ISOLATION, IDENTIFICATION AND ANTAGONISTIC EFFECT OF RHIZOBACTERIA ASSOCIATED WITH COFFEE (*Coffea arabica* L.) RHIZOSPHERE AGAINST COFFEE BERRY DISEASE (*Colletotrichum kahawae)* JIMMA, ETHIOPIA

MSc. Thesis

BY

GEMECHU SENBETO SIRNO

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Isolation, Identification and Antagonistic Effect of Rhizobacteria Associated with Coffee (*Coffea arabica* L.) Rhizosphere Against Coffee Berry Disease (*Colletotrichum kahawae)* Jimma, Ethiopia

MSc. Thesis

Gemechu Senbeto Sirno

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DEDICATION

I dedicate this thesis manuscript to my beloved father **Senbeto Sirno,** my mother **Warite Lemu, my sisters and brother** for their dedicated partnership in the success of my life.

STATEMENT OF THE AUTHOR

I declare that this thesis is my work and all sources of materials used for this thesis have been duly acknowledged. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma or certificate. I have followed all ethical and technical principles for the preparation, data collection, data analysis and compilation of the thesis. Any scholarly matter included in the thesis has been given recognition through citation.

Name: Gemechu Senbeto Sirno Signature: ----------------------------

Place: Jimma University, College of Agriculture and Veterinary Medicine, Jimma

Date of Submission: ---

BIOGRAPHICAL SKETCH

The author, Gemechu Senbeto was born from Senbeto Sirno and his mother Warite Lamu at Dembi Dollo, in Kellem Wallega, Oromia regional state, Ethiopia in June 20, 1987. He attended grade 1 to 8 at Olika Dingil Elementary School (1994-2001), and grade 9 to 12 at Kellem Comprehensive Secondary School (2002-2005), in Dembi Dollo, Wollega, Ethiopia. He joined Haramaya University in September 2006, and graduated with BSc. degree in Crop Production and Protection in June 27, 2008. The author was employed by Ethiopian Ministry of Agriculture in September 2009 and served as expert at Agricultural and Rural development of Kellem Wollaga Zone, for two years. In September 2011, he joined the school of graduate studies of Jimma University to pursue his graduate study in Plant Pathology. Starting from August 2016, he has serving Ethiopian Evangelical Church Mekane Yesus Development Association Social Service Commission West Wollega Bethel Synod Branch Office in program of Green Livelihood and Natural Resources Development (Green LaND) as a manager.

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ABBREVIATIONS AND ACRONYMS

AFMs	Antifungal metabolites
AHL	Acyl homoserine lactone
ANOVA	Analysis of variance
BCA	Biological control agent
CBD	Coffee berry disease
CLR	Coffee leaf rust
CNN	Competition for nutrients and niches
CRD	Complete randomized design
CWD	Coffee wilt disease
DAPG	Diacetylphloroglucinol
DI	Disease index
ECX	Ethiopian commodity exchange
FDREMT	Federal democratic republic of Ethiopian--trade
HCN	Hydrogen cyanide
ISR	Induce systemic resistance
ITC	International trade center
JUCAVM	Jimma University College of Agriculture and Veterinary Medicine
KB	King's B medium
KBHS	Presolidified half-strength King's B medium
KOH	Potassium hydrogen peroxide
NCDC	National Climatic Data Center.
PDA	Potato dextrose agar
PGPR	Plant growth-promoting rhizobacteria
Phl	Phloroglucinols
Phz	Phenazines
SAR	Systemic acquired resistance
TFRR	Tomato foot and root rot
USA	United States of America
WP	Wattable powder

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ABSTRACT

Coffee berry disease caused by fungus Colletotrichum kahawae, is considered as major disease of coffee in Ethiopia. Different control methods practiced at coffee producing farms and farmer levels in the country including chemical control. Hence, the use of chemical fungicide is causes environmental pollution and abortion of natural sustainability. However, the development of biological control helps to improve plant health, yield, reduce health risk and hazardous effects on environments have got great concern. Hereafter, this study was carried out to examine the antagonistic effects of rhizobacteria isolated from Arabica Coffee rhizosphere against Colletotrichum kahawae, that causes coffee berry disease, under in vitro and in vivo conditions. The soil sample used sources of bacteria were taken from Gera, Ela Dalle and around the JUCAVM campus. The isolation result showed that, total of 215 isolated from Gera (104), Ela dale (67) and JUCAVM campus (44) in ten different bacteria species on Kings B medium. The isolated bacteria were in-vitro tested by using CRD with three replications. Out of 215 isolated rhizobacteria antagonistic tested on Half Strength King's B (HSKB) medium, 28 exhibited remarkable range of 8.2% – 91.3% *C. kahawae radial growth inhibition and four rhizobacteria; Bacillus spp. (JU544) by* 91.3%, *Pseudomonas spp. (JU13)* by 82.3%, *Bacillus spp. (JU735 by 74.6%, and Micrococcus spp. (JU533) with* 67% *showed high inhibition percentage. High performed rhizobacteria were tested under in vivo condition against the fungal pathogen on detached green coffee berry, by applying the isolates at the same time of inoculation, 48 hours before and after inoculation of the pathogen. The results at both severity and incidence showed that Bacillus spp. (JU544) and Pseudomonas spp. (JU13) genera were the most effective that significantly reduced ($p<0.0001$) the radial culture growth of the pathogen when compared to the control, respectively. Furthermore, Bacillus spp. (JU544) and Pseudomonas spp. (JU13) genera consistently reduced disease severity and incidence when applied at the same time than applying before 48hrs and 48 hours after inoculation on detached coffee berries. However, the highest bio-control efficiency reduced disease severity and disease incidence was 89% and 76.7% when the coffee berry was treated with Bacillus spp. (JU544) at the same time with inoculation of the pathogen. Thus, considerable efforts should be devoted on isolation, identification of the plant growth promoting rhizobacteria and to evaluate the genera's effectiveness against the coffee berry disease causing pathogen. In order to develop new microbial fungicides as alternative to control and fight berry anthracnose and as well as to formulate integrated disease management schedule and come up with strong recommendation. This study was conducted under laboratory conditions. Moreover, optimum concentration, application methods of rhizobacteria should be studied. Further conformation may be also important.*

Key words: Antifungal Potential, Characterization, *Colletotrichum kahawae*, Isolation.

1. INTRODUCTION

Ethiopia has the longest tradition of coffee production and consumption in the world with inimitable coffee ceremony (Adugna 2004). The country is believed to be the origin of Arabica coffee (*Coffea arabica* L.) that makes over 70% of the world's production (Adugna *et al*., 2009). It was not cultivated as a crop until early 900 B.C in the mountains of Yemen, and it remained an Arab monopoly for hundreds of years (Jaffee, 2007). Hence, it is a single most important cash crop that has been contributing a lion's share to the Ethiopian economy (Girma *et al.,* 2008). Ethiopian coffee is in high demand in the international market, and it is specifically valued for its special aroma and distinct flavor (Abu and Teddy, 2013).

However, it has crucial values in development of the country, substantial constrained of its production were reported. Several factors were contributed to the current low level of coffee production (Bayetta *et al*., 2009). Among these is the lack of adequate knowledge of farmers to properly weeding, stumping, pruning, at harvesting and post-harvest management. The major constraints of coffee production in the country are diseases caused by many etioletic agents, mainly the fungi. The major coffee diseases in Ethiopia are coffee berry disease (CBD), coffee wilt disease (CWD) and coffee leaf rust (CLR), as the most important diseases both in severity and wide distribution are CBD and CWD (Arega, 2006).

Coffee berry disease (CBD) was first detected in 1922 in Kenya around Mt. Elgon, west of the Rift Valley (Mc Donald,1926; Waller *et al*., 2007). In Ethiopia, CBD is the major threat to Arabica coffee production since its outbreak in 1971 (Arega *et al.*, 2008). Tefestewold (1995) reported concomitant outbreak of CBD in southwest and southeastern part of Ethiopia in the early 1970s. Furthermore, the disease was extended to Shewa and Gamugofa in 1977 and Harraghe in 1978 (Vander Graaff, 1981), it also disseminated to other coffee growing countries.

CBD causes significant yield losses. Van der Graaff (1981) and Mardasa (1985) reported that the average national yield losses were about 28% between 1974 and 1978. Additionally, Losses due to CBD on individual farms vary considerably and in high rainfall and high-altitude areas,

losses may reach up to 100% (Van der Graaff, 1981). The average yield losses ranged from 51% to 81% from Wondogent, Gera and Jimma experimental plots (Merdasa, 1985). Eshetu and Girma (1992) estimated coffee yield losses exceeding 40% at Gera. Similarly, in Hararghe, the losses were estimated to be as high as 100% (Tefestewold, 1995). At present, CBD has rapidly spread to all coffee growing areas of Ethiopia and is still inflicting significant crop loss (up to 100% on susceptible land races) although the magnitudes vary from place to place and from time to time (Arega *et al.*, 2006). The overall national average loss due to CBD is estimated to range from 24 to 30% and the loss may reach 100% during favourable season in some areas of Ethiopia (Van der Graaff 1981; Biratu 1995; Derso *et al*., 2000; Derso & Waller 2003; Adugna *et al*., 2009).

Various methods of CBD controlling methods are practicing at farmer's and large scale production farm. Some of them are, agronomic practices such as; pruning, striping off diseased berries, removing old stems, thinning out branches and shading are important cultural practices for the control of coffee diseases. In addition, biological control treatments on green coffee berries with *Epicocum nigrum* achieved good control of CBD when compared to leaves treated with copper-based fungicides (Koomen and Jeffries, 1993). For Arabica, studies carried out in Kenya by Van der Vossen and Walyaro (1980) concluded that coffee resistance to CBD appears to be controlled by major genes on three different loci. Fungicides such as Daconil and Delan were considered as promising against *Colletotrichum kahawae*. Later, however, such products including Dyrene, Octave, Mancozeb and Maneb were promising chemicals (Eshetu *et al.*, 2000; Gertrude and Gichiru, 2014).

Members of the microbiota on coffee plants have been tested against *C. kahawae* and a significant number of them showed strong antagonism (Gichuru, 2005; Rutherford and Phiri, 2006). Biratu (1995) has indicated the existence of competitive and antagonistic microorganisms on the coffee phyllorsphere that could play a vital role in limiting CBD development. Nevertheless, their reduction by fungicidal sprays could lead usually to severe disease outbreaks as indicated by Masaba (1991). Eventually, the use of fungicide against CBD can induce negative effects on indigenous antagonistic microorganisms and subsequently may lead to loss of natural biological control mechanisms (Masaba, 1991). These problems make it

essential to look for alternative strategies that can ensure competitive coffee yields while protecting the health of the environment.

Resistant varieties are by far profitable since they stable cash income for the subsistent farmers. Consequently, growing resistant cultivars are environmentally friendly and can realize organic coffee production (Kumlachew *et al*., 2017). In spite of this fact, 90% of the coffee productions under farmers' field in major coffee growing areas of Ethiopia are local landraces that remain susceptible to coffee berry disease. Thus, effective biological control agents offer great potential to develop supplementary methods that are economical and suited for adoption by the small coffee industries (Pal and Gardener, 2006).

Despite, the fact that antagonistic microorganisms have potentials in disease management, very few records are available regarding biocontrol of coffee berry disease in Ethiopia (Biratu, 1995; Adugna *et al*., 2009a; Kumlachew *et al*., 2017). Some workers have reported successful management of plant disease and increases yield in various horticultural crops including coffee with the application of Plant Growth-Promoting Rhizobacteria (PGPR) (Diriba *et al.,* 2009; Dinesh, 2011; Melkamu *et al.,* 2013; Kumlachew *et al*., 2017). In addition, it has tremendous values in environmental friendly in modern agriculture (Weller, 1988).

The rhizobacteria that can serve such role may be present (1) in the soil surrounding roots, utilizing the metabolites leaked from roots as the growth nutrients; (2) on the root surface or rhizoplane; (3) in the root tissue, inhibiting apices between cortical cells; and (4) inside the cells in specialized root structures or nodules (Gray and Smith, 2005; Diriba *et al.,* 2009). Thus, based on their root proximity and intimacy of association, PGPR are categorized into two different classes: (1) extracellular PGPR (ePGPR), present in rhizosphere; and (2) intracellular PGPR (iPGPR), that exist inside the cells in specialized nodular structures (Gray and Smith, 2005).

The Rhizobacteria are known to induce the systemic resistance (ISR) in plants and restrict the establishment of infection by the pathogens in the host (Van Peer *et al*., 1991; Wei *et al*., 1991). For instance, *Pseudomonas spp*. can protect plants from pathogens through various mechanisms, such as induced systemic resistance in the host (Van Peer *et al*., 1991; Maurhofer

et al., 1994), antibiotic production (Thomashaw and Weller, 1988; Maurhofer *et al*., 1995), growth promotion (Schippers *et al*., 1987) and competition for nutrients (Duijff *et al*., 1993; Leeman *et al*., 1996). In addition, several strains of *P. fluorescens, P. cepacia,* and *P. aeruginosa* have been used for the biological control of various plant diseases in a wide range of horticultural crops (Weller, 1988; Chandel *et al*., 2010).

Multiple species of *Bacillus* and *Paenibacillus* are known to promote plant growth as well. The principal mechanisms of growth promotion include, solubilization and mobilization of phosphate, sidophore production, induce systemic resistance and antibiosis (Gutierrez Manero *et al.,* 2001; Whipps, 2001; Idris *et al*., 2007; Richardson *et al*., 2009).

However, the study regarding the role of PGPR symbiosis in relation to control or antagonistic effect of fungus diseases causes yield loss in coffee Arabica is infant stage. Furthermore, the knowledge regarding the biological suppression of *Colletotrichum kahawae* by application of plant growth promoting rhizobacteria in Ethiopia is very limited. Therefore, present study was initiated with the following objectives:

General objective:

- ✓ To isolate, characterize and test rhizobacteria activities against CBD (*Colletotrichum kahawae)*

Specific objectives:

- ➢ To isolate rhizobacteria associated with Arabica coffee root rhizosphere
- ➢ To identify rhizobacteria associated with Arabica coffee root rhizosphere,
- ➢ To test the antagonistic potentials of characterized rhizobacteria to *Colletotrichum kahawae* in the laboratory condition.

2 LITERATURE REVIEW

2.1 Production status of coffee

Coffee ranks as one of the world's most valuable and widely traded commodity crops. It belongs to the family Rubiaceae and genus Coffea, which include well over 500 genera and 600 species (Wrigley, 1988 sited in Kumlachew *et al.*, 2017). The Coffea plant is native to subtropical Africa and some islands in southern Asia (Wrigley, 1988 sited in Kumlachew *et al.*, 2017). The plant was exported from Africa to countries around the world and coffee plants are now cultivated in over 70 countries, primarily in the equatorial regions of the Americas, Southeast Asia, India, and Africa (ITC, 2017). At present, the two most grown and of real economic importance species are *C. arabica* referred to in the trade as Arabica and accounting for 60% - 70% of world production and *C. canephora piere* ex Froehne called Robusta in the trade and making up 30% - 40% of world production (ITC, 2017). Ethiopia was fifth largest coffee producing countries in 2018/2019 with metric ton after Brazil (2,598,000), Vietnam (2,000,000), Colombia (810,000), Indonesia (700,000) and Ethiopia (4.000.000) (Ryan, 2019).

Coffee is the most important crop in the national economy of Ethiopia and remains the leading export commodity. The total area coverage of coffee land in the country is 1.2 million hectares of which 900,000 hectares of land is estimated to be productive CSA 2003/4-2017/18. According to CSA 2003/4-2017/18 about 92-95% of coffee is produced by 4.7 million small scale farmers and 5-8 % large scale plantations. An annual coffee production in the country is 500,000-700,000 tones and an average national productivity is below 7 quintals per hectare (CSA 2003/4-2017/18). Coffee production system in Ethiopia is forest, semi forest, garden and plantation production methods. Coffee produced by small scale farmers at garden accounts for 70%, 25% is collected in forest and semi forest coffee systems, and merely 5% is plantation coffee (Girma *et al*., 2008). The coffee production of Ethiopia comes from more than 64% Oromia region, 35% from South Nation and Nationalities of People and the remains 1% from Gambella regional states and around 90% based on smallholders (ITC, 2019). Ethiopia is the coffee producing country that consumes around 50% of its production (ITC, 2019).

2.2. Economic importance's of coffee in Ethiopia

Coffee is most important and backbone of Ethiopian economy, which accounts for an average 5% of GDP, 10% of the total agriculture production and 60% of export earnings (Girma, 2011). It is the first major export commodities in the country which contributing 29.5% of the total export in agricultural crops (ITC, 2019). In 2017/18, it has been accounting for trade US$ 866 million, when 221,000 tons were shipped. Also, it was estimated that coffee sector provides job opportunity for about 26 million peoples in 52 producing countries (ITC, 2019).

2.3 Major constraints of coffee production in Ethiopia

Coffee is one of the most important cash crops that have been contributing a lion's share to the country's economy. However, the average coffee productivity in Ethiopia (below 0.7 ton/ha) remains low, compared to the world standard and to other coffee producing countries (CSA, 2016). In Ethiopia over 90% of the coffee is produced by small scale substance farmers (Worako *et al.,* 2008) and is a low input-output crop. It grows under age-old traditional production systems (forest, semi forest and garden) (Kumlachew *et al*., 2017).

Agronomic Practices and crop husbandry remain conventional and the adoption and diffusion of improved technologies, like high yielding cultivars and better pre and postharvest management activities have been slow (Adugna *et al.,* 2009b; Kufa *et al.,* 2011). Coffee disease and Insect pests are also a major limitation to economic coffee production (Kumlachew *et al*., 2017). Among the insect pests the major ones inflicting considerable damage. Antestia alone could cause considerable damage, amounting 9% berry fall and 48% darkened coffee beans (Abebe, 1987). The crop is prone to a number of diseases that attack fruits, leaves, stems and roots and reduce the yield and marketability (Derso and Waller, 2003).

2.4 Major Coffee diseases in Ethiopia

The major coffee disease in Ethiopia are Coffee berry disease caused by fungus (*Calletotricum kawahee),* Coffee wild disease (CWD) caused by fungus (*Gibberella xylarioides*) and coffee leaf rust (*Hemileia vastatrix*) (Girma *et al.,* 2008). Of these, CBD is by far the most economically important disease of coffee causing yield loss up to 100% (Derso and Waller, 2003; Adugna, 2004).

Yield losses caused by coffee diseases remain among the major constraints to increased production in many parts of Ethiopia, where several diseases have been recorded. Following the advent of CBD and modernization of the crop production system leads to the replanting of limited number of CBD resistant cultivars, which brought deforestation and rehabilitation of diverse coffee population. Side by side, modern cultural practices are widely employed (Van der Graaff, 1981). Coffee wilt disease or tracheomycosis caused by *Fusarium xylarioides* Steyaert (teleomorph: *Gibberella xylarioides* Heim and Saccas) is becoming important in some regions of Eastern, Central and West Africa, not only in Robusta but also in Arabica coffee (Girma *et al.*, 2001; Geiser *et al.*, 2005; Silva *et al*., 2006). The diseases severely attack the vascular system of the plant, causing wilting and eventually die-back (Pieters and Van der Graaff *et al*., 1980). Coffee vascular disease was documented in Ethiopia for the first time in the 1970s (Girma *et al.*, 2001) and in 1993 in Uganda, where it is causing significant yield losses in Robusta coffee (Geiser *et al.*, 2005).

In major Arabica coffee growing areas of southwestern Ethiopia, including Bebeka, Teppi, Jimma and Gera, the incidence of *Fusarium* (*Gibberella*) *xylarioides* is reported to be 60 % and the fungus is causing significant yield losses due to very severe damage and death of millions of trees (Girma *et al.*, 2001). Leaf rust (*Hemileia vastatrix*) was reported by Sylvian (1958). CBD *(Colletotrichum kahawae)*, Coffee leaf rust (*Hemileia vastatrix*), CWD (*Gibberella xylarioides*), bean discoloration (*Pseudomonas syringae*), leaf blight (*Ascochyta tarda*), brown-eyespot *(Cercospora coffeicola*), fruit-rot (*Fusarium* spp.), and thread-blight (*Corticium kolleorega*) were associated with coffee in Ethiopia (Merdassa, 1985; Eshetu *et al*., 2000).

2.4.1. Historical development of CBD

Coffee Berry Disease (CBD) was first detected in 1922 in Kenya around Mt. Elgon, west of the Rift Valley (Mc Donald,1926; Waller *et al*., 2007). The disease is specific for green berries and is caused by *Colletotrichum kahawae* (*Colletotrichum coffeanum*), (Hindorf, 1975; Agrios, 2004). By the 1950s CBD had established in the east, the main coffee growing areas (Rodriguez *et al*., 1992). Apparently, the free movement of coffee plant materials from CBD infected areas has been the main factor in distribution of this disease throughout all important

Arabica growing areas in Africa. The disease was reported in Angola around 1930, Zaire in 1937, Cameroon 1955-1957, Uganda in 1959, Tanzania in 1964, Ethiopia 1971(Van der Graaff, 1981) and in Malawi in 1985 (Lutzeyer *et al*., 1993).

CBD was also confirmed in Malawi, Zimbabwe and Zambia in 1985 (Masaba and Waller, 1992). It is not known outside of Africa, although a leaf spot and ripe berry anthracnose caused by related *Colletotrichum* species has been reported from Guatemala and Brazil (Griffiths *et al*., 1991). Then spread to all major coffee producing regions within very short period except to the lower altitude. Big plantations, garden and forest coffee, with and without shade all were infested alike (Tefestwold, 1995). So environmental issues except low altitudes did not make much difference. Merdassa (1985) reported yield losses of 51% at Melko and 81% at Wondo Genet due to CBD.

2.4.1.1 Importance of Coffee Berry Disease

CBD is the major factor threatening Arabica Coffee production in Ethiopia, Kenya, Tanzania, and other African countries. Since the disease affects the harvestable berries, it causes direct yield loss, but has no influence on the vegetative vigor production potential of the plant. In Ethiopia average national loss due to CBD was estimated to be between 24-30% (Eshetu, 1997), losses on individual farms may reach up to 100% during favorable seasons in some areas where altitude and rainfall areas are high (Girma *et al.,* 2008). Holger and Chrispine, 2010 reported that CBD was present mostly in Bonga (40.0%) and Yayu (26.3%), but less frequent in Harenna (18.6%) and Berhane-Kontir (6.0%).

In 1987, the disease spread to almost all the coffee growing regions. In Kenya average yield loss due to CBD was estimated at not less than 30% and reaching 50 to 80% in years of severe fungicide spray programmed (Van der Vossen, 1981). CBD is also severe in Tanzania (31 to 68%), Uganda (35 to 50%) and Cameroon (up to 80% losses) (Van der Vossen, 1981). The importance of CBD can also be judged from the cost incurred to control the disease using chemicals. In Ethiopia, it was estimated that on a national basis, spraying of all coffee farms costs about 150 million birr (US $30 million) (Robinson, 1973). In Kenya the cost of chemical control is estimated to be 30- 35% of total production costs losses (Van der Vossen, 1981).

2.4.1.2 Taxonomy of Colletotrichum Pathogen

Taxonomy of genus *Colletotrichum* classified it to *Eumycota* to the major sub division of *Deutronmycoxin*a, class *Coelomycetes*, order *Melancolianles* and the single family *Melanconiaceae* (Sutton, 1980; Farr *et al*., 1989; Agrios, 2005). The Genus has also been recorded worldwide both as causing pre-harvest and post-harvest crop loss (Jeffries *et al*., 1990). Representatives of the genus *Colletotrichum* are ubiquitous and often criminal causing a variety of disease symptoms, commonly known as anthracnose on fruits, leaves and stems, die-back on branches, root rot, leaf spot, and blossom rot.

The most aggressive species causing CBD are present only in east and central Africa. Gassert (1979) studied occurrence of CBD pathogen in different geographical regions. *Colletotrichum* can be recovered easily from all coffee tissues and have been reported in all coffee growing areas. *C. kahawae* is the only species which is pathogenic to green coffee berries, which colonizes berries of all stages, leaves and maturing bark of the branches (Tefestewold, 1995). Based on analysis of cultural, morphological, biochemical and physiological characteristics, it has been distinguished from other *Colletotrichum* spp. as CBD causing pathogen, associated with Arabica coffee in Ethiopia (Gabisa, 2016).

2.4.1.3 Disease Cycle

The disease cycle of CBD has been reviewed by Vander de Graff (1981; 1992). The CBD pathogen are over winters as conidia on the yellowish to browning bark of the coffee tree, on mummified berries of the previous year and on leaf flecks. The conidia are water borne and require the presence of liquid water or 100% relative humidity as well as an optimal temperature of about 22ºC for germination. Under optimal conditions, the time between infection and lesion development (incubation period) takes two to three or four weeks (Vander de Graff, 1992).

According to Waller *et al*., 1993 the conidia are born directly on hyphae with no acervuli conidia. When humidity is high, conidia are formed massively and a pink layer of conidia covers the lesion. If conditions are adverse (no rain and no favorable humidity and temperature), growth is arrested and cork cambium is formed that seals off the lesion. Such

lesions turn brown and are called 'scab' lesions. Heavily diseased berries are often shed, but some remain throughout the year on the tree if not interfered with by coffee pickers, becoming potential source of inoculums for the next season crop. The development of CBD infection varies through the stages of berry development (Mulling 1970; Muller, 1984 and Van der Graff, 1981, 1992).

2.4.2. Epidemiology of Coffee Berry Disease

2.4.2.1 Climatic Condition and Disease Development

The occurrence and intensity of CBD varies from place to place and from one season to the other, depending largely on host susceptibility, pathogen aggressiveness and favorable weather conditions. The disease is very severe and causes appreciable yield loss in areas where the temperature is relatively low and relative humidity is high, mainly in the rainy seasons (Girma, 1995). Generally, high rainfall, air humidity or wetness and relatively low temperature that persist for long periods favors CBD development and the disease is severe at height altitudes where these condition generally prevail (Cook, 1975).

Severe CBD incidence could also occur in valley bottoms at lower altitude where high humidity and heavy dews are common (Cook, 1975; Vander Graff, 1981). In Ethiopia, CBD development and severity is high above 1750m, moderate between 1500 and 1750 m, while below 1500 m CBD is not resistant. In Jimma where the severity is moderate, the altitude is 1753 m and the respective monthly average temperatures are about 11°c, with 70% relative and a total annual rainfall of 1590 mm (Girma *et al*., 2008).

The first symptoms of CBD are dark brown blotches or streaks on the white tissue that may develop and scab-lesions. Active lesions are characterized by small dark brown slightly sunken patches of lesions/spots, which gradually expand and eventually cover the whole berry, causing rotting of the pulp and the beans side. On the surface of the lesion, pinkish masses of conidia appear. During the final stages, berries which are still attached to the branches, appear as small, black, brittle shells which can easily be crushed between the fingers, the majority of diseased berries drop off, but small proportion remain on the as empty black 'mummies'. Scab lesions are light-tan colored spots which are not sunken, and on them small darker acervuli are formed.

They occur as a result of unfavorable weather condition for disease development and are not serious unless located close to the peduncle, when they might cause the fruit to fall off (Coste, 1992).

Earlier studied lesion formations using detached berries from resistant cultivar and ripe berries are seen as dark sunken patches, which spread vey reaction. A late attack on ripe berries is seen as dark sunken paths, which spread very rapidly and may cover the whole berry. This condition is known as 'Brown blight' of ripe berries. At this stage, the lesion has no impact on yield as the beans are not affected (Wrighley, 1988).

2.4.3 Management of CBD

Various methods of CBD managements were practiced in different coffee cultivating countries.

2.4.3.1 Cultural Control

Cultural practices are a variety of management techniques which may be manipulated by agricultural producers to attain their crop production goals (Bedimo *et al*., 2007). The term may also be used to mean the manipulation of the environment to improve crop production while cultural control is the deliberate alteration of the production system, either the cropping system itself or specific crop production practices, to reduce pest populations or avoid pest injury to crops (Bedimo *et al*., 2007). Cultural practices that can be applied to manage coffee diseases and thereby promote optimum production in small holder farms include mixed cropping with fruit trees, intercropping with food crops and maintenance pruning of coffee trees (Adejumo, 2005). An epidemiological study of CBD carried out in Cameroon by (Adejumo, 2005), showed that the infection rate was significantly lower on coffee trees grown intensively than on coffee trees grown in the traditional manner. Coffee trees located under the shade of fruit trees were significantly less infected than those located in full sunlight (Gertirude, 2014).

In addition, berries on the leafless parts of branches, near the main trunk of the coffee tree, were less infected than those on leafy sections. These results show that maintenance pruning, removal of mummified berries, and mixed cropping with shade plants are cultural practices which create environmental conditions that limit CBD development (Gertirude, 2014). Pruning and shading is an important cultural practice for the control of CBD and CLR. Shade tends to

alter the microclimate and soil properties in coffee plantations, thus directly or indirectly affecting pest and disease development (Avelino, 2010). However, growing coffee under shade may cause an increase or decrease in coffee diseases as the microclimate alterations may be favorable to one species or stage of development and unfavorable to another species or developmental stage (Avelino, 2010).

2.4.3.2 Chemical Control

Timely application of fungicides can provide adequate controls, but it is usually beyond the financial means of small scale farmers who are the main coffee producers in most countries Gichimu and Omondi (2010). The success of chemical control depends on the disease pathogenesis stage at the moment when the fungicide is applied (Ricardo, 2010). The application of a fungicide prior to contact between pathogen and host is considered to be preventative while application after inoculation and just before initial symptoms is curative. Curative fungicides are active against pathogens that have already infected the plant but they tend to have a higher risk of pathogens developing resistance to the fungicide (Bridge *et al*., 2008).

2.4.3.2.1. Organic fungicides

The use of these fungicides began in 1934 and has since played a major role in the world wide control of plant diseases (Masaba *et al*., 1992). They are more effective and less toxic than inorganic ones. The first molecules in this group included carbamates which have been the most important, versatile, and widely used fungicides under trade names such as Mancozeb and Maneb (Gertrude and Gichiru, 2014). They were followed by Dicarboximides such as Captan, Folpet and Captafol which are wide-spectrum fungicides (Masaba *et al*., 1992). Later, new molecules were introduced; Iprodione, Vinclozolin and Chlorothalonil; which have a narrow spectrum and may be prone to development of resistance by the target fungi.

Studies showed that the use of Chlorothalonil as a straight spray to control CBD caused an upsurge of BBC, and it was therefore recommended to be used as a tank mix with copper and Maneb to control both CBD and BBC Melaku and Samuel (2000). (Lutzeyer *et al*., 1993), (Workafes and K. 2000), found out that certain strain of *Colletotrichum coffeanum* (now *C.*

kahawae) in coffee plots sprayed with Carbendazim formulations (Bavistin and Derosal) and with Folicidin (Cypendazol) were resistant to these fungicides as well as Benlate (Benomyl) (Workafes, 2000). The isolates retained the resistance even after stoppage of usage of the fungicides and this led to withdrawal of the molecules.

2.4.3.2.2. Systemic fungicides

These are the most recently developed fungicides as well as the most promising for the future. Those used in coffee to manage CBD include Benzimidazoles (Thiabendazole), sterol inhibitors (Triadimefon, Cyproconazole, Hexaconazole and Propiconazole) and the most recently introduced Strobulins (Trifoxystrobin, Pyraclostrobin and Azoxystrobin). These fungicides are absorbed by the plant and translocated to various parts of the plant. Most of them are eradicative. However, because of their site specific mode of action, the pathogen may readily develop resistance if they are not properly managed (Masaba *et al*., 1992). To reduce the risk of resistance development it is recommended that they are used as mixtures with coppers, used as single spray in one season or alternated with other fungicide formulations (Hidorf, 1970). Some of them are premixed by the manufacturers such as Quadris Opti (Azoxystrobin and Chlorothalonil). In view of the changing climate characterized by off season rains and continuous flowering, these compounds offer the most desired curative action to control off season infections.

In Ethiopia spraying fungicides has limited application since yields are much lower the trees are irregularly spaced and unpruned (Wrigley, 1988). Contributory factionary for less CBD springing in Ethiopia are also the imbalance between fungicide costs and coffee prices. Six fungicides, namely, Daconil '2787' 75% WP, Daconil 75% WDG, Shirlan 50% SC, Nordox 50% WP, Octave super 50% WP and a tank mixture of Daconil '2787' and Nordox are recommended to control CBD in Ethiopia (Eshetu *et al.*, 2000).

2.4.3.3. Resistant variety

The genetic resistance in coffee appeared complete in *C. canephora* and partial in *C. arabica*. The major genes for CBD resistance are conferred to Rume Sudan (R and k genes), Hibrido de Timor (Ck-1 or T gene) and K7 (k gene) (Hein *et al*., 2006). Currently, coffee breeding is

focusing on developing true to type varieties with a target of selecting *C. arabica* L. varieties that have both resistances to major coffee diseases, improved yields and quality (Gertrude and Gichiru, 2014). Five lines, CBU0822/97, CBU2222/97, CBU2322/97, CBU2722/97 and CBU3022/97 were selected for release as commercial cultivars. Subsequently, three of the lines were released as Batian 1, Batian 2 and Batian 3. Their unique features include tall stature, true breeding and resistance to CBD. They are also high yielding with good bean and liquor quality (Tefestewold, 1989). Coffee breeding has been enriched by the introduction of molecular markers and genetic engineering techniques, based on the knowledge of genome, genes structure and function (Gertrude and Gichiru, 2014). Advances in cellular, developmental and molecular genetics, combined with traditional breeding, can target and achieve improvements in specific agronomic, processing and quality traits (Van der Graaff, 1981). Coffee is a major agricultural product, but it has only benefited from technological developments at the cellular and molecular levels in the recent times (Van der Graaff, 1981).

The genetic variability within Arabica coffee populations, in the Southwestern highlands of Ethiopia, is extremely high (Meyer, 1965; Sylvian, 1958; Melaku Werede, 1984; Bayetta Belachew, 2001). Resistance in 741 of the cultivars could be considered as horizontal or non-race specific, whereas 74110 and 744 showed susceptible and mixed (susceptible for some and resistant for others) reactions, respectively (Tefestewold, 1995). 74110 is one of the released resistant cultivars and still in production (Bayetta *et al*., 2000). Arega, (2006) reported that since the inception of CBD resistant selection and breeding program at Jimma Agricultural Research center, 19 CBD resistant cultivars were released to growers, based on regular field observations on the farm, 6 of the cultivars were withdrawn from production from time to time due to their manifestation of either high CBD, rust or wilt diseases and/or low yield. Tefestewold (1997) reported that seeds of CBD resistant coffee selections in Ethiopia and cultivars in Kenya had higher caffeine content than the susceptible ones.

2.4.3.4 Biological control

Biological control (Biocontrol) is the use of living organisms to control disease causing organisms Van der Vossen, *et al*., 1980). Although not highly applied in the coffee industry, it offers a safe and sustainable disease control strategy. There has been a shift in the control of

plant diseases from the regular use of pesticides to alternative and more ecofriendly bio pesticides and plant-based products over the recent years Van der Vossen, *et al*., 1980). Many fungi and bacteria have the potential to act as biological control agents.

Baker and Cook (1974) defined the biocontrol from a plant pathologist perspective as "the reduction of inoculum density by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists." What is so fascinating about biocontrol was brought to light with this concept is the interaction between the host, the pathogen(s), antagonist(s) and the environment. The biocontrol of plant pathogens with bacteria dates back to 1927 when Millard and Taylor used non-pathogenic isolates of *Streptomyces* spp. for the control of common scab of potato (Baker and Cook, 1974). Biological control has also been widely used for the control of plant pathogens and insects (Gnanamanickam *et al*., 2002).

Waller (1988) discusses three possible explanations to the sometimes-erratic results observed by the biocontrol of rhizobacteria. First, loss of ecological competence in which the bacteria fails to acclimatize to the environment. Second, target pathogen absence or non-target interference which suggests that if the pathogen is absent the growth differential will not be observed because no disease was controlled and third, variable root colonization by bacteria denotes that the bacteria fail to establish and reach high populations. The persistence of the bacteria in the environment is an essential factor for biocontrol abilities. However, this colonization might be ignored when disease is not developed.

2.4.3.5. Integrated Management of Coffee Berry Disease

Integrated disease management is a broad ecological approach to control disease in a compatible manner (Kumlachew *et al*., 2017). It advocates control of the diseases through the combination of several control practice without depending on heavily toxic chemicals. The integration of a number of practices with the aim of reducing or eliminating negative side effects caused by chemicals used for controlling major coffee diseases is the most realistic option for solving the problem (Kumlachew *et al*., 2017). Measures to enables adequate ariation of the coffee canopy such as pruning, shade control and adequate spacing will reduce

humidity and wetness duration of berry surfaces and, to some extent hinder the pathogen. This also enable better penetration and coverage of fungicide if this are used (Kumlachew *et al*., 2017). The capping of the taller stems of multiple steamed coffee can also increase the incidence of CBD, at these provide measure source of inoculum for the crop (Waller *et al.,* 2007).

2.5 Plant Growth Promoting Rhizobacteria (PGPR)

PGPR are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and/or indirectly (Joseph *et al*., 2007). Micro-organisms naturally occurring on the surface of coffee trees can be used as biological control agents of coffee berry disease (Gertrude and Gichiru, 2014). A number of bacterial species associated with plant rhizosphere belong to genera *Pseudomonas* spp., *Acinatobacter* spp., *Flavobacterium* spp., *Azospirillum* spp., *Azotobacter* spp., *Klebsiella* spp., *Enterobacter* spp., *Alcaligenes* spp., *Arthrobacter* spp., *Burkholderia* spp., *Bacillus* spp., *Brevibacillus* spp., *Serratia* spp., *Micrococcus* spp. *and Staphylococcus* species (Kloepper *et al*., 1989; Okon and Labandera-Gonzalez, 1994; Glick, 1995; Parvin *et al*., 2011; Rodriguez and Fraga, 1999; Sturz and Nowak, 2000; Bloemberg and Lugtenberg, 2001; Roldan *et al.*, 2009).

Plant growth promotion by PGPR may be an indirect mechanism of biological control, leading to disease escape when the growth promotion results in shortening the time that a plant is in a susceptible state, e.g. in the case where PGPR cause enhanced seedling emergence rate, thereby reducing the susceptible time for pre-emergence damping-off. Hence, PGPR-mediated biocontrol can be extended to foliar and systemic diseases, even when the PGPR are applied only to seeds and roots, if the mechanism for control involves induction of host defenses.

Most popular bacteria studied and exploited as biocontrol agents include the species of *Pseudomonas* and *Bacillus*. Thomashow and Weller (1990), sited in Yamaoka *et al*. (1995) shown that strains of *Bacillus* sp. and *Pseudomonas* sp. have been effective in reducing diseases in field trials. The indigenous strains of *Bacillus subtilis* and *Pseudomonas fluorescens* appear to function as better antagonists in disease control because they are well adapted to local

conditions (Waller et al., 1993). Species of both *Pseudomonas* and *Bacillus* are effective biocontrol agents of several rusts including coffee rust under controlled conditions (Waller et al., 1993). The mixtures of PGPR can enhance disease protection and improve the consistency of the biological control (Raupach and Kloepper, 1998).

2.6 Mechanisms of Biocontrol

The modes of action of beneficial microorganisms can be based on either a direct or an indirect antagonism. However, both mechanisms are not mutually exclusive as they have been frequently described as co-occurring within the activity of the same biological control agent (BCA) (Castoria and Wright, 2009). Direct antagonism results from physical contact and/or from a high degree of selectivity of the mechanism(s) expressed by the BCA(s), in relation to the pathogen, i.e., parasitism and predation, production of antibiotics, and signal interference. In contrast, indirect antagonism results from activities that do not involve sensing or targeting of a pathogen by the BCA(s), i.e., competition for nutrients and niches, production of siderophores, and induced systemic resistance (ISR) (Castoria and Wright, 2009).

2.6.1 Antibiosis

Antibiotics produced by microorganisms have been shown to be particularly effective in suppressing plant pathogens and diseases. Most biocontrol strains of *Pseudomonas* spp. with a proven effect in plant bioassays produce one or several antibiotic compounds, e.g. *P. fluorescens* strains CHAO (Laville *et al*., 1998; Haas *et al*., 2000) and Pf-5 (Thompson *et al*., 1999) produce complex cocktails of these secondary metabolites. In *in vitro*, these antibiotics inhibit the growth of the fungal pathogens, and for this reason, strains acting through antibiosis are usually identified by screening them for antagonistic activity on plates on which the target pathogen is also inoculated (Lugtenberg and Bloemberg, 2004).

Well characterized antibiotics with biocontrol properties include phenazines (Phz), phloroglucinols (Phl), pyoluteorin, pyrrolnitrin, hydrogen cyanide (HCN), cyclic lipopeptides (Perneel *et al.,* 2008; Keel *et al.,*1992; Thomas and Weller, 1988; Haas and Keel, 2003; Raaijmakers *et al*., 2006), 2-hydroxymethyl-chroman-4-one (Kang *et al.,* 2004), D-gluconic acid (Kaur *et al*., 2006), and 2-hexyl-5-propyl resorcinol (HPR) (Cazorla *et al.,* 2006).

Phenazines are analogs to flavin coenzymes, inhibiting electron transport (Ran *et al*., 2003). The best known Phl compound 2-4-Diacetylphloroglucinol, causes membrane damage to *Pythium* spp. and is particularly inhibitory to zoospores of this oomycete (De Souza *et al*., 2003).

Gleeson *et al*., (2010) provided evidence that Phl acts through impairing the function of mitochondria. Dikin *et al*., (2007) reported that pyrrolnitrin causes the loss of mitochondrial activity in the fungal cytoplasm, inhibiting succinate oxidase and NADH cytochrome reductase. Pyrrolnitrin also interferes with cellular processes such as oxidative stress, blockage of electron transport as well as inhibition of DNA and RNA synthesis (Dikin *et al*., 2007). The cyanide ion derived from HCN is a potent inhibitor of many metalloenzymes, especially copper containing cytochrome oxidases (Blumer and Haas, 2000). Finally, cyclic lipopeptides have surfactant properties and are able to insert themselves into membranes and perturb their function, resulting in broad antibacterial and antifungal activities (Haas and De´fago, 2005; Perneel *et al*., 2008).

Recently, Mazzola *et al*. (2009) have shown that in the wheat rhizosphere, the cyclic lipopeptides viscosin and massetolide not only protect the plant against fungi, but also against protozoan predation. In fact, the protozoa *Naegleria Americana* de represses the synthesis of these antibiotics. The synthesis of antifungal metabolites (AFMs) are extremely sensitive to environmental conditions in the rhizosphere, such as soil mineral content, oxygen tension, osmotic conditions, carbon sources, as well as fungal, bacteria, and plant metabolites can all influence the expression of secondary metabolites (Haas and Keel, 2003; Lugtenberg and Bloemberg, 2004; Duffy and De´fago, 1999; Van Rij *et al*., 2005).

2.6.2 Predation and Parasitism

Different microbes can produce lytic enzymes which can result in biocontrol abilities (Markowich and Kononova, 2003). Beta-1, 3-glucanase donates considerably to the biocontrol activities of *Lysobacter enzymogenes* strain C3 against Bipolaris leaf spot caused by *Phytium* spp. (Palumbo *et al*., 2005). Howell *et al*., (1988) reported that *Enterobacter cloacae* were able to produce volatile compounds like ammonia that are involved in the suppression of *Pythium*

ultimum induced damping-off of cotton. Chitinase of *Serratia marcescens* is also involved in the biocontrol of *Sclerotium rolfssi* (Ordentlich *et al*., 1998).

2.6.3. Competition for Nutrients and Niches (CNN)

Microorganisms must effectively compete for the available nutrients to successfully colonize the rhizosphere. CNN between pathogens and beneficial has been shown to be important for limiting the incidence and severity of disease (Kamilova *et al*., 2005). Enrichment for enhanced competitive tomato root tip colonizers was used to select bacteria, not producing antibiotics, which control tomato foot and root rot (TFRR), a disease caused by *Fusarium oxysporum* f. by CNN (Kamilova *et al*., 2005). These authors reported that two *Pseudomonas* spp. strains, *P. alcaligenes* AVO110 and *P. alcaligenes* AVO73, were selected for their efficient colonization abilities.

However, only AVO110 demonstrated significant protection against avocado white root rot. Further analysis revealed that both strains colonize different sites on the root: biocontrol strain AVO110 was observed to colonize the root at preferential penetration sites for *R. necatrix* infection (intercellular crevices between neighboring plant root epidermal cells and root wounds) while *P. alcaligenes* AVO73 was predominantly found forming dispersed micro colonies over the root surface and in the proximity of lateral roots, areas not colonized by this pathogen (Pliego *et al*., 2008). These results strongly suggest that biocontrol bacteria acting through CNN must efficiently colonize the same mini-niche as the pathogen. The competition for ferric iron ions is well documented example of competition of biocontrol bacteria with pathogenic fungi for nutrients (Leong, 1986; Lugtenberg and Bloemberg, 2004).

2.6.4 Induced Systemic Resistance (ISR)

Various PGPR have been identified as potential ISR elicitors, for their ability to induce resistance in plants toward pathogenic fungi, bacteria, and viruses (Van Loon *et al*., 1998; Van Loon, 2007). The inducing rhizobacteria activated a reaction in the plant roots that gave rise to a signal that spread systemically throughout the plant and enhanced the defensive capacity of distant tissues to subsequent infection by the pathogen (Van Loon, 2000). ISR is dissimilar from systemic acquired resistance (SAR) in several key physiological and biochemical

phenotypes that are best defined in *A. thaliana* (Van Wees *et al*., 1997). Studies with *A. thaliana* mutants indicated that the jasmonate/ethylene-inducible defense pathway is important for ISR.

Many bacterial determinants induce ISR. These include flagella, siderophores (pycholin and pyocyanin), salicylic acid (Van Loon, 2007), and cyclic lipopeptides (Ongena *et al*., 2007), *N*-acyl homoserine lactone (AHL) molecules (Shuhegger *et al*., 2006), the bacterial volatile 2, 3-butanediol produced by *Bacillus* spp. (Ryu *et al*., 2003), and antibiotics such as Phl (Lavicoli *et al*., 2003). In several ISR competent strains of *fluorescent pseudomonads*, it has been difficult to identify specific ISR elicitors, possibly because a combination of siderophores, O-antigen, and flagella might account for the ISR effect (Bakker *et al*., 2003). It has also been shown that several *Pseudomonas* spp. are able to induce ISR in a wide range of plants toward different pathogens (Van Loon, 2007). Generalization of the signal transduction pathways that are involved in ISR are further complicated by the fact that an ISR response to a given PGPR depends on the plant species and cultivar. For example, in *Arabidopsis thaliana*, the PGPR strain *P. fluorescens* WCS417r elicited ISR on all ecotypes examined, except ecotypes Wassilewskija (Van Wees *et al*., 1997).

2.6.5 Signal Interference

Gram-negative bacteria have one type of communication system functions via small, diffusible *N*-acyl homoserine lactone (AHL) signal molecules. Such a regulatory system allows bacteria to sense the density of cells of their own kind and to express target genes in relation to their cell density. This cell to cell communication mechanism regulates a variety of physiological processes, including warming, swimming and twitching motilities, production of pathogenicity/virulence factors and rhizosphere colonization (Gray and Garey, 2001; Miller and Bassler, 2001). Several groups of AHL-degradation enzymes have recently been identified in a range of organisms, including bacteria and eukaryotes.

The expression of these enzymes was identified to interfere with the quorum-sensing system of pathogenic bacteria. *E. carotovora* produces and responds to AHL quorum-sensing signals to regulate antibiotic production and expression of virulence genes, whereas *B. thuringiensis*

strains abolished the accumulation of the AHL signal by expression of AHL lactonase, a potent AHL degrading enzyme. In plants, *B. thuringiensis* significantly decreased the incidence of *E. carotovora* infection and symptoms development of potato soft rot caused by the pathogen (Dong *et al*., 2004). The discovery of these enzymes has not only provided promising means to control bacterial infections, but also represents new challenges to investigate their roles in host organisms as well as their potential impacts on ecosystems (Dong *et al.*, 2004).

2.6.6 Competition

From a microbial perspective, soils and living plant surfaces are frequently nutrient limited environments (Pal McSpadden, 2006). To successfully colonize the phytosphere, a microorganism must effectively compete for the available nutrients. On plant surfaces, host-supplied nutrients include exudates, leachates, or senesced tissue. Furthermore, nutrients can be obtained from waste products of other organisms such as insects (e.g. aphid honeydew on leaf surface) and the soil. The most abundant nonpathogenic plant associated microbes are generally thought to protect the plant by rapid colonization and thereby exhausting the limited available substrates so that none are available for pathogens to grow. For example, effective catabolism of nutrients in the sperm sphere has been identified as a mechanism contributing to the suppression of *Pythium ultimum* by *Enterobacter cloacae* (Van Dijk and Nelson, 2000; Kageyama and Nelson, 2003). Blake and Brodie, (1977) reported that delivering of *Pseudomonas* to beet leaves actively compete for amino acids on the leaf surface and inhibited spore germination of *Botrytis cinerea*, *Cladosporium herbarum* and *Phoma betae.*

2.7. Role and success history of rhizobacteria in plant disease management

PGPR are beneficial, naturally occurring micro-organisms, which are environmentally friendly and nontoxic. From an ecological perspective, their application is sustainable (Labuschagne *et al.*, 2010). The use of PGPR offers an attractive way to replace chemical fertilizers, pesticides, and supplements; most of the isolates result in disease control in plants (Saharan and Nehra, 2011), since PGPR exhibited direct and indirect mechanisms as plant growth promoters and biological control agents.

The provision of bioavailable phosphorus for plant uptake, nitrogen fixation for plant use, sequestration of iron for plant by siderophores, production of plant hormones like auxins, cytokinins and gibberellins and lowering plant ethylene levels using ACC deaminase that accumulate during biotic and abiotic stresses are direct mechanism of PGPR (Glick, 1995; Glick *et al.*, 1999; Mayak *et al*., 2004).

Indirect mechanisms of PGPR include production of antibiotics, *viz.* 2,4-Diacetyl phloroglucinol (DAPG), phenazine, pyoluteorin and pyrrolnitrin against pathogenic fungi and bacteria, reduction of iron available to phytopathogens in the rhizosphere, synthesis of fungal cell wall and insect-gut membrane lysing enzymes, chitinase enzyme for hydrolysis of chitin layer of the eggshell of nematode and also competition with detrimental microorganisms for sites on plant roots and induction of systemic resistance against various pathogens and pests in plants (Ramamoorthy *et al*., 2001). Below are presented some genera of rhizobacteria with biological control potential (Table1).

Table 1. Genera of rhizobacteria with biological control potentials of plant diseases

Bacteria	Pathogen	Host	Reference
Bacillus subtilis *	*Hemileia vastatrix*	Coffee	Daivasikamani and Rajanaika (2009)
Burkholderia	Banana fruit pathogen	Banana	De Costa and Erabadupitiya (2004)
*P. fluorescens**	*Hemileia vastatrix*	Coffee	Daivasikamani and Rajanaika (2009)
Bacillus spp	*Piper nigrum*	Piper	Yap Chin, (2012)
*Streptomyces sp.**	*Colletotrichum kahawae*	Coffee	James *et al*., (2010)
AUPB15 (*P. spp)*	*F. stilboide*	Coffee	Diriba *et al*. (2007)
AUBB05 (*B.subtilis)*	*F. stilboide*	Coffee	Diriba *et al*. (2007)
Bacillus	*Sclerotium cepivorum*	Onion	Utkhede and Rahe (1983)
Enterobacter	*Phytophthora cactorum*	Apple	Gupta and Utkhede (1986)
Pseudomonas	*Fusarium oxysporium*	Carnation,	Baker *et al*. (1986)
Alcoligenes	*Fusarium oxysporium*	Carnation	Yen and Schroch (1986)
*Pseudomonas**	*Erwinia carotovora*	Potato	Rhodes and Logan (1986)
*Pseudomonas**	*Gaeumannomyses graminis*	Wheat	Weller and Cook (1986a)
*Pseudomonas**	*Pythium* spp.	Wheat	Weller and Cook (1986b)
*Pseudomonas**	Deleterious microorganisms	Potato	Kloepper *et al*. (1980)
*Pseudomonas**	Deleterious mo	Potato	Schippers *et al*. (1986)
*Bacillus**	*Phytophthora cactorum*	Apple	Gupta and Utkhede (1986)
*Arthrobacteria**	*Fusarium oxysporium*		Sneh (1981)
*Bacillus**	*Gaeumannomyses graminis*	Wheat	Capper and Campbell (1986)

* Significant control was obtained in field trails.

2.8. Drawbacks of rhizobacteria

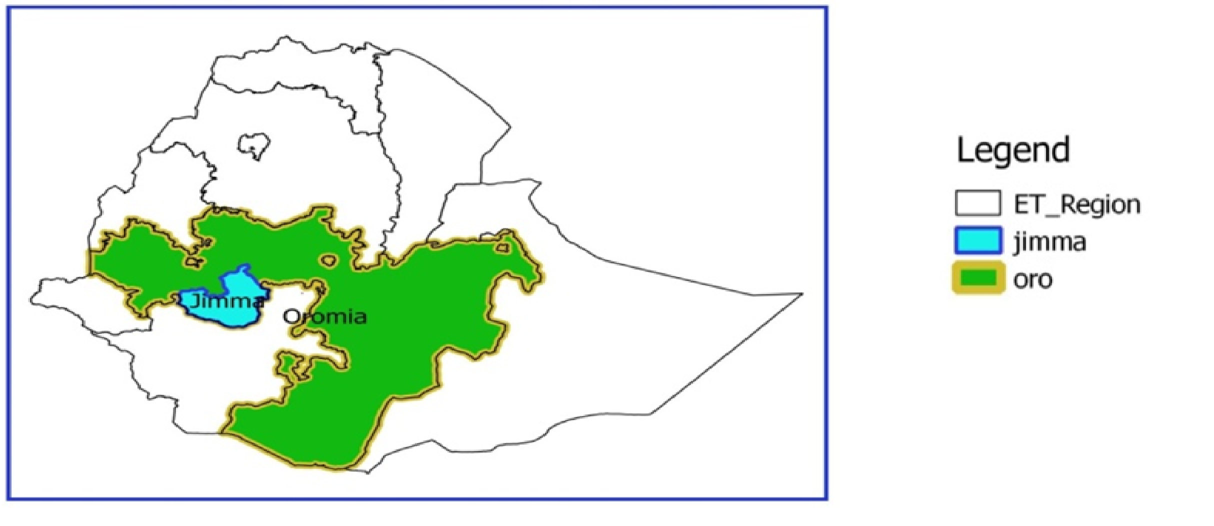
Certain disadvantages of the use of PGPR as biocontrol agents however exist. Since they are live microorganisms, PGPR are more sensitive to environmental conditions such as temperature, soil conditions desiccation, etc., and in addition, the shelf life of the commercial PGPR is shorter than that of the chemical pesticides and fungicides. So far, the efficacy of PGPR and biocontrol agents in general has been inconsistent under field conditions (Labuschegne *et al.,* 2010) and this is the major drawback. Many scientists report effective biocontrol under environmentally controlled conditions *in vitro* or in greenhouses, but the data concerning the efficacy under field conditions remain scarce (Kurabachew and Widra, 2013;

Grobelak *et al*., 2015; Ghosh *et al*., 2015). However, this does not detract from the fact that PGPR as biocontrol agents are constantly becoming more effective as researchers are gaining more knowledge on the factors and mechanisms involved in biological control of plant diseases by means of PGPR and the factors that play a role in the biocontrol of plant diseases (Labuschegne *et al.,* 2010).

3 MATERIALS AND METHODS

3.1 Description of the Study Area

The study was conducted in Plant Pathology laboratory of Jimma University College of Agriculture and Veterinary Medicine (JUCAVM) during 2011/2012. It is located 352 km southwest of Addis Ababa, within the geographic coordinates of 7°, 33′N, and 36°57′ E longitude at an altitude of 1710 m.a.s.l. The mean minimum temperatures are 26.8°C and 11.4°C, respectively and the mean maximum and minimum relative humidity are 91.4% and 39.92%, respectively (NCDC, 2012). The soil samples were taken from Gera, Ela Dale and around JUCAVM campus (Fig1). Gera district is located at 90 km away from the Jimma town in southwestern of the Jimma Zone, Ela dale is under Manna Woreda (Ela Dale-JUCAVM field) is located at 7°42'N and 36°48'E with an altitude of 1710 m. a. s. l and the annual rainfall ranges from 1250 mm in southwest Jimma Zone at around 8 km away from (JUCAVM) (Mulugeta *et al*, 2011). The mean maximum and minimum temperature are 28^0C and 11^0C, respectively. The mean maximum and minimum relative humidity are 91.4% and 39.92% respectively (Mesret, 2012).



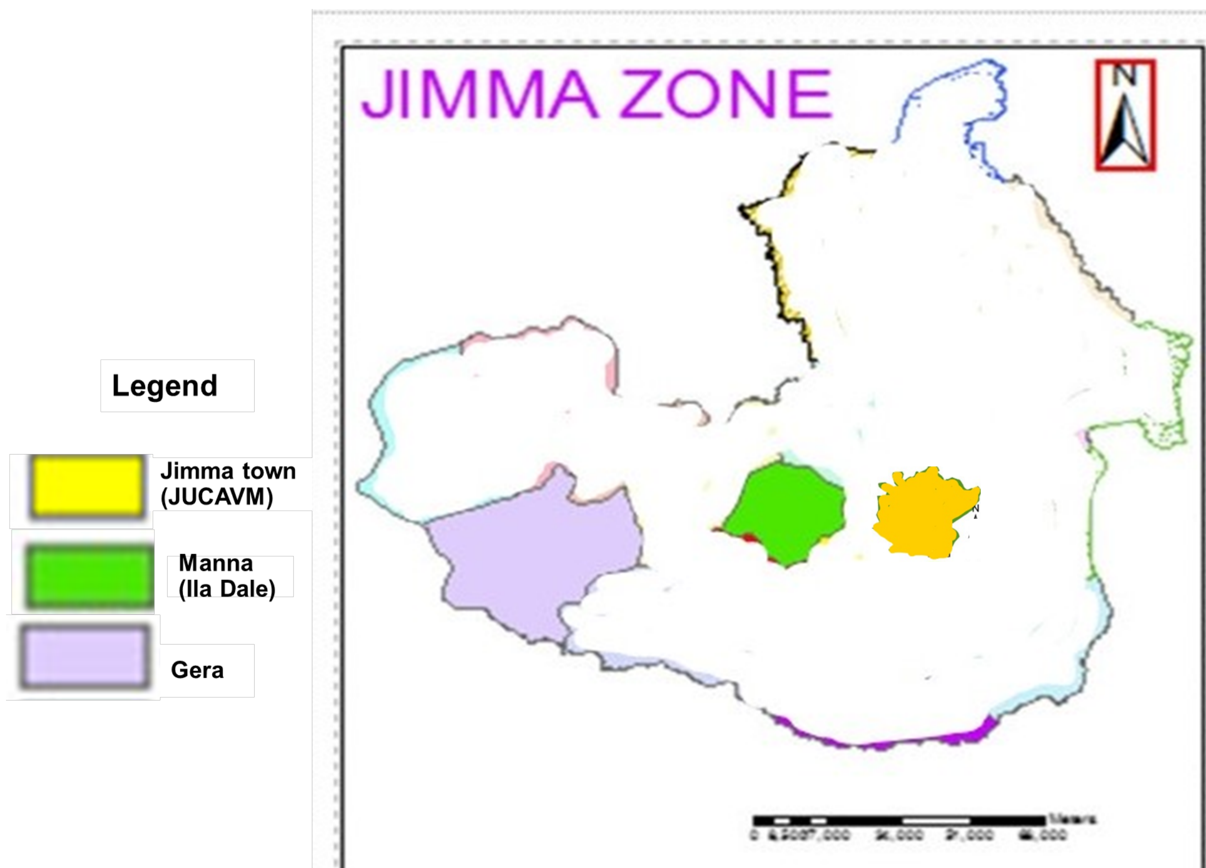


Fig 1: Map of the study area (Jimma town, Ela Dale and Gera), Jimma Ethiopia (2012)

3.2 Methodology

3.2.1 Soil sampling, rhizobacteria isolation and maintenance

The soil samples were collected from Gera, Ela Dale and around JUCAVM campus. Three or four young (2–4 years old) coffee root with stem per sample were randomly uprooted along with a good amount of non-rhizosphere soil and brought intact to the laboratory in sterile plastic bags within two to three days (Diriba *et al.,* 2009). A total of 67 coffee root with stem samples were carefully uprooted and stored at 4^0C prior to further processing. For rhizosphere bacterial isolation, the non-rhizosphere (loosely adhering) soil were removed by vigorous shaking, leaving behind only the rhizosphere soil (strongly adhering to the roots) followed by cutting of the whole roots into segments. Coffee root fragments with strongly adhering soil particles 10g were placed in flasks containing 90 ml sterile saline (0.85% NaCl) solution and shaken for 25 minutes on an orbital shaker at 100 rpm. The subsequent suspensions were serially diluted.

King's B medium was prepared by dissolving commercially formulated dehydrated (powdered) in flask. The 20g Proteose peptone, 10g Glycerol, 1.5g K_2HPO_4, 15g agar was dissolved into 1liter of distilled water (Diriba *et al.,* 2009). The dissolved was boiled and stirred with a magnetic stirrer for 15 minutes for completely mixed with each other. The mixed solution was autoclaved at 121°C for 20 minutes to sterilize the media and maintained under aseptic condition and allowed to cool about 50°C (Diriba *et al.,* 2009). The appropriate serial dilution was spread on King's B (KB) medium for the isolation of Gram-negative rhizobacteria. For the isolation of Gram-positive endospore forming bacteria, the appropriate serial dilutions were heat treated in a water bath at 80^0C for at least 10 minutes, in order to break spore dormancy of the isolates and then plate the suspension on isolation media. Then pour to Petri dishes and incubated for 24h to 48h for further studies (Diriba *et al.,* 2009). Thereafter, for maintenance of isolated rhizobacteria a 0.1ml aliquot was spread onto nutrient agar. Isolates were purified and stored at -70^0C in freezing medium for the subsequent analyses or stored by test tube containing nutrient agar (E. O. King., 1954 sited in Diriba *et al.,* 2009).

3.2.2. Identification and Characterization of Rhizobacteria isolates

3.2.2.1. Morphological Characterization

The colony was observed for colony morphology of rhizobacteria such as gram staining, spore staining, shape, colony characteristics (colony color, form of bacterial colony, elevation and texture) were observed (Kebede *et al*., 2018). The shape of colonies was observed on plates (circular, irregular, filamentous and rhizoid) (Acharya, 2016). The elevation of the rhizobacteria colony was observed by the side view in the media (Flat, raised, convex, con flat) (Acharya, 2016). Based on the procedures of Bergey's manual of determinative bacteriology Holt *et al*. (1994); morphological characterization, motility and gram staining, the isolates were compared with those of standard species (Reddy *et al*., 2000; Yu Zhou *et al*., 2008; Ashrafuzzaman *et al*., 2009; Dastager *et al*., 2010; Seema *et al*., 2011; Talyta *et al*., 2016).

3.2.2.2. Biochemical characterization

3.2.2.2.1. Gram Staining

A smear of the selected strains was prepared on a clean glass slide allowed to air-dry and applied to the heat. The smear was then flooded with crystal violet and after one minute, it was washed with water and again flooded with mordant Gram's iodine. Further, the smear was decolorized with 95 % ethyl alcohol, washed with water and then counter-stained with safranin for 45s. After washed with water, the smear was dried with tissue paper and examined under oil immersion (100 x) [10] Gram +ve were appeared purple and gram –ve were appeared pink (Nanjwade *et al*., 2010).

3.2.2.2.2. KOH test

A few drops of 3% KOH were dropped onto slide. Bacterial colony (24-48 h) were picked with loop; stirred into KOH for 5-10 sec. After pulling up the loop and Gram-negative were show viscous slimes (Sally, 2008).

3.2.2.2.3. Catalase test

Catalase test was performed to study the presence of catalase enzyme in bacterial colonies. Bacterial colonies (24 h old) were taken on glass slides and with one drop of H_2O_2 (30 %)

added. Appearance of gas bubble indicated the presence of catalase enzyme (MacFaddin, 1980). Bubble form was +ve result.

3.2.2.2.4 Oxidase test

For oxidase test, different rhizobacteria isolates were grown on nutrient agar and KB medium plates for 2 days at $28^{\pm}2$°C. Solution of *tetra methyl-p- phenyl-diamine hydrochloride* (1%) was added to the colony surface on the plate or on filter paper (Joseph, 2007). Finally, after 15 seconds the color changes of the colony were examined and change of the colony color to blue /purple were shown oxidase positive.

3.2.2.2.5. Motility Test

For motility test semisolid agar medium were placed in test tube. The isolates were inoculated with straight wire, making a single stab down the center of the tube to about half the depth of the medium. The tubes containing the inoculated isolates were incubated at 37°C. The motility of the bacterial isolates was examined at interval of 6hr, 1 and 2 days depends on germination time of bacteria. Then after, the motility was determined stab line. The non-motile rhizobacteria were give growths that are confused to the stab line, have sharply defined margins and leave the surrounding tube containing medium clearly transparent. For Motile rhizobacteria typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque (Czaban *et al*., 2006).

3.2.2.2.6. Oxidation Fermentation test

Two tubes of medium were boiled in water for 10 minutes to drive off the oxygen, cooled and inoculated by inserting a straight wire vertically. One of the tubes was incubated aerobically, while the second tube was either aerobically incubated or scale the surface with a layer a sterile liquid paraffin oil to create anaerobic conditions. After that the tubes were incubated at 35-37^{0}C for 72 hours. Longer incubation may be required for slowly growing species. Tubes were examined for color change (Snel *et al*., 1999).

3.2.3. Culture Media preparation

Culture of *C. kahawae* was obtained from Jimma Agricultural Research Center (JARC) Jimma, Ethiopia. Potato dextrose agar (PDA) was prepared by dissolving commercially formulated dehydrated (powdered) PDA. The agar was prepared by dissolving 25 g of agar into one liter of distilled water. The Potato dextrose powder were then mixed with sterilized distilled water in a flask at the rate of 39 gm/l and heated until melting. The mixture was boiled while stirring with a magnetic stirrer for 15 minutes to completely dissolve the powdered agar. The solution was then autoclaved at 121°C atmospheric pressure of 15psi for 15 minutes to sterilize the media. The liquid media were maintained under aseptic condition and allowed to cool to about 50°C. Streptomycin sulphate powder were added to the PDA media at the rate of l gm/1 to avoid bacterial contamination and the media were poured into sterilized Petri dishes and the agar were then allowed cooling and solidifying before being used for maintaining fungal cultures.

For sub-culture, blocks of fungal agar were cut out with a sterile surgical blade from the leading edge of the actively growing portion and transfer to fresh agar medium and incubate at 25^0C for 3-5 days. The fungal pathogen was maintained by storing the pure culture of the pathogen in sterile distilled water (Van der Graaf, 1981).

3.2.4. *In vitro* test

The effect of rhizobacteria on the radial growth of *C. kahawae* were determined using the method described by Diriba *et al*., (2007) with some modification on dual culture experiment (Melkamu *et al*., 2013). On dual culture antagonistic study, first the pathogen was cultured on Potato Dextrose Agar (PDA) medium for 5 to 7 at 25°C from preserved stock culture. Then small fungal agar block (1 × 2 cm^2), from the leading margin of cultures was propagated on potato dextrose agar for 5-7 days at 25 °C and was centrally placed on (KBHS) (Diriba *et al*., 2007; Melkamu *et al*., 2013). Thereafter, one hundred fifty (150) isolates were randomly selected for the test. Then a loop of actively grown (24h old) bacterial cultures (two isolates/plate) were streaked as a broad band (making a straight short bar) approx. 3 cm away from the mycelia block at two opposite edges of duplicate Petri dishes (90 mm diameter). The plates were incubated with pathogen and without bacteria were used as control. Finally, plates

were incubated at 25°C for 15 days and potent rhizobacteria isolates were selected depending on their degree of inhibition. Fungal radial growth inhibition was calculated as described in (Montealegre *et al*., 2003). The potent rhizobacteria were then chosen for further studies.

$$\text{Inhibition \%} = \frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100$$

3.2.5 In vivo test

3.2.5.1 Detached berry test

Four potent rhizobacteria isolates under *in-vitro* were selected and tested for their potential to inhibit the targeted fungal coffee pathogen on detached green coffee berry *in-vivo* condition. This test was performed using the methods of Van der Graaf (1981). Accordingly, berries having the same size and development stage were collected from the susceptible coffee variety (selection 370). The harvested berries were surface sterilized with 5% Sodium hypochlorite for two minutes and were rinsed three times each for 3 min with sterilized distilled water. Then, the berries were placed on clean Petri- dish covered with sterilized water saturated sponge to obtain 100% humidity. Ten (10) berries were used for each three replications.

Mycelia colonies of fungal pathogen were carefully removed with a sterile scalpel from the PDA medium and washed with sterile distilled water to harvest conidia from 10 days old cultures. The suspension was stirred with magnetic stirrer for 10-15 min and filtered through double layers of cheese cloth. After repeating the procedure, 25 µl conidial suspension with 2×10^{6} conidial /ml were dropped at the center of the berries; and an equal amount of sterilized water were dropped on control berries (Van der Graaf 1981).

3.2.6 Bacterial inocula preparation and application

The rhizobacteria isolates that resulted in significant inhibition to *C. kahawae* in *in-vitro* were selected for the *in-vivo* experiment. The cultures were initiated by streaking the isolates culture into nutrient agar plates and incubated at 24^{0}C for 24 h (Bhai *et al*., 2005). Serial dilutions were made, and 100 µl (0.1 ml) were spread on the surface of a NA medium (Englerbrecht, 1994; Diriba *et al*., 2009). After this isolates colonies were counted and rhizobacteria suspension

adjusted to1×10^8 CFU/ml) (Diriba *et al*., 2009; Zhan *et al*., 2013). Then, the berries were placed on clean Petri- dish covered with sterilized water saturated sponge to obtain 100% humidity. Ten (10) berries were used for each three replications.

The isolated bacteria were sprayed on berries by using hand sprayer per the following schedule:

- Forty-eight hours before inoculation of the pathogen
- Forty-eight hours after inoculation of the pathogen
- At the same time

The interaction between isolates and inoculated pathogen were recorded starting from 15 days after inoculation every 2 day until 21 days after inoculation. The disease assessment on coffee berry were performed using 0-5 scale (Table2)

The percentages of disease incidence were calculated by using the following formula suggested by Cooke *et al.* (2006).

$$\text{Incidence (\%)} = \frac{\text{No. of diseased berry}}{\text{Total no of berry assessed}} \times 100$$

Disease index on berries were calculated using the following equation (Tefestewold, 1995).

DI = for berries $100\,(b1+2b2+3b3+4b4+5b5)/5(b0+b1+b2+b3+b4+b5)$

B0= number of berries in class 0

B1= number of berries in class 1

B2= number of berries in class 2

B3= number of berries in class 3

B4= number of berries in class 4

B5= number of berries in class 5

Where, b0, b1, b2, b3, b4, b5 are number of berries in class 0, 1, 2, 3, 4, 5 respectively (Tefestewold, 1995).

Table 2. Classification for CBD assessment on detached green berries

Class	Symptom	Code for DI
0	no symptom	b0
1	minute brown spot lesion 1mm in diameter	b1
2	black lesion of 1-5mm in diameter	b2
3	black lesion of 5-10mm in diameter	b3
4	black lesion of 10-20mm in diameter	b4
5	black lesion of greater than 20mm in diameter	b5

Source; Van der Graaf (1981)

Biological control efficacy was calculated according to Guo *et al*. (2004) as;

$$\text{Efficacy (\%)} = \frac{(\text{Disease of control} - \text{Disease of the treatment}) \times 100}{\text{Disease of control}}$$

3.2.7 Statistical Data analysis

The experiments were laid out in Complete Randomized Design (CRD) and data were subjected to analysis of variance using SAS software (SAS Institute, 2009). The mean values of significant parameters were compared by Tuckey's test (Montgomery, 2008) at $p < 0.05$.

4. RESULTS

4.1. Isolation of Rhizobacteria Cultures from the Coffee Root Rhizosphere

In the present investigations, a total of 67 coffee root samples were collected randomly from Arabica coffee (*Coffea arabica* L.) root rhizosphere of Gera, Ela Dalle and around JUCAVM campus (Table 3). From 67 coffee root sampled, two hundred fifteen (215) of rhizobacteria were identified using morphological and biochemical characterization by serial dilution plate method using King's B medium. The identified isolates were belonging to genera of *Bacillus* spp, *Pseudomonas* spp, *Acinatobacter* spp, *Bulkaholdera* spp, *Enterobacter* spp, *Micrococcus* spp, *Enterococcus* spp, S*taphylococcus* spp, *Flavobacter* spp and *Arthrobacter* spp (Appendix 8.).

Table 3. Amounts of samples and rhizobacteria isolates in numbers

Site	No of Sample	No of Isolates
Gera	26	104
Ila Dalle	22	67
JUCAVM campus	19	44
Total	67	215

Table 4: Summary of Rhizobacteria species isolated from sampled area

Genus	**Site and number of isolates**				**Typical characterization**
	Gera	Ila Dalle	JUCAVM campus	Total	
***Bacillus* spp.**	33	22	15	70	+ve rod
***Pseudomonas* spp.**	14	18	9	41	-ve rod
***Micrococcus* spp.**	15	6	2	23	+ve cocci
***Bulkaholdera* spp.**	9	4	4	17	+ve rod bipolar
***Enterococcus* spp.**	10	4	2	16	-ve cocci chain
***Enterobacter* spp**	2	2	4	8	-ve rod
***Flavobacter* spp.**	6	1	3	10	-ve rod
***Acinatobacter* spp.**	5	2	3	10	-ve rod
***Arthrobacter* spp.**	7	4	2	13	+ve cocci rod
***Staphylococcus* spp.**	3	3	1	7	+ve cocci cluster
Total	**104**	**66**	**45**	**215**	

4.2. Characterizations of Rhizobacteria Isolates

In morphological parameter, the bacterial colonies were selected based on morphological characteristics like colony color, shape, gram staining, spore staining, elevation, texture and formation of bacterial colony for further characterization (Fig.2). In the biochemical characterization, KOH test, Catalase test, Oxidase test, Motility test and Oxidation/Fermentation test was used. One hundred twenty-four (124) rhizobacteria were positive to gram staining and belonged to genera of *Bacillus* spp., *Micrococcus* spp., *Arthrobacter* spp., *Enterococcus* spp., and *Staphylococcus* spp., whereas, 91 isolates were gram negative which belonged to *Pseudomonas* spp., *Flavobacter* spp., *Acinatobacter* spp., *Bulkaholdera* spp. and *Enterobacter* spp.(Fig.4).

For the KoH test, 26 were negative and belonged to *Pseudomonas* spp., *Arthrobacter* spp. and *Bulkaholdera* species (Fig.5). For Catalase test 203 rhizobacteria isolates were positive to catalase test and belonged to *Bacillus* spp., *Micrococcus* spp., *Arthrobacter* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Flavobacter* spp., *Acinatobacter* spp., *Bulkaholdera* spp. and *Enterobacter* spp.(Fig.6). Ninety-seven isolates were found oxidase positive and belonged to *Pseudomonas* species, *Arthrobacter* spp., *Micrococcus* spp. as well as some of *Bacillus* species and 118 rhizobacteria were oxidase positive then belonged to *Micrococcus* spp., *Flavobacter* spp., *Acinatobacter* spp., *Bulkaholdera* spp. and *Enterobacter* spp., *Bacillus* spp. and *Staphylococcus* spp. (Fig.7). For Motility test, 91 isolates were non motile and belonged to genera of *Arthrobacter* spp., *Micrococcus* spp., *Flavobacter* spp., *Acinatobacter* spp., *Staphylococcus* spp., and *Enterococcus* spp., while 124 isolates were motile and they are under genera of *Pseudomonas* spp., and *Bulkaholdera* species, *Bacillus* spp., and *Enterobacter* spp.(Fig.8). For Oxidation/Fermentation test, out of 215 coffee associated rhizobacteria isolates tested, 116 were Oxidative and belonged to *Flavobacter* spp., *Micrococcus* spp., *Pseudomonas* spp., *Acinatobacter* spp., *Bulkaholdera* spp. and *Bacillus* spp. whereas, *Enterobacter* spp., *Enterococcus* spp., *Staphylococcus* spp., *Bacillus* spp. and *Arthrobacter* spp., 91 were fermentative and 8 were inert from *Bacillus* spp. (Fig.9).

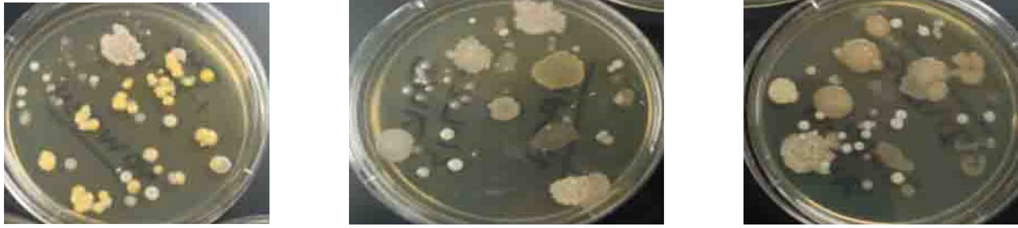


Fig 2. Colony morphology after 3-day growth of different Rhizobacteria on King's B medium

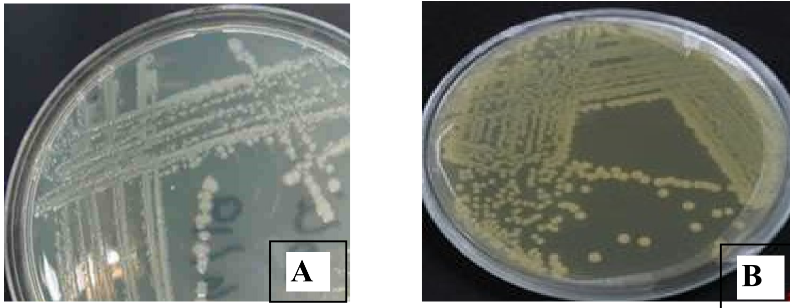


Fig 3 Streak plate of Rhizobacteria from coffee root rhizosphere: *a*) ***Bacillus*** **spp. (JU544)** *b*) ***Pseudomonas*** **spp. (JU13)**

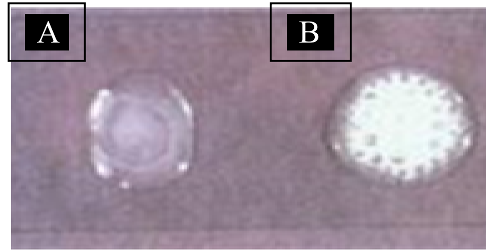
A) 124 isolates (Gram +ve)

B) 91 isolates (gram –ve)

Fig 4. Gram stain results of rhizobacteria isolated from coffee root rhizosphere: *a*) **Indicated Gram-positive** *b*) **Gram-negative**

26 isolates (–ve)

Fig 5. KOH test results of rhizobacteria isolated from coffee root rhizosphere that indicated Gram-negative rhizobacteria



A) 12 Isolates (Catalase –ve) b) 203 Isolates (Catalase +ve)

Fig 6. Catalase test results of rhizobacteria isolated from coffee root rhizosphere; *a*) Indicated Gram-negative and *b*) indicated Gram-positive rhizobacteria

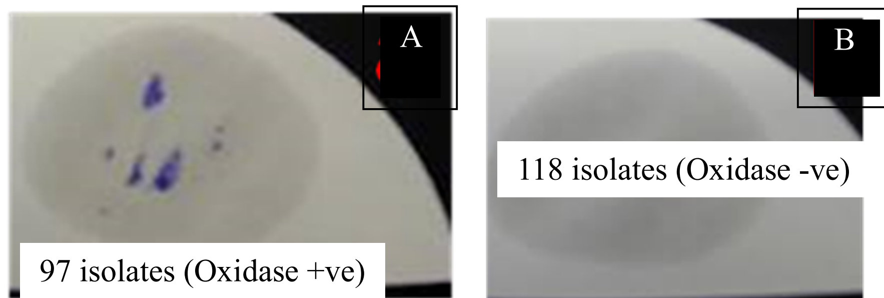


Fig 7. Oxidase test result of rhizobacteria isolates, left side *(a)* indicated positive to Oxidase test (*b)* Indicated negative to Oxidase test

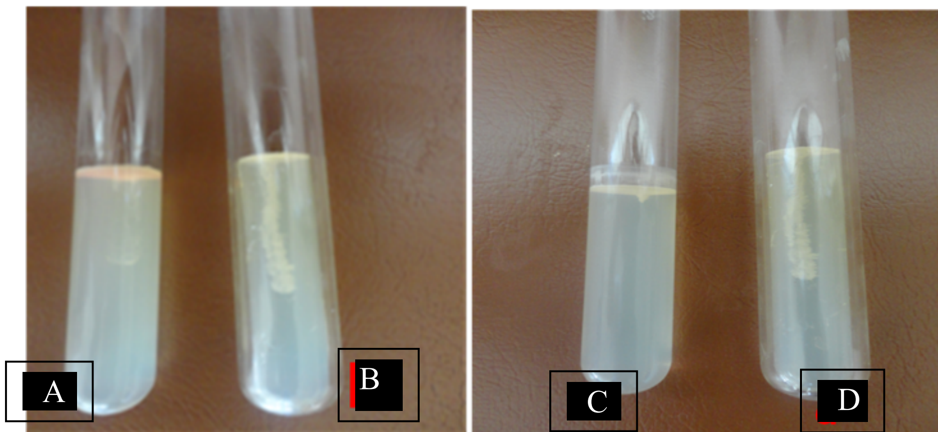


Fig 8. Motility test of rhizobacteria isolated from coffee root rhizosphere, a and c are motile, b and d are non-motile

Totally 91 isolates (Non motile) and 124 isolates (Motile).

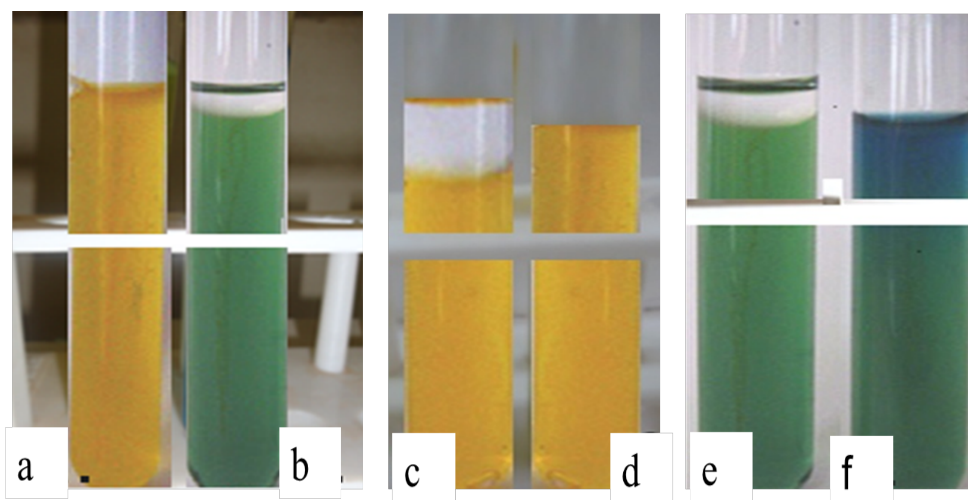


Fig 9. Oxidative-fermentative test inoculated with coffee root associated rhizobacteria.
The side tubes of (b, c, and e) are sealed tubes and the tubes of (a, d and f) are open tubes. Yellow color in both tubes is fermentative result (91strain). Yellow color only in the open tubes indicates oxidative (116 strain). The lack of yellow color in both tubes indicated inert (8 strain)

4.3. In Vitro Antagonistic Activity of Rhizobacteria Isolates against *C. Kahawae*

There were significant differences ($p < 0.0001$) between rhizobacteria antagonist and free growth of fungus in inhibiting the mycelia expansion of *C. kahawae* ranging from 8.2% – 91.3 % radial growth inhibition, respectively (Table 5). Out of 215 rhizobacteria isolates, 150 were randomly selected and tested for their potential to inhibit the targeted fungal coffee pathogen. Of them 150 rhizobacteria, 28 rhizobacteria were antagonists exhibited remarkable fungal radial growth inhibition. Some of the efficient rhizobacteria isolates also clearly showed discernible inhibition of *C. kahawae.* The rhizobacteria antagonists showed highly significant variations ($p <.0001$) in restricting the fungal mycelia growth. Four out of the 28 antagonists, namely *Bacillus* (JU544) inhibited the fungal growth by 91.3%, *Pseudomonas* (JU13) inhibited the fungal growth by 82.33%, inhibited the fungal growth *Bacillus* (JU735) by 74.6 and *Micrococcus* (JU533) inhibited the fungal growth by 67% were the most aggressive inhibitors (Fig.10) and were then chosen for further studies. Overall, isolate *Bacillus* (JU544) inhibited the fungal growth by 91.3% and *Pseudomonas* (JU13) by 82.3% and were the most efficient inhibitors against the fungal pathogen *Colletotrichum kahawae* tested *in vitro.*

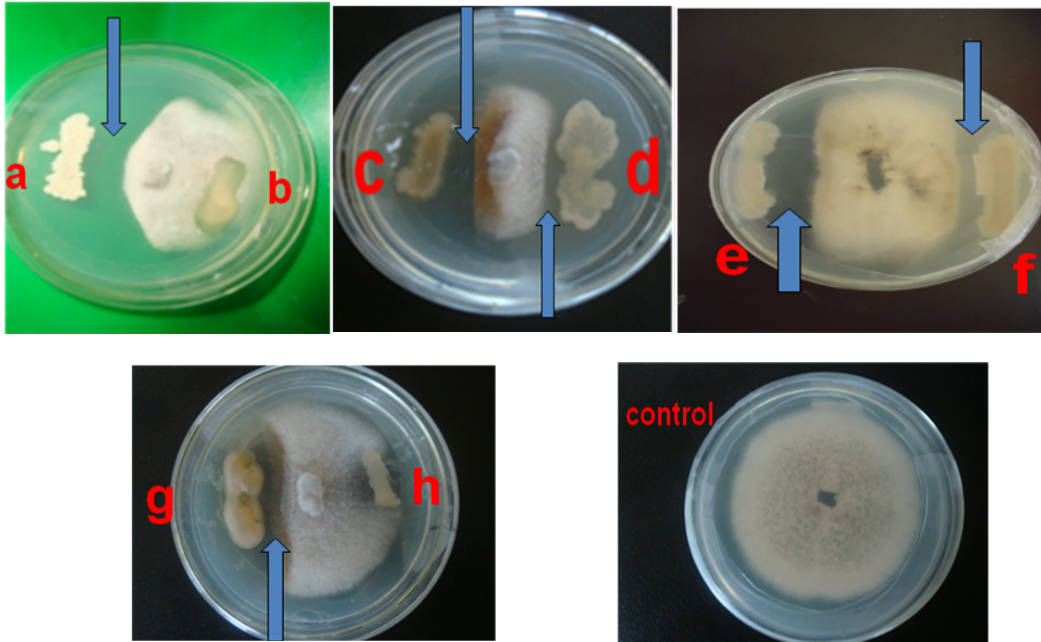


Fig 10. *In vitro* antagonistic activity of the most rhizobacteria isolates:
Control plates and dual culture media showing rhizobacteria and coffee pathogen interactions: (A) *Bacillus* spp. (JU544), (B) *Acinatobacter* (JU631) (no inhibition), (C) *Pseudomonas* spp.(JU13) (D) *Bacillus* spp. (JU133), (E) *Bacillus* spp. (JU735), (F) *Flavobacter* spp. (JU621), (G) *Micrococcus* spp. (JU533) and (H) *Arthrobacter* spp. (JU727) (no inhibition).

Table 5. Mean Percentage inhibition of the radial growth of fungal coffee pathogen (*Colletotrichum kahawae*) due to coffee-associated rhizobacteria antagonists

S. No	Rhizobacteria	Radial growth (mm)	Inhibition%
1	*Arthrobacter* (JU612)	56.3	43.7^{def}
2	*Bacillus* spp. (JU133)	68.1	31.9^{ghi}
3	*Bacillus* spp. (JU312)	91.8	8.2^{lm}
4	*Bacillus* spp. (JU431)	58.5	41.5^{def}
5	*Bacillus* spp. *(*JU433)	50.7	49.3^{d}
6	*Bacillus* spp. (JU544)	8.7	91.3^{a}
7	*Bacillus* spp. (JU614)	75.4	24.6^{hij}
8	*Bacillus* spp. *(*JU635)	82.2	17.8^{jk}
9	*Bacillus* spp. (JU638)	54.1	45.9^{de}
10	*Bacillus* spp. (JU715)	50.4	49.6^{d}
11	*Bacillus* spp. (JU735)	25.4	74.6^{bc}
12	*Bacillus* spp. (JU813)	56.7	43.3^{def}
13	*Micrococcus* spp. (JU833)	91.5	8.5^{lm}
14	*Bacillus* spp. (JU835)	60.8	39.2^{efg}
15	*Bacillus* spp. (JU847)	76.9	23.1^{ijk}
16	*Bulkaholdera* spp. (JU342)	63.7	36.3^{fg}
17	*Bacillus* spp. (JU431)	83.9	16.1^{jkl}
18	*Enterobacter* spp. *(*JU732)	79.4	20.67^{jk}
19	*Enterococcus* spp. (JU432)	82.7	17.3^{jk}
20	*Enterococcus* spp. (JU816)	76.8	23.2^{ijk}
21	*Flavobacter* spp. (JU422)	52.9	47.1^{de}
22	*Micrococcus* spp. (JUD121)	55.8	44.2^{def}
23	*Micrococcu*s spp. (JU531)	76.8	23.2^{ijk}
24	*Micrococcus* spp. (JU533)	33	67^{c}
25	*Pseudomonas* spp. (JU122)	58.7	41.3^{def}
26	*Pseudomonas* spp. *(*JU543)	67.7	32.3^{gh}
27	*Pseudomonas* spp. (JU13)	17.7	82.3^{b}
28	*Pseudomonas* spp. (JU726)	85.5	14.5^{kl}
29	Untreated/ control	100	0^{m}
	CV (%) 7.52		

Means with the same letter are not significantly different (α= 0.05)

4.4. In vivo Antifungal Activity of Rhizobacteria Isolates against *C. kahawae*

4.4.1 Detached green coffee berries

4.4.1.1 Disease severity on detached green coffee berries

Four out of the 28 antagonists, namely *Bacillus* (JU544)*, Pseudomonas* (JU13), *Bacillus* (JU735) and *Micrococcus* (JU533) were the most aggressive inhibitors in *in-vitro* test were chosen for detached green coffee berries to evaluate their *in vivo* antifungal activities against *C. kahawae*. There was significant ($p < 0.0001$) interaction effect between type of rhizobacteria isolates and time of application of the rhizobacteria (Appendix 2). The disease incidence reached 100% in the untreated control treatment. The application of *Bacillus* spp. (JU544) significantly reduced the disease severity to 10.3%, 14.6% and 24% respectively when applied at the same time, 48h before and 48h after inoculation of the pathogen. Likewise, application of *Pseudomonas* spp. (JU13) suspension on green berries at the same time and 48h before inoculations of the test pathogen was significantly reduced the severity of coffee berry disease on the berries to 22.3 % and 20.7 respectively (Table 6). *Bacillus* spp. (JU735) and *Micrococcus* spp. (JU533) did not reduce the severity of the disease when applied on green berries 48h, at the same time and before inoculations of the pathogen.

Table 6. Effect of the type of rhizobacteria isolates and time of application on detached green coffee berries in reducing disease severity percentage

Isolates	Application time	**Mean Severity%**	**Efficacy %**
Bacillus spp. (JU544)	48hB	14.6^{cd}	85.4
	48hA	24^{cd}	76
	AT	10.3^{d}	89.7
Pseudomonas spp. (JU13)	48hB	20.7^{c}	79.3
	48hA	58.7^{b}	41.3
	AT	22.3^{c}	77.7
Bacillus spp. (JU735)	48hB	97.5^{a}	2.5
	48hA	96.2^{a}	3.8
	AT	96.5^{a}	2.7
Micrococcus spp. (JU533)	48hB	98.8^{a}	1.2
	48hA	97.7^{a}	2.3
	AT	97.3^{a}	2.7
Control	48hB	100^{a}	0
	48hA	100^{a}	0
	AT	100^{a}	0
CV (%) 4.85			

Means with different letters are significantly different (*p<0.05*). *Bacillus* spp. (JU544) = highly antagonistic during, *Pseudomonas* spp. (JU13) = antagonistic, *Bacillus* spp. (JU735) and *Micrococcus* spp. (JU533) = non-significant. Inoculation time 28hB= Treating the rhizobacteria suspension with berry 28hours before pathogen inoculation; 28hA= Treating the rhizobacteria suspension with berry before 28hours before pathogen inoculation; Treating the rhizobacteria suspension with berry at the same time with the pathogen inoculation.

4.4.1.2 Disease incidence on detached green coffee berries

The rhizobacteria isolates of *Bacillus* spp. (JU544), *Bacillus* spp. (JU735), *Pseudomonas* spp. (JU13) and *Micrococcus* spp. (JU533) were tested on detached green coffee berries to evaluate their *in vivo* antifungal activities against *C. kahawae* because of their high antifungal effect observed under *in vitro* conditions. There was significant ($p < 0.0001$) interaction effect between type of rhizobacteria isolates and time of application of the rhizobacteria (Appendix 3). The disease incidence reached 100% in the untreated control treatment. The application of *Bacillus* spp. (JU544) significantly reduced the disease incidence to 23.3%, 26.6% and 36% respectively when applied at the same time, 48h before and 48h after inoculation of the pathogen. Likewise, application of *Pseudomonas* spp. (JU13) suspension on green berries at the same time inoculations of the test pathogen was significantly reduced the severity of coffee berry disease on the berries to 40.3% respectively (Table 7). *Bacillus* spp. (JU735) and *Micrococcus* spp. (JU533) did not reduce the incidence of the disease when applied on green berries 48 h after, before and at the same time of inoculations.

Table 7. Effect of the type of rhizobacteria isolates and time of application on detached green coffee berries in reducing disease incidence percentage.

Isolates	Application time	Mean Incidence %	Efficacy %
Bacillus spp. (JU544)	48hB	26.6[b]	73.4
	48hA	36.6[b]	63.4
	AT	23.3[b]	76.7
Pseudomonas spp. (JU13)	48hB	80.6[a]	19.4
	48hA	93.3[a]	6.7
	AT	40.3 [b]	59.7
Bacillus spp. (JU735)	48hB	100[a]	0
	48hA	100[a]	0
	AT	100[a]	0
Micrococcus spp. (JU533)	48hB	100[a]	0
	48hA	100[a]	0
	AT	100[a]	0
Control	48hB	100[a]	0
	48hA	100[a]	0
	AT	100[a]	0
CV (%) 6.5			

Means with different letters are significantly different (*p<0.05*). *Bacillus* spp. (JU544) = highly antagonistic during, *Pseudomonas* spp. (JU13) = antagonistic, *Bacillus* spp. (JU735) and *Micrococcus* spp. (JU533) = non influential. Inoculation time 28hB= Treating the rhizobacteria suspension with berry 28hours before pathogen inoculation; 28hA= Treating the rhizobacteria suspension with berry before 28hours before pathogen inoculation; Treating the rhizobacteria suspension with berry at the same time with the pathogen inoculation.

5. DISCUSSION

The concept of plant growth promoting rhizobacteria mediated plant growth promotion has gained worldwide importance and acceptance in recent years. PGPR also naturally occur in soil microorganisms that colonize roots and stimulate plant growth. Such bacteria have been applied to a wide range of plants for the purpose of plant growth enhancement and disease control (Barka *et al.*, 2000 and Chakraborty *et al.*, 2005). PGPR may be suppressing disease due to iron sequestration, production of antibiotics, production of antimicrobials or induction of systemic resistance (Chakraborty *et al.*, 2005).

The aim of this study was to investigate the isolation, characterization and antagonistic effect of rhizobacteria against *C. kahawae* both *in-vitro* and *in-vivo* conditions. A total of 67 of Arabica coffee root were sampled from the different locations of Gera, Illa Dalle and around JUCAVM campus and 215 rhizobacteria were isolated and identified. These isolated rhizobacteria belonged to genera of 10 *Acinatobacter,* 13 *Arthrobacter,* 70 *Bacillus,* 17 *Bulkholdera,* 8 *Enterobacter,* 16 *Enterococcus,* 10 *Flavobacter,* 23 *Micrococcus,* 41 *Pseudomonas and* 7 *Staphylococcus.* Similar results on the occurrence and isolation of *Pseudomonas, Acinetobacter* spp.*, Flavobacter* spp.*, Azospirillum* spp.*, Azotobacter* spp.*, Klebsiella* spp.*, Enterobacter* spp.*, Alcaligenes* spp.*, Arthrobacter* spp.*, Burkholderia* spp.*, Bacillus* spp.*, Brevibacillus* spp.*, Serratia* spp.*, Micrococcus* spp. and *Staphylococcus* species from the rhizosphere of different plants have been reported by various workers Suryakala *et al.*, 2004; Mishra *et al.*, 2005; Chung *et al.*, 2005; Babalola and Akindolire, 2012). spp.

In this study *Bacillus* was a dominant group and also a major component of the microbial flora, living in close association with various plant crops. Lilinares *et al*. (1994) report that predominance of *Bacillus* is due to its ability to efficiently use the nutrients provided by the plant through exudates. In addition, *Bacillus* can inhibit the growth of other strains. Moreover, many strains of *Bacillus* have been reported to produce substances that act as growth inhibitors for other microorganisms. *Bacillus* sp. produces large, spreading, grey white colonies with irregular margins and a unique characteristic of this bacterium is its ability to produce endospores, when environmental conditions are stressful (Ambreen *et al.,* 2002).

Morphological and biochemical characterization of PGPR in this study is in agreement with work done by (Ramos *et al.,*1998; Chandrashekharaiah, 2005; Olivera *et al*., 2005; Silke *et al.,* 2006; Priyanthi, 2007; Muhammed *et al*., 2007; Yu *et al*., 2008; Babalola and Akindolire, 2012) who reported that *Bacillus* species were white, yellow and ash in color, irregular and circle in colony formation, flat and opaque or smooth, Gram-positive rod in Gram reaction and cell shape, also endospores forming bacteria, in oxidation/fermentation, fermentative or non-fermentative or inert, oxidase negative, catalase positive and motile.

Correspondingly (Michael and Richard, 1989; Chandrashekharaiah, 2005; Priyanthi, 2007; Mishra *et al*., 2009; Ambreen Akhtar *et al.,* 2012; Babalola and Akindolire, 2012) reported that *Pseudomonas* species colony were white, yellow, creamy, convex, dull white, off white, smooth in appearance, irregular or circle, flat, raised center, opaque or smooth, oxidase positive/negative, catalase positive, aerobic, motile and the differential staining and microscopic examination revealed that the isolate was Gram-negative rod. Similarly, Naureen *et al*. (2005) and Kirankumar, (2007) reported genera of *Pseudomonas* were whitish, creamy, circular or slimy circular, smooth, Gram-negative rod, positive for oxidase test and catalase activity as well as genera of *Enterobacter* were whitish, creamy circular, Gram-negative rod positive for biochemical test oxidase and catalase.

Chaitanya, (2011) also suggested that Genus *Enterobacter* is a Gram-negative, straight rod which is motile with peritrichous flagella and is facultative anaerobic. The colonies of *Arthrobacter* were either white, orange or yellow in color, round, smooth, convex, rod-coccus, Gram-positive, no spore forming, aerobic, catalase positive and non-motile (Reddy *et al*., 2000). The *Bulkholdera* colonies were yellow, circular, convex, entire margin, shiny, Gram-negative rod Oxidase negative/positive, catalase positive/negative and non-fluorescent under UV light (Kamaruzaman and Dikin, 2005; Babalola and Akindolire, 2012).

Bacteria belonging to the genus *Acinetobacter* are strictly aerobic, non-fermenting, Gram-negative, non-motile, cocco-bacillary/short rod, smooth, pink microorganisms with a negative oxidase and a positive catalase reaction (Juni, 2005; Dijkshoorn and Nemec, 2008; Babalola and Akindolire, 2012). *Flavobacterium* was a genus of Gram-negative, non-motile and motile,

rod-shaped, aerobic with no flagella that were 0.4–0.6 μm in width, 1.4–2.2 μm in length, and the colonies were yellow and not circular (Sang and Kim, 2012). Bacteria which belong to the genus *Micrococcus* were catalase positive, oxidase negative and non-motile (Kumar *et al*., 2012). Similarly, Syed *et al*., (2010) reported that the *Micrococcus* bacterial strain was Gram-positive, non-motile coccus, with circular, smooth, convex, entire and pale yellow in color. The genus *Enterococcus* comprises Gram-positive cocci that are oxidase and catalase negative, usually facultative, anaerobic bacteria (Winton and Jiam, 1998; Maero and Blanch, 1999).

In the present study, out of 215 rhizobacteria isolated 150 were randomly selected and evaluated for their antagonism against *Colletotrichum kahawae* affecting coffee berry. Of them, 28 rhizobacteria isolates were inhibited the radial growth of *Colletotrichum kahawae* the causal agent of coffee berry disease in the *in-vitro* condition with a bigger range of inhibition i.e (8.2% –91.3 %) on dual culture media during screening. These bio-antagonistic bacterial isolates belonged to the genus of *Bacillus* spp., *Arthrobacter* spp., *Bulkaholdera* spp., *Enterobacter* spp., *Enterococcus* spp., *Flavobacter* spp., *Micrococcus* spp., *Pseudomonas* spp. The majority did not show any antagonistic effects. Such variation in the level of radial growth inhibition can be influenced by the character of bacterial isolates. Landa *et al*. (1997) indicated that the ability of four bacterial isolates to inhibit different races of *F. oxysporium* differed significantly. This suggested that the type of antifungal metabolites produced by the isolates may vary and that the bacterial isolates are taxonomically different from each other (Williams and Asher, 1996).

Bacillus spp. (JU544), *Pseudomonas* spp. (JU13), *Bacillus* spp. (JU735) and *Micrococcus* spp. (JU533) were the most efficient antagonistic bacteria with 91.3%, 82.3%, 74.6% and 67% inhibition against *C. kahawae* under *in vitro* conditions respectively. Different studies support the antagonistic effects of rhizobacteria under *in vitro* study of this research. Melkamu *et al.* (2013) was took isolates from those rhizobacteria identified in this work and was tested their antagonistic potential under *in vitro* and *in vivo* conditions against *Gibberella xylarioides.* From tested rhizobacteria, strain of *Bacillus* spp. (JU544) and *Pseudomonas* spp. (JU13) showed strong antagonism under *in vitro* and *in vivo* conditions against *Gibberella xylarioides*. Kumlachew *et al*. (2017) reported that the *B. cereus, B. mycoids* and *P. spinosa* had significantly ($p<0.05$) reduced mycelial growth of the *C. kahawae under in vitro* test. Other

investigation by Kamaruzaman and Dikin, (2005) reported that dual cultures were tested on two isolates of *B. cepacia (*BC-S and BC-TM) and BC-S inhibited the mycelial growth of *S. commune* (70.8%) and *Colletotrichum dematium* (77%); while, BC-TM was more effective in inhibiting the mycelial growth of *F. oxysporum* f.sp. *lycopersici* (62%), *F. solani* (72.5%). The bacterial antagonists tested in this research showed a significant reduction of mycelia growth under *in vitro* condition without physical contacts with the test pathogen indicating that the bacterial antagonists could release certain antifungal metabolites and hydrolytic enzymes as suggested by Montealagre *et al*. (2003).

Four potential rhizobacteria species that showed greatest disease reduction under *in vitro* were also significantly reduced the disease incidence and severity better than untreated control on detached coffee berry. Among the tested bacteria antagonists *Bacillus* spp. (JU544) and *Pseudomonas* spp. (JU13) showed significant reduction on detached coffee berry. *Bacillus* spp. (JU544) significantly reduced the severity and incidence of CBD on detached coffee berry when applied at the same time of inoculation of the pathogen, 48 h before and after inoculation of the pathogen. *Pseudomonas* spp. (JU13) significantly reduced the severity of CBD on detached coffee berry when applied at the same time of inoculation of the pathogen and 48 h before inoculation of the pathogen and reduced the incidence of CBD on detached coffee berry when applied at the same time of inoculation of the pathogen. Several studies have demonstrated that *Bacillus* species possess antifungal activity against coffee pathogens (Jacobsen *et al*., 2004; pedro *et al*., 2004; Kildea *et al*., 2008; Kumlachew *et al.,* 2017).

When the detached coffee berry inoculated at the same time with CBD pathogen and before the inoculation of the pathogen, the highest biocontrol efficiency (89% and 85%,) was encountered with *Bacillus* spp. (JU544) followed by *Pseudomonas* spp. (JU13) with their control efficiency of 77% and 79% respectively. The bacterial antagonists were effective when they were applied before and at the same time of the pathogen inoculation. Different scholars also demonstrated that bacterial antagonists are effective when they are applied prior to inoculation of the pathogen (Melkamu *et al*., 2013; Kumlachew *et al.,* 2017). Likewise, Melkamu *et al*. (2013) reported that when the coffee seedlings inoculated seven days before CWD pathogen, the

highest biocontrol efficacy (72.64%) was encountered with *Bacillus* spp. (JU544) followed by *Pseudomonas* spp. (JU13) with their control efficacy of 61.87% respectively.

Hinton and Bacon (1995) reported that isolate of *Enterobacter cloacae* associated as endophyte with corn roots, stems and leaves are antagonistic to *F. moniliforme* and other toxic fungi associated with corn. Cavaglieri *et al*. (2005) *Arthrobacter globiformis RC5* and *Azotobacter armeniacus RC2* were able to exert an effective control on *in vitro* conditions. Burkhead *et al.* (1994) reported that *Bulkholdera cepacia* strain B37w inhibited the growth of a bioherbicide fungus, *Colletotrichum truncatum* and *F. sambuinum*, the agent causing dry rot of potato. *Flavobacterium johnsoniae* GSE09 inhibited pathogen development (mycelial growth, sporulation, and zoospore germination) in a plastic plate and effectively colonized on pepper root, rhizosphere, and bulk (pot) soil, which reduced the pathogen colonization in the roots and disease severity in the plants (Sang and Kim, 2012).

Williams and Asher (1996); Landa *et al*., (1997); Commare *et al*., (2002) reported that several members of the genus *Bacillus* are effective in controlling a variety of fungal plant diseases. Similarly, Hong (2005) reported that strain of *Bacillus Subtilis* (TL2) showed strong antagonism under *in vitro* conditions against *Pestalotiopsis theae.* Diriba *et al*. (2007) reported that isolates of AUPB15 (*Pseudomonas* sp.) and AUBB05 (*B. subtilis*) have high maximum inhibitory effect against *F. stilboide,* due to their production of antibiotics. Other workers (Chakraborty *et al*.,1998) who tested the antagonistic effect of *Micrococcus in vitro*, reported that the bacterium *Micrococcus luteus* showed good antagonism to *G. cingulatam* and an antifungal compound extracted from *M. luteus* inhibited growth of *G. cingulata* and *Pestalotiopsis theae*.

Pre-harvest foliar application of talc based fluorescent *Pseudomonas* strain Fp7 supplemented with chitin at fortnightly intervals (5g/plant; spray volume 20 L/tree) on to mango tree from pre-flowering to fruit maturity stage induced flowering to the maximum, reduced the latent infection by *Colletotrichum gleosporioides* besides increasing fruit quality and yield (Vivekanathan *et al.*, 2004).

Thomashow and Waller, (1990), sited in Yamaoka *et al*. (1995) shown that strains of *Bacillus* sp. and *Pseudomonas* sp. have been effective in reducing diseases in field trials. Both *Bacillus* and *Paenibacillus* species express antagonistic activities by suppressing the pathogens and numerous reports covering this aspect both under *in vitro* and *in vivo* conditions are available (Arrebola *et al.* 2010; Chen *et al.* 2009). Yap Chin, (2012) investigated *Bacillus* sp. against pathogenic fungi of pepper (*Piper nigrum*).

The result of rhizobacteria of pepper (*Piper nigrum*) and their antifungal activities showed that strains WW6 (*B. amyloliquefaciens),* WW14 and WW15 (*B. vallismortis)* and CBF (*B. subtilis)* can be used at field level to biocontrol the fungus disease in pepper vine. *Bacillus* spp. (JU544) and *Pseudomonas* spp. (JU13) has been reported to inhibit the growth of *C. kahawae*. Stanghellini and Miller, (1997) reported that bacteria in the genera *Pseudomonas, Bacillus, Arthrobacter, Rodococcus, Acinatobacter and Corynebacterium* have been reported to produce rhamnolipid biosurfactants, non-ionic surfactants that destroy zoospores of fungi.

Likewise, Hass and Keel, (2003) also reported that *Pseudomonas s*pp. produces a number of antifungal metabolites and provides effective biocontrol mechanisms. *Bacillus cereus* UW85 suppressed alfalfa damping off consistently in the field (Handelaman *et al*., 1990). The antagonistic effects of *Pseudomonas* and *Bacillus* spp. on fungal pathogen may be due to induction of systemic resistance, and production of siderophores or antibiotics. Ambreen *et al.,* (2002) reported that *Bacillus subtilis* has been used for industrial production of proteases, amylases, antibiotics and chemicals. *B. subtilis* strain QST713 has natural fungicidal activity, and is employed as a biocontrol agent. *Bacillus* sp. produces large, spreading, grey white colonies with irregular margins. A unique characteristic of this bacterium is its ability to produce endospores, when environmental conditions are stressful *(*Ambreen *et al.,* 2002).

Interestingly, the present study suggests that *Pseudomonas* and *Bacillus* spp. have the potential to be applied as a control measure against infection of CBD caused by *C. kahawae.* The application of *Bacillus* spp. (JU544) and *Pseudomonas* spp. (JU13) seem promising, as it is an effective and cheap alternative means of *C. kahawae* management for the majority of Ethiopian subsistence farmers, who cannot afford synthetic chemicals. Moreover, the risk associated with

the synthetic chemicals as well as consumers' towards its application in agriculture makes the product more attractive for organic agriculture.

6. SUMMARY AND CONCLUSION

Coffee (*Coffea arabica* L.) is extremely important sources of foreign exchange and commodity in international trade. Majorities of its production obtained from forest and semi forest. However, recently its highly severed by abiotic factors such as climate change, biotic factors such as diseases and insect pest. The Coffee berry disease caused by fungus *Colletotrichum kahawae,* is considered as major disease of coffee berry in Ethiopia. Different control methods are practiced at coffee producing farms and farmer levels like resistant variety, cultural practices in the country including chemical control. However, the use of chemical fungicide is causes environmental pollution and abortion of natural sustainability. Hence the development of biological control which helps to optimum yield, reduce health risk and hazardous effects on environments is highly important in buffering this climate change.

The aim of this study was to isolate, characterization and antagonistic effect of rhizobacteria associated with coffee (*Coffea arabica* L.) rhizosphere against *C. kahawae* both *in-vitro* and *in-vivo* conditions. The soil sample used sources of rhizobacteria were taken from Gera, Ela Dalle and around the JUCAVM campus. The isolation result showed that, total of 215 isolated from Gera (104), Ela dale (67) and JUCAVM area (44) with ten different bacteria species. The KB half strength (KBHS) media was used for antagonistic rhizobacteria growth. The isolated bacteria were *in vitro* tested by using CRD with three replications. From 215 isolated bacteria, 28 exhibited remarkable range of 8.2% – 91.3 % fungal radial growth inhibition. Four rhizobacteria; *Bacillus* spp. (JU544) by 91.3%, *Pseudomonas* spp. (JU13) by 82.3%, *Bacillus* spp. (JU735 by 74.6%, and *Micrcoccus* spp. (JU533) with 67% showed best performances. The results four isolates were the most effective that significantly reduced ($p<0.0001$) the radial culture growth of the pathogen when compared to the control, respectively.

The four best performed rhizobacteria under *in vitro* were tested under *in vivo* condition and was highly against the fungal pathogen on detached green berry, by applying the isolates at the same time and 48 hours before and 48h after inoculation of the pathogen. The rhizobacteria isolates from rhizosphere of coffee root have been the potential to suppress coffee berry disease (*Colletotrichum kahawae)* pathogen. The application of *Bacillus* spp. (JU544) at the same time,

48 h before and 48 h after inoculations of the pathogen was significantly reduced the severity and the incidence of CBD on detached green berries. *Pseudomonas* spp. (JU13) significantly reduced the severity of the disease on the detached green berries when applied at the same time and 48 h before the inoculations of the pathogen.

In conclusion, *Bacillus spp.* (JU544) showed high performance under *in vitro* fungal radial growth inhibition as well as significantly reduced the disease severity to 10.3%, 14.6% and 24% respectively when applied at the same time, 48h before and 48h after inoculation of the pathogen respectively. Likewise, *Pseudomonas* spp. (JU13) showed high performance under *in vitro* fungal radial growth inhibition. application of *Pseudomonas* spp. (JU13) suspension on green berries at the same time and 48h before inoculations of the test pathogen was significantly reduced the severity of coffee berry disease on the berries to 22.3 % and 20.7 respectively.

Thus, considerable efforts should be devoted on isolation, identification of the plant growth promoting rhizobacteria and to evaluate the genera's effectiveness against the coffee berry disease causing pathogen, in order to develop new microbial fungicides as alternative to control and fight berry anthracnose and as well as to formulate integrated disease management schedule and come up with strong recommendation. This study was conducted under laboratory conditions. Moreover, optimum concentration, application methods of rhizobacteria should be studied. Further conformation may be also important.

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8. APPENDICES

Appendix 1: Analysis of variance of rhizobacteria isolated antagonistic on radial growth of *C. kahawae* percentage

Source	**DF**	**SS**	**MS**	**F value**	**Pr > F**
Rhizobacteria	28	41231.93416	1472.56908	195.36	<.0001
Error	58	437.18160	7.53761		
Corrected total	86	41669.11576			

Appendix 2: Analysis of variance of rhizobacteria isolated and time of application on detached green berries test severity percentage

Source	**DF**	**SS**	**MS**	**F value**	**Pr > F**
Rhizobacteria	4	54840.40410	13710.10103	1438.81	<.0001
Time	2	863.58923	431.79462	45.31	<.0001
Rhizobacteria*time	8	1969.25744	246.15718	25.83	<.0001
Error	22	209.63333	9.52879		
Corrected total	38	57901.01077			

Appendix 3: Analysis of variance of rhizobacteria isolated and time of application on detached green berries test incidence percentage

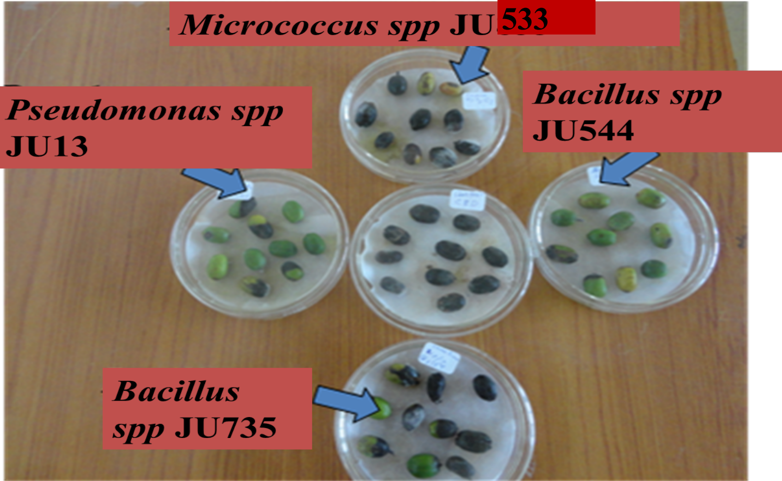
Source	**DF**	**SS**	**M S**	**F Value**	**Pr > F**
Rhizobacteria	4	32252.99145	8063.24786	322.53	<.0001
Time	2	1953.84615	976.92308	39.08	<.0001
Rhizobacteria*time	8	4769.44444	596.18056	23.85	<.0001
Error	22	550.00000	25.00000		
Corrected total	38	39630.76923			

Appendix 4. Step of sample collection up to preservation of rhizobacteria isolates.

Appendix 5. ***In vivo*** **arrangement of detached coffee berries and application of rhizobacterial suspension**

Appendix 6. Detached green berries by (1-5) scale disease management

Appendix 7. Detached berries tested by different rhizobacterial suspension



Appendix 8. Morphological and biochemical characterization of rhizobacteria isolates from Arabica Coffee (*C. arabica* L.) rhizosphere

S. No	Area	Sample Code	Isolation	Morphological characteristics								Bio-chemical characteristics					Genus/ species
				bacteria colony color	Isolate Code	Form of bacteria	Texture	Elevation of Bacteria	Shape	Endo-spore	Gram stain	H_2O_2	KOH	O/ F	M	Ox	
1	Gera	Q1T1	G1	Y-orange	JU111	Circle	Dull	Con-flat	Short rod	no	-	+	+	O	-	+	*Flavobacter*
2	Gera	Q1T1	G2	Y-Cream	JU112	Circle	Smooth	Flat	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
3	Gera	Q1T2	G1	Cream	JU122	Circle	Smooth	Flat	Rod	no	-	+	-	O	+	+	*Pseudomonas*
4	Gera	Q1T2	G3	Yellow	JU123	Circle	Smooth	Flat	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
5	Gera	Q1T2	G4	Light yellow	JU124	Circle	Smooth	Convex	Rod	form	+	+	+	F	+	-	*Bacillus*
6	Gera	Q1T3	G	Cream	(JU13)	Circle	Smooth	Flat	Rod	no	-	+	-	O	+	+	*Pseudomonas*
7	Gera	Q1T3	G1	White-cream	(JU13)1	Circle	Smooth	Convex	Cocci bacillary	no	-	+	+	O	-	-	*Acinatobacter*
8	Gera	Q1T3	G2	Yellow	(JU13)2	Circle	Smooth	Convex	Rod bipolar	no	-	+	-	O	+	-	*Burkoholderia*
9	Gera	Q1T3	G3	White-cream	(JU13)3	Circle	Rough	Flat	Rod	form	+	+	+	F	+	-	*Bacillus sp.*
10	Gera	Q1T3	G4	White-Cream	(JU13)4	Circle	Rough	Raised	Rod	form	+	+	+	F	+	-	*Bacillus sp*
11	Gera	Q1T3	G5	Yellow	(JU13)5	Circle	Smooth	Convex	Rod	no	-	+	-	O	+	-	*Burkholderia*
12	Gera	Q1T3	G6	White-cream	(JU13)6	Circle	Smooth	Raised	Rod	form	+	+	+	F	+	+	*Bacillus sp.*
13	Gera	Q3T1	G1	Light Yellow	JU311	Irregular	Smooth	Flat	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
14	Gera	Q3T1	G2	Light yellow	JU312	Circle	Smooth	Raised	Rod	form	+	+	+	F	+	-	*Bacillus*
15	Gera	Q3T2	G1	Y-orange	JU321	Circle	Dull	Con-flat	Short rod	no	-	+	+	O	-	+	*Flavobacter*
16	Gera	Q3T3	G1	White	JU331	Irregular	Smooth	Raised	Rod	form	+	+	+	I	+	-	*Bacillus*
17	Gera	Q3T3	G2	Light Yellow	JU332	Circle	Smooth	Raised	Rod	no	-	+	+	O	+	-	*Burkholderia*
18	Gera	Q3T3	G3	Yellow	JU333	Circle	Smooth	Flat	Rod short	no	-	+	+	O	-	+	*Flavobacter*
19	Gera	Q4T1	G1	Light-yellow	JU411	Circle	Smooth	Convex	Cocci rod	no	+	+	-	F	-	+	*Arthrobacter*
20	Gera	Q4T1	G2	Y-orange	JU412	Circle	Dull	Con-flat	Short rod	no	-	+	+	O	-	+	*Flavobacter*
21	Gera	Q4T1	G3	Light-yellow	JU413	Circle	Smooth	Convex	Cocci rod	no	+	+	-	F	-	+	*Arthrobacter*
22	Gera	Q4T1	G4	White-Cream	JU414	Circle	Rough	Raised	Rod	form	+	+	+	O	+	-	*Bacillus sp.*
23	Gera	Q4T2	G1	Cream yellow	JU421	Circle	Smooth	Raised	Cocci cluster	no	+	+	+	F	-	-	*Staphylococcus*

24	Gera	Q4T2	G2	Y-orange	JU422	Circle	Dull	Con-flat	Short rod	no	-	+	+	O	-	+	*Flavobacter*
25	Gera	Q4T2	G3	Cream	JU423	Circle	Rough	Flat	Rod	form	+	+	+	O	+	+	*Bacillus*
26	Gera	Q4T2	G4	Cream	JU424	Irregular	Smooth	Flat	Rod	form	+	+	+	F	+	-	*Bacillus*
27	Gera	Q4T3	G1	White	JU431	Circle	Rough	Raised	Rod	form	+	+	+	O	+	-	*Bacillus sp.*
28	Gera	Q4T3	G2	Whit-Cream	JU432	Circle	Rough	Raised	Cocci chain	no	+	-	+	F	-	-	*Enterococcus*
29	Gera	Q4T3	G3	White	JU433	Circle	Smooth	Raised	Rod	form	+	+	+	I	+	-	*Bacillus*
30	Gera	Q4T3	G4	White	JU434	Circle	Rough	Raised	Cocci chain	no	+	-	+	F	-	-	*Enterococcus*
31	Gera	Q5T2	G1	Light yellow	JU521	Circle	Smooth	Raised	Rod	form	+	+	+	F	+	-	*Bacillus*
32	Gera	Q5T2	G2	Y-Cream	JU522	Circle	Smooth	Flat	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
33	Gera	Q5T3	G1	Y-Cream	JU531	Circle	Smooth	Flat	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
34	Gera	Q5T3	G2	White	JU532	Irregular	Smooth	Flat	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
35	Gera	Q5T3	G3	Cream	JU533	Irregular	Smooth	Convex	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
36	Gera	Q5T4	G2	Yellow-cream	JU542	Irregular	Smooth	Flat	Rod chain	no	-	+	-	O	-	+	*Pseudomonas*
37	Gera	Q5T4	G3	White-cream	JU543	Irregular	Smooth	Flat	Rod	no	-	+	+	O	+	+	*Pseudomonas*
38	Gera	Q5T4	G4	White	(JU544)	Circle	Smooth	Raised	Rod	form	+	+	+	I	+	-	*Bacillus*
39	Gera	Q5T4	G5	White-cream	JU545	Circle	Smooth	Raised	Rod	form	+	+	+	F	+	-	*Bacillus sp*
40	Gera	Q6T1	G1	White-cream	JU611	Circle	Rough	Raised	Cocci chain	no	+	-	+	F	-	-	*Enterococcus*
41	Gera	Q6T1	G2	Light yellow	JU612	Irregular	Rough	Flat	Rod Cocci	no	+	+	+	F	-	-	*Arthrobacter*
42	Gera	Q6T1	G3	White	JU613	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
43	Gera	Q6T1	G4	Cream	JU614	Circle	Smooth	Convex	Rod	form	+	+	+	O	+	-	*Bacillus*
44	Gera	Q6T1	G5	Light-yellow	JU615	Circle	Smooth	Convex	Cocci rod	no	+	+	-	F	-	+	*Arthrobacter*
45	Gera	Q6T2	G1	White-cream	JU621	Circle	Dull	Raised	Cocci bacilli	no	-	+	+	F	-	-	*Flavobacter*
46	Gera	Q6T2	G2	White	JU622	Circle	Rough	Raised	Rod	no	-	+	+	F	+	+	*Bacillus*
47	Gera	Q6T2	G3	Light-yellow	JU623	Circle	Smooth	Convex	Cocci rod	no	+	+	-	F	-	+	*Arthrobacter*
48	Gera	Q6T2	G4	Cream	JU624	Circle	Smooth	Raised	Rod	no	-	+	+	O	+	+	*Pseudomonas*
49	Gera	Q6T2	G5	Yellow	JU625	Circle	Rough	Convex	Rod	no	-	+	-	O	+	-	*Burkoholderia*
50	Gera	Q6T3	G1	Pink	JU631	Circle	Smooth	Raised	Cocci bacilli	no	-	+	+	O	-	-	*Acinetobacter*
51	Gera	Q6T3	G2	White	JU632	Circle	Rough	Raised	Rod	no	-	+	+	F	+	+	*Bacillus*
52	Gera	Q6T3	G3	Cream	JU633	Circle	Smooth	Raised	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
53	Gera	Q6T3	G4	White - yellow	JU634	Circle	Smooth	Raised	Cocci rod	no	+	+	+	F	+	-	*Arthrobacter*

54	Gera	Q6T3	G5	Light Pink	JU635	Circle	Rough	Flat	Cocci bacilli	form	+	+	+	F	+	-	*Bacillus*
55	Gera	Q6T3	G6	White-cream	JU636	Circle	Smooth	Raised	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
56	Gera	Q6T3	G7	White	JU637	Circle	Rough	Raised	Coccibacillary	no	-	+	+	O	-	-	*Acinetobacter*
57	Gera	Q6T3	G8	White	JU638	Circle	Rough	Raised	Rod	no	-	+	+	F	+	+	*Bacillus*
58	Gera	Q7T1	G1	Cream	JU711	Irregular	Rough	Convex	Rod	form	+	+	+	F	+	-	*Bacillus*
59	Gera	Q7T1	G2	White-Cream	JU712	Circle	Smooth	Flat	Rod	form	+	+	+	O	+	-	*Bacillus sp*
60	Gera	Q7T1	G3	Light-pink	JU713	Circle	Smooth	Raised	Cocci bacillary	no	-	+	+	O	-	-	*Acinetobacter*
61	Gera	Q7T1	G4	White	JU714	Circle	Smooth	Flat	Rod	form	+	+	+	O	+	+	*Bacillus*
62	Gera	Q7T1	G5	White	JU715	Circle	Rough	Raised	Rod	no	-	+	+	F	+	+	*Bacillus*
63	Gera	Q7T1	G6	White	JU716	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
64	Gera	Q7T2	G1	Cream-yellow	JU721	Circle	Smooth	Raised	Cocci cluster	no	+	+	+	F	-	-	*Staphylococcus*
65	Gera	Q7T2	G2	Cream-yellow	JU722	Circle	Smooth	Raised	Cocci cluster	no	+	+	+	F	-	-	*Staphylococcus*
66	Gera	Q7T2	G3	White-cream	JU723	Circle	Smooth	Raised	Rod	form	+	+	+	F	+	-	*Bacillus sp*
67	Gera	Q7T2	G4	C - Yellow	JU724	Circle	Rough	Convex	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
68	Gera	Q7T2	G5	White-Cream	JU725	Circle	Rough	Raised	Rod	form	+	+	+	O	+	-	*Bacillus sp.*
69	Gera	Q7T2	G6	White-cream	JU726	Irregular	Smooth	Flat	Rod	no	-	+	+	O	+	+	*Pseudomonas*
70	Gera	Q7T2	G7	W-yellow	JU727	Circle	Rough	Raised	Cocci –rod	no	+	+	+	F	-	-	*Arthrobacter*
71	Gera	Q7T3	G1	White	JU731	Circle	Rough	Raised	Rod	no	-	+	+	F	+	+	*Bacillus*
72	Gera	Q7T3	G2	White	JU732	Circle	Rough	Convex	Rod	no	-	+	+	F	+	-	*Entrobacter sp*
73	Gera	Q7T3	G3	Yellow	JU733	Circle	Smooth	Convex	Rod bipolar	no	-	+	-	O	+	-	*Burkoholderia*
74	Gera	Q7T3	G4	White	JU734	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
75	Gera	Q7T3	G5	Light Pink	(JU735)	Circle	Smooth	Flat	Rod	form	+	+	+	F	+	-	*Bacillus*
76	Gera	Q8T1	G1	White-cream	JU811	Irregular	Smooth	Flat	Coccibacillar	no	-	+	+	O	+	+	*Pseudomonas*
77	Gera	Q8T1	G2	Cream	JU813	Irregular	Smooth	Flat	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
78	Gera	Q8T1	G3	White	JU813	Circle	Smooth	Raised	Rod	form	+	+	+	F	+	-	*Bacillus*
79	Gera	Q8T1	G4	White	JU814	Irregular	Rough	Flat	Coccibacillary	form	+	+	+	F	+	-	*Bacillus*
80	Gera	Q8T1	G5	Cream	JU815	Circle	Smooth	Raised	Rod	no	+	+	+	O	-	+	*Pseudomonas*
81	Gera	Q8T1	G6	Yellow C	JU816	Irregular	Smooth	Flat	Cocci chain	no	+	-	+	F	-	-	*Enterococcus*
82	Gera	Q8T1	G7	Cream	JU817	Circle	Smooth	Raised	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
83	Gera	Q8T2	G1	White-cream	JU821	Circle	Smooth	Flat	Cocci chain	no	+	-	+	F	-	-	*Enterococcus*
84	Gera	Q8T2	G2	Yellow	JU822	Circle	Smooth	Convex	Rod	no	-	+	-	O	+	-	*Burkoholderia*
85	Gera	Q8T2	G3	White	JU823	Circle	Rough	Raised	Cocci chain	no	+	-	+	F	-	-	*Enterococcus*

86	Gera	Q8T2	G4	Yellow	JU824	Circle	Rough	Convex	Cocci chain	no	+	+	+	O	-	-	*Micrococous*
87	Gera	Q8T2	G5	White	JU825	Circle	Smooth	Raised	Rod	form	+	+	+	I	+	-	*Bacillus*
88	Gera	Q8T2	G6	Yellow	JU826	Circle	Smooth	Convex	Rod	no	-	+	-	O	+	-	*Burkoholderia*
89	Gera	Q8T2	G7	White-cream	JU827	Flat	Rough	Unbonat	Rod	form	+	+	+	O	-	+	*Bacillus sp*
90	Gera	Q8T2	G8	Cream	JU828	Circle	Smooth	Unbonat	Cocci chain	no	+	-	+	F	-	-	*Enterococcus*
91	Gera	Q8T3	G1	Cream	JU815	Circle	Smooth	Raised	Rod	no	+	+	+	O	-	+	*Pseudomonas*
92	Gera	Q8T3	G2	Yellow C	JU816	Irregula	Smooth	Flat	Cocci chain	no	+	-	+	F	-	-	*Enterococcus*
93	Gera	Q8T3	G3	Light-yellow	JU833	Circle	Smooth	Convex	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
94	Gera	Q8T3	G4	Cream	JU817	Circle	Smooth	Raised	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
95	Gera	Q8T3	G5	White-cream	JU835	Circle	Smooth	Convex	Rod	form	+	+	+	F	-	-	*Bacillus*
96	Gera	Q8T4	G1	White-cream	JU821	Circle	Smooth	Flat	Cocci chain	no	+	-	+	F	-	-	*Enterococcus*
97	Gera	Q8T4	G2	Yellow	JU822	Circle	Smooth	Convex	Rod	no	-	+	-	O	+	-	*Burkoholderia*
98	Gera	Q8T4	G3	White	JU823	Circle	Rough	Raised	Cocci chain	no	+	-	+	F	-	-	*Enterococcus*
99	Gera	Q8T4	G4	Yellow	JU824	Circle	Rough	Convex	Cocci chain	no	+	+	+	O	-	-	*Micrococous*
100	Gera	Q8T4	G5	White	JU825	Circle	Smooth	Raised	Rod	form	+	+	+	I	+	-	*Bacillus*
101	Gera	Q8T4	G6	Yellow	JU826	Circle	Smooth	Convex	Rod	no	-	+	-	O	+	-	*Burkoholderia*
102	Gera	Q8T4	G7	White	JU831	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
103	Gera	Q8T4	G9	White	JU832	Circle	Rough	Convex	Rod	no	-	+	+	F	+	-	*Entrobacter sp*
104	Gera	Q8T4	G10	Off White	JU834	Circle	Rough	Raised	Coccibacillary	no	-	+	+	O	-	-	*Acinetobacter*
105	I/Dal	D1T1	G1	White	JUD111	Circle	Rough	Convex	Rod	no	-	+	+	F	+	-	*Entrobacter sp*
106	I/Dal	D1T1	G2	Yellow	JUD112	Circle	Smooth	Convex	Rod	no	-	+	-	O	+	-	*Burkoholderia*
107	I/Dal	D1T1	G3	White-cream	JUD113	Irregular	Smooth	Flat	Rod	no	-	+	+	O	+	+	*Pseudomonas*
108	I/D	D1T2	G1	Cream	JUD121	Circle	Smooth	Raised	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
109	I/D	D1T2	G2	White	JUD122	Circle	Smooth	Raised	Coccibacillary	form	+	+	+	F	+	-	*Bacillus sp*
110	I/D	D1T3	G1	Cream	JUD131	Circle	Smooth	Convex	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
111	I/D	D1T3	G2	White	JUD132	Irregular	Smooth	Flat	Rod curve	no	-	+	+	O	+	+	*Pseudomonas*
112	I/D	D1T3	G3	White-cream	JUD133	Irregular	Smooth	Flat	Rod	no	-	+	+	O	+	+	*Pseudomonas*
113	I/D	D1T4	G1	White-cream	JUD141	Irregular	Smooth	Flat	Cocci bacilli	no	-	+	+	O	-	-	*Acinatobacter*
114	I/D	D1T4	G2	Cream-white	JUD142	Irregular	Smooth	Raised	Rod	form	+	+	+	O	-	-	*Bacillus*
115	I/D	D2T1	G1	White	JUD211	Circle	Smooth	Flat	Cocci chain	no	+	+	+	F	-	-	*Enterococcus*
116	I/D	D2T1	G2	Light-yellow	JUD212	Circle	Smooth	Convex	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*

117	I/D	D2T2	G1	White-purpl	JUD221	Circle	Smooth	Raised	Rod chain	no	-	+	+	O	+	+	*Pseudomonas*
118	I/D	D2T2	G2	Cream	JUD222	Circle	Smooth	Raised	Coccibacillary	no	-	+	+	O	+	+	*Pseudomonas*
119	I/D	D2T3	G1	White	JUD231	Circle	Smooth	Flat	Rod	form	+	+	+	O	+	+	*Bacillus*
120	I/D	D2T3	G2	White	JUD232	Circle	Rough	Raised	Rod	no	-	+	+	F	+	+	*Bacillus*
121	I/D	D2T4	G1	White	JUD241	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
122	I/D	D2T4	G2	Creamyellow	JUD242	Circle	Smooth	Raised	Cocci cluster	no	+	+	+	F	-	-	*Staphylococcus*
123	I/D	D2T5	G1	Creamyellow	JUD251	Circle	Smooth	Raised	Cocci cluster	no	+	+	+	F	-	-	*Staphylococcus*
124	I/D	D2T5	G2	White-cream	JUD252	Circle	Smooth	Raised	Rod	form	+	+	+	F	+	-	*Bacillus sp*
125	I/D	D3T1	G1	CreamYellow	JUD311	Circle	Rough	Conve	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
126	I/D	D3T1	G2	White-Cream	JUD312	Circle	Rough	Raised	Rod	form	+	+	+	O	+	-	*Bacillus sp.*
127	I/D	D3T1	G3	White-cream	JUD313	Irregular	Smooth	Flat	Rod	no	-	+	+	O	+	+	*Pseudomonas*
128	I/D	D3T2	G1	W-yellow	JUD321	Circle	Rough	Raised	Cocci –rod	no	+	+	+	F	-	-	*Arthrobacter*
129	I/D	D3T2	G2	White	JUD322	Circle	Rough	Raised	Rod	no	-	+	+	F	+	+	*Bacillus*
130	I/D	D3T2	G3	White	JUD323	Circle	Rough	Convex	Rod	no	-	+	+	F	+	-	*Entrobacter sp*
131	I/D	D3T3	G1	Yellow	JUD311	Circle	Smooth	Convex	Rod bipolar	no	-	+	-	O	+	-	*Burkoholderia*
132	I/D	D3T3	G2	White	JUD332	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
133	I/D	D3T3	G3	Light Pink	JUD333	Circle	Smooth	Flat	Rod	form	+	+	+	F	+	-	*Bacillus*
134	I/D	D3T4	G1	White-cream	JUD341	Irregular	Smooth	Flat	Rod	no	-	+	+	O	+	+	*Pseudomonas*
135	I/D	D3T4	G2	Light yellow	JUD342	Irregular	Rough	Flat	Rod Cocci	no	+	+	+	F	-	-	*Arthrobacter*
136	I/D	D3T4	G3	White	JUD343	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
137	I/D	D4T1	G1	Cream	JUD411	Circle	Smooth	Convex	Rod	form	+	+	+	O	+	-	*Bacillus*
138	I/D	D4T1	G2	Light-yellow	JUD412	Circle	Smooth	Convex	Cocci rod	no	+	+	-	F	-	+	*Arthrobacter*
139	I/D	D4T1	G3	White-cream	JUD413	Circle	Dull	Raised	Cocci bacilli	no	-	+	+	F	-	-	*Flavobacter*
140	I/D	D4T1	G4	White	JUD414	Circle	Rough	Raised	Rod	no	-	+	+	F	+	+	*Bacillus*
141	I/D	D4T2	G1	Light-yellow	JUD421	Circle	Smooth	Convex	Cocci rod	no	+	+	-	F	-	+	*Arthrobacter*
142	I/D	D4T2	G2	Cream	JUD422	Circle	Smooth	Raised	Rod	no	-	+	+	O	+	+	*Pseudomonas*
143	I/D	D4T3	G1	Yellow	JUD431	Circle	Rough	Convex	Rod	no	-	+	-	O	+	-	*Burkoholderia*
144	I/D	D4T3	G2	White	JUD432	Circle	Rough	Raised	Cocci chain	no	+	+	+	F	-	-	*Enterococcus*
145	I/D	D4T3	G3	White	JUD433	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*

146	I/D	D4T3	G4	Cream	JUD434	Circle	Smooth	Flat	Rod	form	+	+	+	F	-	+	*Bacillus*
147	I/D	D5T1	G1	Pure white	JUD511	Circle	Smooth	Raised	Coccibacillary	form	+	+	+	F	-	-	*Bacillus sp*
148	I/D	D5T1	G2	White	JUD512	Circle	Rough	Flat	Coccibacillary	no	-	+	+	O	-	-	*Acinetobacter*
149	I/D	D5T2	G1	Y -cream	JUD521	Circle	Smooth	Unbonat	Cocci chain	no	+	+	+	F	-	-	*Enterococcus*
150	I/D	D5T2	G2	White	JUD522	Circle	Rough	Convex	Rod	form	+	+	+	F	+	-	*Bacillus*
151	I/D	D5T2	G1	Light-yellow	JUD521	Circle	Smooth	Convex	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
152	I/D	D5T2	G2	Cream	JUD522	Circle	Smooth	Raised	Coccibacillary	no	-	+	+	O	+	+	*Pseudomonas*
153	I/D	D5T2	G3	White-cream	JUD523	Irregular	Smooth	Flat	Rod	form	+	+	+	I	-	-	*Bacillus*
154	I/D	D5T3	G1	Light cream	JUD531	Circle	Smooth	Flat	Rod	form	+	+	+	F	+	+	*Bacillus*
155	I/D	D5T3	G2	Yellow	JUD532	Circle	Smooth	Convex	Rod bipolar	no	-	+	-	O	+	-	*Burkoholderia*
156	I/D	D5T3	G1	White	JUD531	Circle	Rough	Convex	Coccibacillary	no	-	+	+	F	+	-	*Entrobacter sp*
157	I/D	D5T3	G2	White	JUD532	Circle	Smooth	Raised	Coccibacillary	form	+	+	+	F	-	-	*Bacillus sp*
158	I/D	D5T3	G3	Light white	JUD533	Irregular	Rough	Flat	Rod	form	+	+	+	F	+	-	*Bacillus sp*
159	I/D	D5T4	G5	White-cream	JUD545	Irregular	Smooth	Flat	Rod	no	-	+	+	O	+	+	*Pseudomonas*
160	I/D	D5T4	G6	White	JUD546	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
161	I/D	D5T4	G7	Light Pink	JUD547	Circle	Smooth	Flat	Cocci bacilli	form	+	+	+	F	+	-	*Bacillus*
162	I/D	D5T4	G8	White-cream	JUD548	Irregular	Smooth	Flat	Rod	no	-	+	+	O	+	+	*Pseudomonas*
163	I/D	D5T5	G6	White-cream	JUD556	Irregular	Smooth	Flat	Rod	no	-	+	+	O	+	+	*Pseudomonas*
164	I/D	D5T5	G7	White-cream	JUD557	Circle	Smooth	Raised	Rod	form	+	+	+	F	+	+	*Bacillus sp*
165	I/D	D5T5	G8	White	JUD558	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
166	I/D	D5T5	G9	White	JUD559	Circle	Smooth	Raised	Rod	form	+	+	+	F	-	-	*Bacillus*
167	I/D	D5T6	G6	White	JUD566	Circle	Rough	Raised	Cocci chain	no	+	+	+	F	-	-	*Enterococcus*
168	I/D	D5T6	G7	White	JUD567	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
169	I/D	D5T6	G8	Cream	JUD568	Circle	Smooth	Flat	Rod	form	+	+	+	F	-	+	*Bacillus*
170	I/D	D5T6	G9	White	JUD569	Circle	Smooth	Flat	Cocci cluster	no	+	+	+	F	-	-	*Staphylococcus*
171	I/D	D5T6	G10	White	JUD560	Irregular	Rough	Flat	Rod	form	+	+	+	O	+	+	*Bacillus*
172	JUC	J1T1	G1	White-cream	JUC111	Circle	Rough	Raised	Rod	no	-	+	-	O	+	+	*Pseudomonas*
173	JUC	J1T1	G2	White	JUC112	Circle	Rough	Raised	Coccibacillary	no	-	+	+	O	-	-	*Acinetobacter*
174	JUC	J1T1	G3	White-cream	JUC113	Irregular	Smooth	Flat	Rod	form	+	+	+	F	+	-	*Bacillus*
175	JUC	J1T2	G1	White-cream	JUC121	Irregular	Smooth	Flat	Coccibacillar	no	-	+	+	O	+	+	*Pseudomonas*
176	JUC	J1T2	G2	White	JUC122	Circle	Smooth	Convex	Coccibacillary	no	-	+	+	F	+	-	*Entrobacter sp*

177	JUC	J1T2	G3	Light cream	JUC123	Irregular	Smooth	Flat	Coccibacillary	form	+	+	+	F	+	-	*Bacillus*
178	JUC	J1T3	G2	Cream	JUC132	Circle	Smooth	Unbonat	Cocci chain	no	+	-	+	F	-	-	*Enterococcus*
179	JUC	J1T4	G1	Creamyello w	JUC141	Circle	Smooth	Raised	Cocci cluster	no	+	+	+	F	-	-	*Staphylococcus*
180	JUC	J1T4	G2	White	JUC142	Circle	Rough	Flat	Cocci chain	no	+	-	+	F	-	-	*Enterococcus*
181	JUC	J1T5	G1	Cream	JUC151	Circle	Smooth	Flat	Rod	form	+	+	+	I	-	-	*Bacillus*
182	JUC	J1T5	G2	White	JUC152	Irregular	Rough	Flat	Coccibacillary	form	+	+	+	F	-	-	*Bacillus*
183	JUC	J1T5	G3	White	JUC153	Circle	Rough	Raised	Coccibacilli	form	+	+	+	O	+	+	*Bacillus*
184	JUC	J1T6	G1	White	JUC161	Circle	Smooth	Flat	Coccibacilli	form	+	+	+	F	+	-	*Bacillus*
185	JUC	J1T6	G2	White	JUC162	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
186	JUC	J2T1	G1	Yellow	JUC211	Circle	Smooth	Convex	Rod	no	-	+	-	O	+	-	*Burkoholderia*
187	JUC	J2T1	G2	White	JUC212	Irregular	Rough	Flat	Rod	form	+	+	+	F	+	-	*Bacillus*
188	JUC	J2T2	G1	White-cream	JUC221	Irregular	Smooth	Flat	Rod	no	-	+	+	O	+	+	*Pseudomonas*
189	JUC	J2T2	G2	White	JUC222	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
190	JUC	J2T3	G1	Light Pink	JUC231	Circle	Smooth	Flat	Cocci bacilli	form	+	+	+	F	+	-	*Bacillus*
191	JUC	J2T3	G2	White-cream	JUC232	Irregular	Smooth	Flat	Rod	no	-	+	+	O	+	+	*Pseudomonas*
192	JUC	J2T4	G1	White	JUC241	Circle	Smooth	Convex	Rod short	no	-	+	+	O	+	+	*Pseudomonas*
193	JUC	J2T4	G2	White	JUC242	Circle	Rough	Convex	Rod	no	-	+	+	F	+	-	*Entrobacter sp*
194	JUC	J2T5	G1	Light-yellow	JUC251	Circle	Smooth	Convex	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
195	JUC	J3T1	G1	White	JUC311	Circle	Rough	Convex	Rod	no	-	+	+	F	+	-	*Entrobacter sp*
196	JUC	J3T1	G2	Yellow	JUC312	Circle	Smooth	Convex	Rod	no	-	+	-	O	+	-	*Burkoholderia*
197	JUC	J3T1	G3	White-cream	JUC313	Irregular	Smooth	Flat	Rod	no	-	+	+	O	+	+	*Pseudomonas*
198	JUC	J3T2	G1	Cream	JUC321	Circle	Smooth	Raised	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
199	JUC	J3T2	G2	Yellow	JUC322	Circle	Smooth	Convex	Rod	no	-	+	-	O	+	-	*Burkholderia*
200	JUC	J3T2	G3	White-cream	JUC323	Circle	Smooth	Raised	Rod	form	+	+	+	F	+	+	*Bacillus sp.*
201	JUC	J3T3	G1	Light Yellow	JUC331	Irregul	Smooth	Flat	Cocci chain	no	+	+	+	O	-	-	*Micrococcus*
202	JUC	J3T3	G2	Light yellow	JUC332	Circle	Smooth	Raised	Rod	form	+	+	+	F	+	-	*Bacillus*
203	JUC	J3T4	G1	White	JUC341	Irregular	Smooth	raised	Rod	form	+	+	+	I	+	-	*Bacillus*
204	JUC	J3T3	G3	Y-orange	JUC333	Circle	Dull	Con-flat	Short rod	no	-	+	+	O	-	+	*Flavobacter*
205	JUC	J3T4	G2	Light Yellow	JUC342	Circle	Smooth	Raised	Rod	no	-	+	+	O	+	-	*Burkholderia*
206	JUC	J3T4	G3	Yellow	JUC343	Circle	Smooth	Flat	Rod short	no	-	+	+	O	-	+	*Flavobacter*
207	JUC	J3T5	G1	Light-yellow	JUC351	Circle	Smooth	Convex	Cocci rod	no	+	+	-	F	-	+	*Arthrobacter*

208	JUC	J3T5	G2	Y-orange	JUC352	Circle	Dull	Con-flat	Short rod	no	-	+	+	O	-	+	*Flavobacter*
209	JUC	J4T1	G1	Light-yellow	JUC411	Circle	Smooth	Convex	Cocci rod	no	+	+	-	F	-	+	*Arthrobacter*
210	JUC	J4T1	G2	White	JUC412	Circle	Rough	Raised	Coccibacillary	no	-	+	+	O	-	-	*Acinetobacter*
211	JUC	J4T2	G1	White	JUC421	Circle	Rough	Raised	Rod	no	-	+	+	F	+	+	*Bacillus*
212	JUC	J4T2	G2	Cream	JUC422	Irregular	Rough	Convex	Rod	form	+	+	+	F	+	-	*Bacillus*
213	JUC	J4T3	G1	White-Cream	JUC431	Circle	Smooth	Flat	Rod	form	+	+	+	O	+	-	*Bacillus sp*
214	JUC	J4T3	G2	Light-pink	JUC432	Circle	Smooth	Raised	Cocci bacillary	no	-	+	+	O	-	-	*Acinetobacter*
215	JUC	J4T3	G3	White-cream	JUC433	Circle	Smooth	Raised	Rod	form	+	+	+	F	-	+	*Bacillus*