain *et al.,* 2014; Nautiyal, 1999).

The *T pinophilus* isolates showed higher Zn solubilization in ZnO supplemented medium in comparison to ZnCO3 amended medium. The mechanisms of zinc solubilization by microorganisms are very complex and are not completely known yet. Solubilisation of zinc can be accomplished by a range of mechanisms, which include excretion of metabolites such as organic acids, proton extrusion, or production of chelating agents (Nahas. 1996; Sayer *et al.,* 1997). It is apparent from the zinc solubilization data that the solubilization potential varied with each isolate. Organic acid production by microbial isolates has been reported to be a major mechanism of solubilization.

All the isolates showed reduction the pH of the broth cultures amended with ZnO and $ZnCO_3$. A negative correlation between drop in pH and Zn solubilization was noted for all isolates. It could be assumed that Zn solubilization by the isolates is

probably attributed to the production of organic acids. Organic acids play an important role in zinc solubilization processes, which can help the release of Zn by providing protons and complexing anions, or ligand exchange reactions or complexion of metal ions release to solution.

In the present study, the isolates JCM 9928 and SI-17URAgr showed the highest efficiency in Zn solubilization in medium supplemented with ZnO and $ZnCO_3$, respectively. The zinc solubilization in my *in vitro* study could be due to production of organic acids, like oxalic, citric, tartaric, malic, lactic, formic and acetic acids. Desai *et al.* **(**2012) reported that higher availability of Zn is directly proportional to acidic pH of the culture broth. However, in some potent strains, pH did not fall drastically suggesting that in those strains other mechanisms may be active and this aspect is being accentuated.

CONCLUSION

This study showed that all the legume species could be cultivated in subtropical Okinawa and they were different in biomass production with the season. Dhaincha and mungbean grew best in summer season (June to August), and chickpea and grasspea in winter season (November to January). On red soils in subtropical regions, low inputs of chemical fertilizer and high inputs of organic matter derived from legume may be a more sustainable agricultural system than systems based on high chemical fertilizer inputs.

Hairy vetch is a more appropriate legume as a green manure than tested legume species in a subtropical region considering its higher contribution of organic matter and nutrient accumulation. The result of the study revealed that incorporation of hairy vetch with soil as a green manure reduced soil bulk density and increased water content, soil organic carbon, total soil N, available P, K, Ca, Mg, Na and S, which resulted in higher growth and yield of turmeric. In addition, hairy vetch also increased mineral contents in rhizome of turmeric but decreased its curcuminoids (curcumin, bis-methoxy and methoxy curcumin) and the key reason is still now unknown. Further research should be conducted to clarify the effects of hairy vetch on curcumin content in rhizome of turmeric. Therefore, the growers who desire to cultivate turmeric with higher production in organic farming system can use hairy vetch as an organic amendment to reduce chemical fertilizer, maximize nutrient use efficiency, and minimize nutrient losses to the environment as well as production costs.

This study concluded that turmeric yield might be increased due to higher mineralization and nutrient uptake by enhancing soil microbial activities. The microbiological study showed that hairy vetch legume significantly increased soil microbial populations, and which might improve turmeric health directly or indirectly through improving soil biological properties. Hairy vetch enriched fungi, identified as *Talaromyces pinophilus*, SI-17URagr and SI-19URAgr, which possessed interesting traits such as phosphate and zinc solubilization with organic acids production. However, further trials are needed to determine the capability of these strains for P and Zn solubilization in sustainable soil management as well as crop production under field condition.

REFERENCES

Abdel-Gawad, K. M., Abdel-Mallek, A. Y., Hussein, N. A., Abdel-Rahim, I. R. 2017. Diversity of mycobiota associated with onion (*Allium cepa* L.) cultivated in Assiut, with a newly recorded fungal species to Egypt. *J Microbiol Biotech Food Sci*. 6(5): 1145-1151.

Abdel-Hafez, S. I. I., Abo-Elyousr, K. A., Abdel-Rahim, I. R. 2015. Leaf surface and endophytic fungi associated with onion leaves and their antagonistic activity against *Alternaria porri. Czech Mycol.* 67(1): 1-22.

Abdul Wahid, O. A., Mehana. T. A. 2000. Impact of phosphate-solubilizing fungi on the yield and phosphorus-uptake by wheat and faba bean plants. *Microbiol Res.* 155(3): 221-227.

Adekiya, A. O., Agbede, T. M., Aboyeji, C. M., Dunsin, O., Ugbe, J. O. 2017. Green manures and NPK fertilizer effects on soil properties, growth, yield, mineral and vitamin C composition of okra (*Abelmoschus esculentus* (L.) Moench). *Journal of the Saudi Society of Agricultural Sciences.* 18(2): 218-223.

Agbede, O. O. 2009. Understanding soil and plant nutrition**.** Petra Digital Press, Abuja, Nigeria. p. 49.

Ahmed, S., Muthuswamy, H., Muthuswamy, S. 1981. Studies on the influence of nitrogen on the yield and yield components of turmeric. *Indian Cocoa Arecanut Species.* 5: 9–10.

Akhtar, N., Mohan, P. M. 1995. Bioremediation of toxic metal ion from polluted lake waters and industrial effluents by fungal biosorbent. *Curr. Sci*. 69: 1028-1030.

Alam, S., Khalil, S., Ayub, N. Rashid, M. 2002. In vitro solubilization of inorganic phosphate by phosphate solubilizing microorganisms (PSM) from maize rhizosphere. *Int J Agric Bio.* l 4: 454-458.

Alloway, B.J. 2009. Soil factors associated with zinc deficiency in crops and humans. *Environ. Geochem. Health*. 31. 537–548.

Badaruddin, M., Meyer, D. W. 1990. Green-manure legume effects on soil nitrogen, grain yield, and nitrogen nutrition of wheat. *Crop Sci.* 30: 819–825.

Badr El-Din, S. M. S., Khalafallah, M. A., Moawad, H. 1986. Response of soybean to dual inoculation with *Rhizobium japanicum* and phosphate dissolving bacteria. *Z. Pflanzenemaehr*. 149: 130-135.

Balogh-Brunstad, Z., Keller, C. K., Dickinson, J. T., Stevens, F., Li, C. Y., Bormannm, B. T. 2008. Biotite weathering and nutrient uptake by ectomycorrhizal fungus, *Suillus tomentosus,* in liquid-culture experiments. *Geochimica et Cosmochimica Acta*, 72: 2601-261

Bardgett, R. D., van der Putten, W. H. 2014. Belowground biodiversity and ecosystem functioning. *Nature*. 515: 505–511.

Barea, J. M., Pozo, M. J., Azcon, R., Azcon, A. 2005. Microbial co-operation in the rhizosphere. *Journal of Experimental Botany.* 56: 1761-1778

Benjawan, C., Chutichudet, P., Kaewsit S. 2007. Effects of green manures on growth, yield and quality of green okra (Abelmoschus esculentus L.) Har Lium Cultivar. *Pak J Biol Sci.* 10 (7): 1028–1035.

Brady, N. C. , Weil, R. R. 1999. Soil organic matter. The nature and properties of soils. (Eds NC Brady, RR Weil) pp. 446–490.

Buommino, E. De., Filippis, A., Nicoletti. R., Menegozzo, M., Menegozzo, S., Ciavatta, M. L., Rizzo, A., Brancato, V., Tufano, M. A., Donnarumma, G. 2012. Cell-growth and migration inhibition of human mesothelioma cells induced by 3-O-Methylfunicone from *Penicillium pinophilum* and cisplatin. *Invest New Drug*. 30: 1343-1351.

Cakmak, I. 2000. Role of zinc in protecting plant cells from reactive oxygen species. *New Phytol*., 146, pp. 185-205

Cakmak, I., 2008. Enrichment of cereal grains with zinc: agronomic or genetic biofortification. Plant Soil. 302: 1–17.

Cakmak, I., Hoffland, E., 2012. Zinc for the improvement of crop production and human health. *Plant Soil.* 361: 1–2.

Caro, Y., Venkatachalam, M., Lebeau, J., Fouillaud, M., Dufosse, L. 2017. Pigments and colorants from filamentous fungi. J.-M. Mérillon, K. G. Ramawat (Eds.), Fungal Metabolites, Springer International Publishing, *Cham*. 499-568.

Caro, Y., Venkatachalam, M., Lebeau, J., Fouillaud, M., Dufossé, L. 2017. Pigments and colorants from filamentous fungi. J.-M. Mérillon, K.G. Ramawat (Eds.), Fungal Metabolites, Springer International Publishing. *Cham.* 499-568

Chai, B., Yan, W., Pengming, L., Biao, L., Meiying, G. 2011. Isolation and phosphate solubilizing ability of a fungus, *Penicillium* sp. from soil of alum mine. *JBM.* 51: 5-14.

Chapin, F. S., Maton, P. A., Vitousel, P. M. 2012. *Principles of terrestrial ecosystem ecology.* Springer, New York.

Chen, Y., Rekha, P., Arun, A., Shen, F., Lai, W.A., Young, C. 2006. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. *Appl. Soil Ecol*. 34: 33– 41.

Cherr, C., Scholberg, J., McSorley, R. 2006. Green manure approaches to crop production. *Agronomy Journal*. 98(2): 302-319.

Choi, B., Daimon, H. 2008. Effects of hairy vetch incorporated as green manure on growth and N uptake of Sorghum crop. *Plant Prod. Science*. 11: 211−216.

Cunningham, J., Kuiack C. 1992. Production of citric and oxalic acids and solubilization of calcium phosphate by Penicillium bilaii. *Appl Environ Microbiol* 85:1451–1458

Desai, S. G., Praveen Kumar, U., Sultana. 2012. Potential microbial candidate strains for management of nutrient requirements of crops," *African Journal of Microbiology Research.* 6(17): 3924–3931.

Dhakar, K., Jain, R., Tamta, S., Pandey, A. 2014. Prolonged laccase production by a cold and pH tolerant strain of *Penicillium pinophilum* (MCC 1049) isolated from a low temperature environment. *Enzyme Res. p. 1-6.*

Elfstrand, S., Båth, B., Mårtensson, A. 2007. Influence of various forms of green manure amendment on soil microbial community composition, enzyme activity and nutrient levels in leek. *Appl. Soil Ecol*., 36: 70-82

Elfstrand, S., Bath, B., Martensson, B. 2007. Influence of various forms of green manure on soil microbial community composition, enzyme activity and nutrient levels in leek. Applied Soil Ecology. 36: 70−82.

El-Naggar, N. E. A., Haroun, S. A, Owais, E. A., Sherief, A. A. 2015. Identification of newly isolated *Talaromyces pinophilus* and statistical optimization of (β-glucosidase production under solid-state fermentation. *Preparative Biochemistry Biotechnology.* 45: 712-729.

Eriksen, J. 2005. Gross sulphur mineralisation-immobilization turnover in soil amended with plant residues. *Soil Biol. Biochem*. 37 (12): 2216-2224

Fajri, A., Kitou, M., Fujio, N., Kinjo, K., Jayasinghe, G. Y. P. 2010. Potential growth of hairy vetch as a winter legume cover crops in subtropical soil conditions. *Soil Science and Plant Nutrition.* 56: 254–262.

Fasim, F., Ahmed, N., Parsons, R., Gadd, G. M. 2002. Solubilization of zinc salts by a bacterium isolated from the air environment of a tannery. *FEMS Microbiology Letters*. 213(1): 1–6.

Fomina, M., Alexander, I. J., Hillier, S., Gadd, G. M., 2004. Zinc phosphate and pyromorphite solubilization by soil plant-symbiotic fungi. *Geomicrobiol J.* 21: 351–366.

Fujii, T., Hoshino, T., Inoue, H., Yano, S. 2014. Taxonomic revision of the cellulose-degrading fungus *Acremonium cellulolyticus* nomen nudum to *Talaromyces* based on phylogenetic analysis. *FEMS Microbiol. Lett.* 351: 32-41.

Gaind, S., Gaur, A. C. 1991. Thermotolerant phosphate solubilizing microorganisms and their interaction with mung bean. *Plant Soil.* 133: 141-149.

Gerretsen FC. 1948. The influence of microorganisms on the phosphorus uptake by the plants. *Plant Soil*, 1:51–81

Glick, B. R. 1995. **T**he enhancement of plant growth by free-living bacteria. *Can. J. Microbiol*. 41(2): 109-117.

Goto, S., S. Nagata. 2000. Effect of crotalaria, sorghum and pampas grass incorporation as green manure on the yield of succeeding crops and soil physical and chemical properties. *J. Soc. Soil. Sci. Plant Nutrition*. 71: 337-344.

Hansen, G. H., Lübeck, M., Frisvad, J. C., Lübeck, P. S., Andersen, B. 2015. Production of cellulolytic enzymes from ascomycetes: Comparison of solid state and submerged fermentation. Process Biochem. 50: 1327-1341.

Haug, A. 1984. Molecular aspects of aluminum toxicity. CRC Crit. Rev. *Plant. Sci.* 1: 345–373.

Herrera, M. A., Salamanka, C. P., Barea, J. M. 1993. Inoculation of woody legumes with selected arbuscular mycorrhizal fungi and rhizobia to recover desertified Mediterranean ecosystems. *App. Env. Microbiol*. 59: 129-133.

Holford, I. C. R. 1997. Soil phosphorus: its measurement, and its uptake by plants. *Aust. J. Soil Res.* 35(2): 227-240.

Hossain, M. A., Akamine, H., Tamaki, M. 2015. Green manure plants influence growth, yield and curcumin content of turmeric (*Curcuma longa* L.) in dark-red soil in Okinawa, *Japan. Trop. Agr. Develop.* 59 (2): 63–75.

Hossain, M. A., Ishimine, Y. 2005. Growth, yield and quality of tu rmeric (*Curcuma longa L.*) cultivated in dark-red soil, gray soil and red soil. *Plant Prod. Sci.* 8: 482–486.

Hossain, M. A., Ishimine, Y., Akamine, H., Motomura, K. 2005a. Effects of seed rhizome size on growth and yield of turmeric (*Curcuma longa* L.). *Plant Prod. Sci.* 8: 86–94.

Hossain, M. A., Ishimine, Y., Motomura, K., Akamine, H. 2005b. Effects of planting pattern and planting distance on growth and yield of turmeric (*Curcuma longa* L.). *Plant Prod. Sci.* 8: 95 –105.

Hotz, C., Brown, K. H., 2004. Assessment of the risk of zinc deficiency in populations and options for its control. *Food Nutr. Bull.* 25: 94–204.

Iqbal, U. N., Jamil, N., Ali, I., Hasnain, S. 2010. Effect of zinc-phosphate-solubilizing bacterial isolates on growth of *Vigna radiata*. *Annals of Microbiology*, 60(2): 243-248.

Ismail, R. Abdel-Rahim, Kamal, A. M. Abo-Elyousrb. 2018. *Talaromyces pinophilus* strain AUN-1 as a novel mycoparasite of Botrytis cinerea, the pathogen of onion scape and umbel blights. *Microbiological Research*, 212-213: 1-9.

Ismail, M. A., El-Maali, N. T. A., Omran, G. A., Nasser, G. A. 2016. Biodiversity of mycobiota in peanut seeds, corn and wheat grains with special reference to their aflatoxigenic ability. *J Microbiol. Biotechnol. Food Sci*. 5: 314.

Jain, R., Saxena, J., Sharma, V. 2012. Solubilization of inorganic phosphates by *Aspergillus awamori* S19 isolated from agricultural soil of semi-arid region. *Annals Microbiol*. 62: 725-735.

Jain, R., Saxena, J., Sharma, V. 2014. Differential effects of immobilized and free forms of phosphate solubilizing fungal strains on the growth and P uptake of mungbean plants. *Annals Microbiol.* 64: 1523-1534.

Jain, R., Saxena, J., Sharma, V. 2017. The ability of two fungi to dissolve hardly soluble phosphates in solution. *Mycology*. 8 (2): 104-110.

Jiff R. C., Saranya K., Jang H. K., Young-Eun Y., Song Y. K. , Yong B. L. 2018. Hairy Vetch Incorporated as Green Manure Inhibits Sulfathiazole Uptake by Lettuce in Soil. *Water Air Soil Pollut*. 229: 104.

Jose, M. S., Milton, P.M., Ivana, D.M., Marina, R., Nubia, S.M. and Alicia, G. 2010. Soil fungal isolates produce different organic acid patterns involved in phosphate salts solubilization. *Biol Fertil Soils.* 46: 755-763.

Kavanagh, K. 2011. Fungal fermentation systems and products, in Fungi: biology and applications. *Wiley-Blackwell*.

Koul. M, S., Meena, A., Kumar, P. R., Sharma, V., Singamaneni, S., Riyaz-Ul-Hassan, A., Hamid, A., Chaubey, A., Prabhakar, P., Gupta, P. 2016. Secondary metabolites from endophytic fungus *Penicillium pinophilum* induce ROS-mediated apoptosis through mitochondrial pathway in pancreatic cancer cells. *Planta Med.* 82: 344-355.

Kulpapangkorna, W., Mai-leang, S. 2011. Effect of plant nutrition on turmeric production. *Procedia Engineering*. 32: 166–171.

Kuo, S., Jellum, E. J. 2000. Long term winter cover cropping effects on corn (zea mays L.) production and soil nitrogen availability. *Biol. Fertil. Soil.* 31: 470–477.
land corn production. *J. Prod. Agric*. 4: 62–67.

Lehmann A, Veresoglou SD, Leifheit EF, Rillig MC. 2014. Arbuscular mycorrhizal influence on zinc nutrition in crop plants - a meta-analysis. *Soil Biol Biochem*. 69:123–131.

Li, C. X., Zhao, S., Zhang, T., Xian, L., Liao, L. S., Liu, J. L., Feng, J. X. 2017. Genome sequencing and analysis of *Talaromyces pinophilus* provide insights into biotechnological applications. *Sci. Rep*. 7: 490.

Liao, H., Wan, H., Shaff, J. 2006. Phosphorus and aluminum interactions in soybean in relation to aluminum tolerance: exudation of specific organic acids from different regions of the intact root system. *Plant Physiology*. 141: 674–684.

Lindow, S. E., Leveau, J. H. 2002. Phyllosphere microbiology. *Curr. Opin. Biotechnol.* 13: 238-243.

Lundquist, E. J., Jackson, L. E., Scow, K. M., Hsu. C. 1999. Changes in microbial biomass and community composition, and soil carbon and nitrogen pools after incorporation of rye into three California agricultural soils**.** *Soil Biol. Biochem*. 31 (2): 221-236

Ma, Y. B., Uren, N. C., 1997. The fate and transformations of zinc added to soils. *Aust. J. Soil Res.* 35: 727–738.

Mappaona, Yoshida, S., Kitou, M. 1994. Yield Response of cabbage to several tropical green manure legumes incorporated into soil. *Soil Sci. Plant Nutri.* 40 (3): 415–424.

Margesin, R., Schinner, F. 2005. Manual for soil analysis-monitoring and assessing soil bioremediation. Publisher: Springer-Verlag Berlin Heidelberg. Volume: 5, ISBN: 978-3 540-28904–3.

McGill, W.B., Cole, C.V. 1981. Comparative aspects of cycling of organic C, N, S and P through soil organic matter. *Geoderma*, 26: 267-286.

Mehrotra, B. S., Kumar, D. A. 1962. A new species of Penicillium from India. *Canadian Journal of Botany*. 40(10): 1399-1400.

Miltner, A., Bombach, P., Schmidt-Brücken, B., Kastner, M. 2012. SOM genesis: microbial biomass as a significant source. *Biogeochemistry*. 111: 41–55.

Mittal, V., Singh, O., Nayyar, H., Kaur, J., Tewari, R. 2008. Stimulatory effect of phosphate-solubilizing fungal strains (*Aspergillus awamori* and *Penicillium citrinum*) on the yield of chickpea (*Cicer arietinum* L. cv. GPF2). *Soil Biol Biochem* 40 (3):718–727.

Miyamaru, N., Gima, Y., Yonamine, Y., Kameya, S. 2008. Growth and decomposition property of several green manures in Okinawa. Okinawa Agriculture Research. 42: 21−27.

Morgan, J. A., Bending, G. D., White, P. J. 2005. Biological costs and benefits to plant–microbe interaction in the rhizosphere. *Journal of Experimental Botany*. 56: 1729-1739

Murphy, J., Riley, J. P. 1962. A modified single solution method for the determination of phosphate in natural waters. *Anal Chim Acta*. 27: 31-36.

Myers, S. S., Wessells, K. R., Kloog, I., Zanobetti, A., Schwartz, J. 2015. Effect of increased concentrations of atmospheric carbon dioxide on the global threat of zinc deficiency: a modelling study. *Lancet Glob. Health*. 3: e639–e645

Nahas, E. 1996. Factors determining rock phosphate solubilization by microorganisms isolated from soil. *World J Microbiol Biotechnol*. 12: 567– 572.

Nakano, M., Miyazaki, T., Shiozawa, S., Nishimura, T. 1995. Physical and environmental analysis of soil. University of Tokyo Press. Japan.

Narsian, V., Patel, H. H. 2000. *Aspergillus aculeatus as a rock phosphate solubilizer*. *Soil Biol. Biochem*. 32: 559–565

Nautiyal, C. S. 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol Lett*. 170(1): 265-270.

Nguyen, C., Yan, W., Tacon, F. Le and Lapeyrie, F. 1992. Genetic variability of phosphate solubilizing activity by monocaryotic and dikaryotic mycelia of the ectomycorrhizal fungus Laccaria bicolor (Maire) P.D. Orton. *Plant and Soil*. 143(2): 193–199.

Nicoletti, R., De Stefano, M., De Stefano, S., Trincone, A., Marziano, F. 2004. Antagonism against *Rhizoctonia solani* and fungitoxic metabolite production by some *Penicillium* isolates. *Mycopathologia*. 158: 465-474.

Nicoletti, R., Trincone, A. 2016. Bioactive compounds produced by strains of Penicillium and Talaromyces of marine origin. *Mar. Drugs*. 14: 37

Ohshiro, M., Hossain, A. M., Ichiro, N., Akamine, H., Tamaki, M., Bhowmikc, P. C., and Akihiro, N. 2016. Effects of soil types and fertilizers on growth, yield, and

quality of edible Amaranthus tricolor lines in Okinawa, Japan. *Plant Production Science*. VOL. 19 (1): 61–72.

Panday, S., Ceballos, S. M, Magnavaca, R., Bahia Filho, A. F. C., Duquevargas, J., Vinasco, L. E. 1994. Genetic tolerance to soil acidity in tropical maize. *Crop Sci*. 34: 1511-1514.

Pandey, A., Das, N., Kumar, B., Rinu, K., Trivedi, P. 2008. Phosphate solubilization by Penicillium spp. isolated from soil samples of Indian Himalayan region. World J *Microbiol Biotechnol*. 24: 97-102.

Parr M., Grossman J. M., Reberg-Horton S. C., Brinton, C., Crozier, C. 2011. Nitrogen delivery from legume cover crops in no-till organic corn production. *Agron J*. 103: 1578−1590.

Pawar,V. C., Thaker,V. S. 2009. Acid phosphatase and invertase activities of *Aspergillus niger*. *Mycoscience*, 50, 323–330.

Pitt, J. 2014. Penicillium and Talaromyces: Introduction, Penicillium, Encyclopedia of Food *Microbiology* (2nd edn). *p*. 6-13.

Pol, D., Laxman, R. S., Rao, M. 2012. Purification and biochemical characterization of endoglucanase from *Penicillium pinophilum* MS 20. *Indian J Biochem Biophys*. 49: 189-194.

Power, J. F., Doran, J. W., Koerner, P. T. 1991. Hairy Vetch as a Winter Cover Crop for Dryland Corn Production. *J. Prod. Agric.* 4 (1).

Pradhan, N., Sukla, L. B. 2005. Solubilization of inorganic phosphate by fungi isolated from agricultural soil. *African J Biotechnol.* 5:850–854.

Prasad, S., Subash C. Gupta, Amit, K. Tyagi, Bharat B. Aggarwal. 2014. Curcumin, a component of golden spice: From bedside to bench and back. *Biotechnology Advances*. 32: 1053–1064.

Pratibha, V., Arvind G. 2009. Organic acid production *in vitro* and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent *Pseudomonas*. *BMC Microbiol*. 9: 174.

Premono M. E., Moawad A. M., Vlek P. L. G. 1996. Effect of phosphate-solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. *Indonesian Journal of Crop Science*. 11: 13–23.

Ram, H., Malik, S. S., Dhaliwal, S. S., Kumar, B., Singh, Y. 2015. Growth and productivity of wheat affected by phosphorus solubilizing fungi and phosphorus levels. *Plant Soil Environ.* 61(3): 122-126.

Randhawa, P. S., Condron, L. M., Di, H. J., Sinaj, S., McLenaghen, R. D. 2005. Effect of green manure addition on soil organic phosphorous mineralisation. *Nutr. Cycl. Agroecosyst*. 73 : 181-189.

Requena, B. N., Jimenez, I., Toro, M., Barea, J. M. 1997. Interactions between plant-growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and *Rhizobium* spp. in the rhizosphere of *Anthyllis cytiisoides*, a model legume for revegetation in Mediterranean semi-arid ecosystems. *New Phytol*. 136: 667-677.

Rochester, I., Peoples, M. 2005. Growing vetches (Vicia villosa Roth) in irrigated cotton systems: Inputs of fixed N, N fertilizer savings and cotton productivity. *Plant Soil*. 271: 251-264.

Rodriguez, H., Fraga, R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv*. 17, 319– 339.

Sainju, U. M., Singh, B. P., Whitehead, W.F. Long-term effects of tillage, cover crops, and nitrogen fertilization on organic carbon and nitrogen concentrations in sandy loam soils in Georgia, USA. *Soil Tillage Res*. 63: 167–179.

Salahin, N., Alom, M. K., Islam, M. M., Nahar, L., Majid, N. M. 2013. Effects of green manure crops and tillage practice on maize and rice yields and soil properties. *Aus. J. crops Sci.* 7 (12): 1901–1911.

Samson, R. A., Yilmaz, N., Houbraken, J., Spierenburg, H., Seifert, K. A., Peterson, S. W., Varga, J., Frisvad, J. C. 2011. Phylogeny and nomenclature of the genus Talaromyces and taxa accommodated in Penicillium subgenus Biverticillium. *Studies in Mycology*. 70: 159-183.

Saravanan, V. S., Kalaiarasan, P., Madhaiyan, M., Thangaraju, M. 2007. Solubilization of insoluble zinc compounds by Gluconacetobacter diazotrophicus and the detrimental action of zinc ion (Zn2+) and zinc chelates on root knot nematode Meloidogyne in cognita. *Letters in Applied Microbiology*. 44(3): 235–241.

Sarker, J. R., Singh, B. P., Dougherty, W. J., Fang, Y., Badgery, W., Hoyle, Dalal, F. C., Cowie, A. L. 2018. Impact of agricultural management practices on the nutrient

supply potential of soil organic matter under long-term farming systems. *Soil Till. Res.* 175: 71-81.

Sarrantonio, M., Scott, T.W. 1988. Tillage effects on availability of nitrogen to corn following a winter green manure crop. *Soil Sci. Soc. Am. J.* 52: 1661–1668.

Sauer, M., Porro, D., Mattanovich, D., Branduardi, P. 2008. Microbial production of organic acids; expanding the markets. *Trends Biotechnol*. 26: 100-80

Saxena, J., Basu, P., Jaligam, V., Chandra, S. 2013. Phosphate solubilization by a few fungal strains belonging to the genera *Aspergillus* and *Penicillium. Afr J Microbiological Res*. 7: 4862-4869.

Sayer, J. A., Gadd, G. M. 1997. Solubilization and transformation of insoluble inorganic metal compounds to insoluble metal oxalates by *Aspergillus niger*," *Mycological Research*. 101(6): 653–661.

Scervino, J. M., Mesa, M. P., Mónica, I. D., Recchi, M., Moreno, S., Godeas, A. 2013. Soil fungal isolates produce different organic acid patterns involved in phosphate salts solubilization. *Biol Fertil Soils*. 49(6): 779–779.

Sembiring, M., Fauzi. 2017. Bacterial and fungi phosphate solubilization effect to increase nutrient uptake and potatoes (*Solanum tuberosum* L.) production on Andisol Sinabung area. *J. Agron*. 16(3): 131-137.

Senthil Kumar, C. M., Jacob, T. K., Devasahayam, S., Stephy, T., Geethu, C. 2018. Multifarious plant growth promotion by an entomopathogenic fungus *Lecanicillium psalliotae*. *Microbiol. Res.* 207: 153–160.

Siddique, M. T., J. S. Robinson. 2003. Phosphorus sorption and availability in soils amended with animal manures and sewage sludge. *J. Environ. Qual.* 32: 1114-1121.

Sillanpää, M., Vlek, P. L. G., 1985. Micronutrients and the agroecology of tropical and Mediterranean regions. *Fert. Res.* 7: 151–167.

Singh, B., Natesan, S. K. A., Singh, B. K., Usha, K. 2005. Improving zinc efficiency of cereals under zinc deficiency. *Curr. Sci.* 88: 36–44.

Singh, M. S., Yadav, L. S., Singh, S. K., Singh, P., Singh, P.N., Ravindra. 2011. Phosphate solubilizing ability of two Arctic *Aspergillus niger strains*. *Polar Research*, 30: 72-83.

Singh, V. B., Swer, B., Singh, P. P. 1992. Influence of nitrogen and potassium on yield andquality of turmeric cv. Lakadong. *Indian Cocoa Arecanut Species J.* 15 (4): 106–108.

Smith, M. S., Frey, W. W., Varco, J. J. 1987. Legume winter cover crops. *Advances in Soil Science*. 7: 95−139.

Son, H. J., Park, G. T., Cha, M. S., Heo, M. S. 2006. Solubilization of insoluble inorganic phosphates by a novel salt- and pH-tolerant *Pantoea agglomerans* R-42 isolated from soybean rhizosphere. *Bioresource Technol*. 97(2): 204-210.

Subba Rao, N. S. 1999. Soil Microbiology (Fourth Edition of Soil Microorganisms and Plant Growth). Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi. 407.

Sullivan, P. 2003. Overview of cover crops and green manures. Appropriate technology transfer for rural areas. *p.* 1−16.

Sultani, M. I., Gill, M. A., Anwar, M. M., Athar, M. 2007. Evaluation of soil physical properties as influenced by various green manuring legumes and phosphorus fertilization under rain fed conditions. *Int. J. Environ. Sci. tech*. 4: 109−118.

Tao, G. C., Tian, S. J., Cai, M. Y., Xie, G. H. 2008. Phosphate-solubilizing and mineralizing abilities of bacteria isolated from soils. *Pedosphere,* 18: 515-523

Tejada, M., Gonzalez, J. L., García-Martínez, A. M., Parrado, J. 2008. Effects of different green manures on soil biological properties and maize yield. *p.* 1758-1767.

Thom, C. 1910. Cultural studies of species of Penicillium. U. S. D. A. Bureau of Animal Industry Bulletin. 118: 1-107

Varco, J. J., Frye, W. W., Smith, M. S., Mackown, C. T. 1989. Tillage effect on nitrogen recovery by corn from a nitrogen-15 labeled legume cover crop. *Soil Sci. Soc. Am. J.* 53: 822–827.

Venkateswarlu, B., Rao, A. V., Raina, P. 1984. Evaluation of phosphorus solubilization by microorganisms isolated from arid soil. *J. Ind. Soc. Soil Sci.* 23: 273-277.

Visser, E. M., Falkoski, D. L., de Almeida, M. N., Maitan-Alfenas, G. P., Guimarães, V. M. 2013. Production and application of an enzyme blend from *Chrysoporthe cubensis* and *Penicillium pinophilum* with potential for hydrolysis of sugarcane bagasse. *Bioresource Technol.* 144: 587-594.

Wang, G. H., Zhou, K. Q., Jin, J., Pan, X. W., Zhao, Y. 2004. Effect of different C sources on the solubilization of rock phosphate by three phosphate solubilizing fungi (PSF). *Chinese J. Ecol.* 23: 32–36.

Wang, M. H., Li, X. M., Li, C. S., Ji, N. Y., Wang, B. G. 2013. Secondary metabolites from *Penicillium pinophilum* SD-272, a marine sediment-derived fungus. *Mar. Drugs.* 11: 2230-2238.

Wani, Z. A., Mirza, D. N., Arora, P., Riyaz-Ul-Hassan, S. 2016. Molecular phylogeny, diversity, community structure, and plant growth promoting properties of fungal endophytes associated with the corms of saffron plant: An insight into the microbiome of *Crocus sativus* Linn. *Fungal Biol.* 120: 1509–1524.

Watanabe, T. 2010. Pictorial atlas of soil and seed fungi morphologies of cultured fungi and Key to species, Third Edition. CRC press, Florida.

Whitelaw M. A. 2000. Growth promotion of plants inoculated with phosphate-solubilizing fungi. *Adv Agron*, 69: 99–151

Wissuwa, M., Ismail, A.M., Yanagihara, S. 2006. Effects of zinc deficiency on rice growth and genetic factors contributing to tolerance. *Plant Physiol.* 142: 731–741.

Xian, L., Wang, F., Luo, X., Feng, Y-L., Feng, J-X. 2015. Purification and characterization of a highly efficient calcium-independent alpha-amylase from *Talaromyces pinophilus* 1–95. *PLoS ONE.* 10(3): e0121531.

Yamgar, V. T., Kathmale, D. K., Belhekar P. S., Patil R. C., Patil P. S. 2001. Effect of different levels of nitrogen, phosphorus and potassium and split application of N on growth and yield of turmeric (Curcuma longa). *Indian J. Agron*. 46: 372–374.

Yao, Y. Q., Lan, F., Qiao, Y. M., Wei, J. G., Huang, R. S., Li, L. B. 2017. Endophytic fungi harbored in the root of *Sophora tonkinensis* Gapnep: diversity and biocontrol potential against phytopathogens. *Microbiology Open*. 1-17.

Zhai, M. M., Niu, H. T., Li, J., Xiao,H., Shi, Y. P., Di, D. L., Crews, P., Wu. Q. X. 2015. Talaromycolides A–C, novel phenyl-substituted phthalides isolated from the green Chinese onion-derived fungus *Talaromyces pinophilus* AF-02. *J. Agric. Food Chem*. 63: 9558-9564.

Zhang, M., Alva, A. K., Li, Y. C., Calvert, D. V. 2001. Aluminium and iron fractions affecting phosphorus solubility and reactions in selected sandy soils. *Soil Sci.* 166: 940-948.

Zhang, X., Jiang, B., Ma, Y., 2017. Aging of zinc added to soils with a wide range of different properties: factors and modeling. Environ. Toxicol. *Chem.* 36: 2925–2933.

Zhang, Y., Chen, F-S., Wu, X-Q., Luan, F-G., Zang, L-P., Fang, X-M., Wan, S-Z., hu, X-F., Ye, J-R. 2018. Isolation and characterization of two phosphate solubilizing fungi from rhizosphere soils of moso bamboo and their functional capacities when exposed to different phosphorus sources and pH environment. *PLOS ONE*. 13(7): e0199625.

Zhen, Li., Bai, T., Dai, L., Wang, F., Tao, J., Meng, S., Hu, Y., Wang, S., Hu, S. 2016. A study of organic acid production in contrast between two phosphate solubilizing fungi: *Penicillium oxalicum* and *Aspergillu niger*. *Sci.Rep*. 6: 25313.

Acknowledgment

Firstly, my sincere gratitude is to ALLAH, the Most Merciful and the Most Compassionate, for his continuing help and support.

My heartiest appreciation and gratitude is addressed to my respected major advisor Prof. Dr. Md. Amzad Hossain, Faculty of Agriculture, University of the Ryukyus for his endless contribution, invaluable guidance and encouragement.

I would like to express gratefulness to my respected co-advisor Dr. Hikaru Akamine, Associate Professor, Faculty of Agriculture, University of the Ryukyus and Dr. Michio Onjo, Professor, Faculty of agriculture, Kagoshima University for their insightful comments, inspiration and encouragement.

I would like to express utmost appreciation to respected sensei, Professor Dr. Ayako Sano, Faculty of Agriculture, University of the Ryukyus for her endless support and encouragement. She gave me kind consent to access her laboratory and provided research facilities.

I would also like to express my thanks to respected sensei, Jun-Ichi Sakagami, Faculty of agriculture, Kagoshima University for critical judgment and valuable comments on research.

I give thanks to respected sensei Dr. Toshihiro KONNO, Associate Professor, Faculty of Agriculture, University of the Ryukyus, Japan for his cordial help and cooperation during molecular study.

I am extremely grateful to the Minister of Education, Culture, Sports, Science and Technology (Monbukagakusho) Japan, for financial support throughout the whole period of my study.

My sincere thanks also goes to all lab members, past and present, and staff of Subtropical Field Science Center, Faculty of Agriculture, University of the Ryukyus, for their cooperation and encouragement both on and off work.

I would like to express my deepest gratitude to my beloved mother, and respected elder brother, Md. Mazharul Islam.

Finally, special thanks to my wife, Israt Sultana, for her sacrifices, patience, inspiration, encouragement and understanding me at all the times.