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*Full Length Research Paper*

# Molecular characterization of *Mycobacterium tuberculosis complex* in Gambella region, Southwest Ethiopia

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**The information on the genetic diversity of *Mycobacterium tuberculosis* in certain geographic region is needed for a better understanding of epidemiology of TB and could have implications for development of new diagnostics, drugs, and vaccines. The aim of this study was to provide information on the strains of *M. tuberculosis* circulating in Gambella region. Cross-sectional study was conducted for six months (November 2012 and April, 2013) in order to generate a primary data on the genetic diversity of *M. tuberculosis.* Clinical examination, Ziehl Neelsen staining, Mycobacterium culturing and molecular typing were used. *M. tuberculosis* isolates were characterized using region of difference 9 (RD9) and spoligotyping. The patterns of strains identified by spoligotyping were compared with the patterns registered in the SpoIDB4 database of the Pasteur Institute. Culture positivity was confirmed in 53.49 % (46/86) of the smear positive cases. Upon molecular characterization, 86.95% (40/46) of culture positive samples were *M. tuberculosis.* The spoligotyping of the 40 isolates lead to the identification of 24 spoligotype patterns. Seven of the patterns were clustered and consisted of 2-3 isolates while the remaining 17 patterns were non-clustered consisting of a single isolate. The most prevalent strain was Spoligotyping International Typing (SIT) 289 with a proportion of 20.0% (8/40). Grouping of the isolates into lineages showed that 32.50% (13/40) were Euro-American, 17.50% (7/40) Indo-Oceanic, and amazingly the remaining 50.0% (20/40) of the isolates could not be grouped into the already known lineages and hence they were new. Findings from this study show that most of strains of *M. tuberculosis* circulating in the region are different from those reported from the other areas of the country. Hence, TB control program should give attention to the control of TB in Gambella region. In addition, characterizing the strains of *M. tuberculosis* circulating in the region would play a great role towards the mapping of molecular epidemiology of TB in Ethiopia.**

**Key words:** Mycobacterium tuberculosis, molecular typing, strain, lineage, Gambella region.

## INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease mainly caused by *Mycobacterium tuberculosis Complex (MTBC).*

The main source of infection is untreated smear-positive pulmonary TB (PTB) patient discharging the bacilli. It mainly spreads by an airborne route when the infectious patient expels droplets containing viable bacilli. It is alsotransmitted by consumption of raw milk containing *Mycobacterium bovis (M. bovis)* (Harries and Dye, 2006; Palomino et al., 2007).

According to a recent WHO report the case detection rate of TB trend in Ethiopia was in increasing manner which was 11% in 1995, and increased to 72% in 2011 (WHO, 2012). A ten year TB trend epidemiological analysis of TB conducted in Ethiopia, by Nigatu and Abraha (2010), indicated that the incidence rate of TB is increasing in at a rate of 5 new TB cases per 100,000 populations per year. Urban agro-ecological zones have been more affected by the disease throughout the ten-year period. Extra-pulmonary rate and smear-negativity has shown a modest increment and males were disproportionately affected by TB within the ten-years. On the other hand case detection rate and treatment success rate are found to be increasing at a rate of 0.5% per year. Federal Ministry of Health of Ethiopia also indicated the rising of detection rate of TB was seen since 2004 to 2009 (FMoH, 2011). WHO (2009) report that the status of TB in Gambella Region was the highest from all the Ethiopian Regions, with the notification rate (new and relapse) 261-421/100, 000 population (WHO, 2009). In high TB incidence settings, like Ethiopia, determination of distinct transmission patterns is often hard to define, but may be greatly enhanced by the use of both molecular and conventional epidemiological tools.

Using genotyping methods and markers for molecular characterization of TB have been applied extensively in the epidemiological study worldwide in the last decades (Candia et al., 2007; Sharma et al., 2008; Valcheva et al., 2008).

Some of the more popular MTBC typing methods being used include IS*6110*-based restriction fragment length polymorphism (RFLP) (van Embden et al., 1993) and PCR based methods like spoligotyping (Kamerbeek et al., 1997), and others like mycobacterial interspersed repetitive units - variable number of tandem repeats (MIRU-VNTR), single-nucleotide polymorphisms and large-sequence polymorphism analysis (Frothingham et al., 1998; Le Fleche et al., 2002; Savine et al., 2002). In this study, we set out an objective to investigate the circulating MTBC isolates, and use spoligotyping typing to distinguish MTBC isolates from Gambella regional hospital, located southwest Ethiopia. The present study has been based on the assumption that patients with genotypically clustered strains are epidemiologically linked and represent recent transmissions. In contrast, patients infected with different types of strains are not considered indicative of recent transmission.

## MATERIALS AND METHODS

### Study area

The study was conducted in Gambella Peoples National Regional State, Gambella town. The region is located in the South Western part of Ethiopia and borders two other regions - Oromia to the North and east and the Southern Nations, Nationalities and Peoples" Regional State to the south and South Sudan to the west. The Gambella is a name for both the region and the city, which is located about 753 km West of Addis Ababa perched at an elevation of 526 m above sea level. The town is founded on the banks of the Baro River, Ethiopia's widest and the only navigable river. Geographically, the portion of Gambella Region is situated in what is known as the western lowland has coordinates 60 28'38" to 80 34' North Latitude and 330 to 350 11'11" East Longitude. Gambella town is located inside the boundary of the Gambella Zuriya woreda, which again this is bordered on the south by the Abol Woreda, on the North and East by Oromia Regional state, on the West by Itang woreda (Figure 1). Based on the 2007 Ethiopian Statistical projections estimate the total population estimated was 390, 593 by the end of 2012 (CSA, 2012). All the people of the region estimated at 390, 593 assumed to be the catchment of the hospital, while around 44, 269 people visit in the hospital and gets service annually (Gambella Regional Health Bureau, 2012).

### Study design

An institutional based cross-sectional study was conducted for six month (November 2012 to April, 2013) to see the period prevalence of TB and molecularly characterize *M. tuberculosis* complex species along with strain diversity and lineages. Besides, TB positive individuals were requested to take HIV testing during sample collection period.

### Study population

The study populations were those who were attending Gambella Regional Hospital TB clinic from November 2012 to 10 April 2013.

### Sample collection and processing

Three times (Spot-morning-spot) sputum samples were collected from each study participant and transported to laboratory for immediate process.

### Laboratory investigations

#### *Microscopic examination*

Smeared on clean slides in the hospital laboratory independently, by the ZN staining technique and positive samples were transported to Aklilu Lemma Institute of Pathobiology for further diagnosis by putting under the ice box at 4°C.

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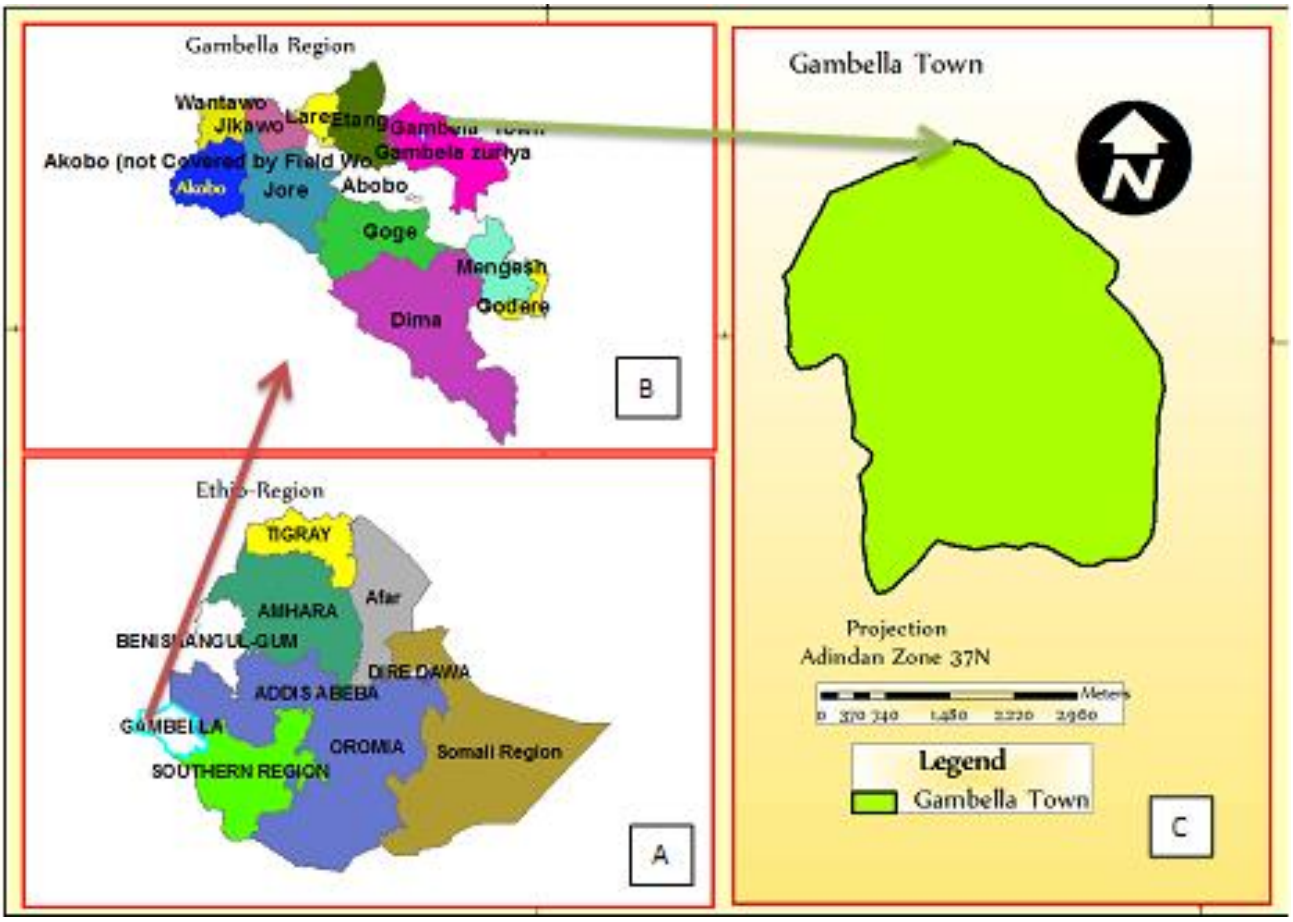


**Figure 1.** Map of study area.. A) Map of Ethiopia. B) Gambella Regional state. C) Gambella town located inside the Gambella Zuriya woreda. Source: Designed by ArcGIS arc map 10 software based on the CSA 2007 data of Ethiopia by region.

***Control Quality control of the slides***

During sputum sample examination positive and negative controls were run each day, when new reagents changed, in order to monitor the quality of reagents. Sputum samples collecting cups and slides were labeled carefully.

**Culture**

The three samples from each patient were pooled, diluted with normal saline and concentrated (Bruchfeld et al., 2000). Mycobacterial culturing was done on conventional Löwenstein-Jensen (LJ) egg medium containing 0.6% sodium pyruvate and 5% glycerol was performed with all sputum samples separately. Before culturing, the samples were digested and decontaminated of non-mycobacterial microorganisms by 4% NaOH (Groothuis and Yates, 1999). This is done by transferring the collected sputum into centrifuge tube and adding equal volume of 4% NaOH to centrifuge tube. Then it is mixed and centrifuged for 15 min at 3000 rpm at 4°C. Then the supernatant decanted and the sediment mixed very well. Following decontamination, we added a phenol red indicator and well-mixed sediment until became red, then 2N HCl added drop by drop until the color changed from purple to yellow. Following neutralization inoculation was conducted by adding one to three drop of neutralized sediment of sputum into two LJ media (pyruvate and glycerol containing), followed by incubation at 37□C with 10% $CO_2$ air at slant position for one week and upright for 8 weeks by monitoring every week for growth at the same temperature and air condition until complete growth.

**Molecular typing**

Heat killed cells were tested by PCR based RD9 deletion typing for the presence or absence of region of difference-9 (RD9) so as to identify *M. tuberculosis* from other species of *M. tuberculosis* complex species. For this deletion typing, a procedure described by Brosch et al. *(*2002) was used. In brief: reaction mixtures were made in a total volume of 20 µl consisting of 10 µl HotStarTaq Master Mix (Qiagen, UK), 7.1 µl distilled water, 0.3 µl of each of the three oligonucleotid primer (100 µM), and 2 µl DNA template samples or controls. *M. tuberculosis* H37Rv and *M. bovis* 2122/97, and water were used as positive and negative controls, respectively. The primers used for RD9 deletion typing were RD9flankF, 5'-GTG TAG GTC AGC CCC ATC C-3'; RD9intR, 5'-CTG GAC CTC GAT GAC CAC TC-3'; and RD9falnkR, 5'-GCC CAA CAG CTC GAC ATC-3'. The reaction mixture was then heated in Thermal Cycler using the following amplification program: 95°C for 10 min for enzyme activation; 95°C for 1 min for denaturation; 55°C for 0.5 min for annealing; 72°C for 2 min for extension, involving

35 cycles all in all; and final extension at $72^0$C for 10 minutes. The product was electrophoresed by Agarose Gel Electrophoresis System (BIO RAD, UK) in 1.5% agarose gel in 1X TAE running buffer. Ethidium Bromide at a ratio of 1:1000, 100 bp DNA reference ladder and orange 6x loading dye were used in agarose gel electrophoresis. The gel was visualized using Multi Image Light Cabinet (Alpha Innotech Corporation, UK) and the photograph was taken. The results were interpreted as *M. tuberculosis* (RD9 present) when a band size of 396 bp was observed.

Finally, PCR products were electrophoresed in 1.5% agarose gel in 10x TAE running buffer with ethidium bromide at a ratio of 1:10. A 100 bp DNA (Promega Cooperation, USA) ladder and Orange 6x loading dye were also used for visual tracking of DNA migration during electrophoresis. The gel was visualized in a Multi- image TM light cabinet using Alpha Innotech version 1.2.0.1 (Alpha Innotech Corporation). The presence of RD9 (i.e. *M. tuberculosis*) gives a product size of 396bp (RD9 FlankF + RD9 Internal) and its absence (*M. africanum*, *M. bovis*) gives a product size of 575bp (RD9 FlankF+RD9 FlankR).

#### Spoligotyping

Spoligotyping was performed as previously described by Kamerbeek et al. (1997) and according to the spoligotype kit supplier's instructions (Ocimum Biosolutions Company, Ijsselstein, The Netherlands). The DR region was amplified by PCR using oligonucleotide primers (DRa: 5' GGT TTT GGG TCT GAC GAC 3' and DRb: 5' CCG AGA GGG GAC GGA AAC 3') derived from the DR sequence. The DRa is biotinylated at the 5'-end.

#### Data processing and analysis

The collected data was checked for any inconvenience and inconsistence and entered to excel spread sheet, coded, and transferred to stata version 11. A p-value less than 0.05 was considered statistically significant. The laboratory result of spoligotype was fed to web based international database of *M. tuberculosis* SITVITWEB version (Dermay et al., 2012) and lineage of mycobacteria, was obtained by SPOTCLUST online software (http://www.tbinsight.cs.rpi.edu/).

#### Ethical clearance

Ethical clearance was obtained from Addis Ababa University, Aklilu Lemma Institute of Pathobiology. A working permission was also obtained from Gambella Regional State Health bureau and Gambella town district health office. Finally written informed consent was signed by study participants.

## RESULTS

### Prevalence of TB cases among the study participants

During the cross-sectional study period, there were 651-suspected individuals registered as TB cases from November 2012 to April 2013 at the Gambella Regional Hospital. Table 1 indicates the proportion of sex, smear result, patient type and age category relative to TB status. The smear positive period prevalence was 13.21% (86/651) among the suspected cases.

### *RD9 deletion typing result*

Majority (87%) of the culture positive samples gave positive signal to RD9 deletion typing as shown in Figure 2 and all the isolates were *M. tuberculosis*. The remaining did not show any band signal to RD9 typing implying that they are not members of the *M. tuberculosis* complex.

### Strain characterization

Identification of the strains and assigning their respective lineages made for 40 *M. tuberculosis* isolates. Accordingly, 57.5% (23/40) of the isolates were found in seven clustered patterns while the remaining 42.50% (17/40) were non-clustered patterns (Table 2). Assigning the isolates to the lineages showed 32.50% (13/40) of the isolates belongs to Euro-American and 17.50% (7/40) Indo-Oceanic. Amazingly, 50.00% (20/40) of the isolates could not be assigned to the presently defined lineages (Table 2). The spolitotyping result shown in Figure 3, with controls and samples. The black squares indicate the presence of spacers while the spaces loss of spacers. The spoligotyping patterns of the isolated strains showed that there is no significant change in their distribution in sex, age residential area or HIV status of the patients (Table 3).

The prevalent strain was SIT289 that comprised of 20.00% (8/40) of the total isolates, while 40% (16/40) did not match the international spoligotyping data base (Table 4).

This figure shows some of the isolates samples; briefly positive (M. tuberculosis H37Rv 2X at 1st and last position) control), negative (distilled H2O at the second position) control; positive (*M. bovis* 2X at 3rd and 21th position) control; 15 unique isolates and 6 clustered isolates

## DISCUSSION

TB has long been recognized as a major public health problem since the 1950s worldwide. By considering the impact, WHO initiated implementation of DOTS strategy in the 1990s to minimize the problem. Advent of HIV / AIDS epidemic makes TB to, remain a major public health problem in endemic developing countries including Ethiopia (WHO, 2005).

In this study, a higher sputum smear positive (SSP) prevalence of TB 69 (14.50) was observed in the age group of 15-44 years while a lower percentage of 2 (12.50%) was seen in age groups of 65 years and above. This finding was similar to observation of Kolifarhood et al. (2012) who reported that 11.2 - 14.5% while, opposite in the age groups of 65≥ years which is 33.5% cases. Proportionally cases residing in Gambella

**Table 1.** Prevalence of TB in the Gambella Regional Hospital from November 2012 to April 2013.

| Variable | Status of TB | | | | Total (N=651) | $\chi^{2}$/fisher Exact | p-value |
|---|---|---|---|---|---|---|---|
| | SSP (n=86) | SSN (n=119) | EPTB (n=32) | Non-TB (n=414) | | | |
| Sex | | | | | | | |
| Male | 51 (13.49) | 77 (20.37) | 23 (6.08) | 227 (60.05) | 378 (100.00) | 6.49 | 0.09 |
| Female | 35 (12.82) | 42 (15.38) | 9 (3.30) | 187 (68.50) | 273 (100.00) | | |
| Total | 86 (13.21) | 119 (18.27) | 32 (4.90) | 414 (63.19) | 651 (100.00) | | |
| Smear result | | | | | | | |
| Positive | 86 (100.00) | - | - | - | 86 (100.00) | | 0.000 |
| Negative | - | 119 (21.06) | 32 (5.66) | 414 (73.27) | 565 (100.00) | | |
| Total | 86 (13.21) | 119 (18.27) | 32 (4.90) | 414 (63.19) | 651 (100.00) | | |
| Age category | | | | | | | |
| 0-14years | - | 24 (36.36) | 7 (10.61) | 35 (53.03) | 66 (100.00) | | 0.000 |
| 15-44years | 69 (14.50) | 84 (17.65) | 24 (5.04) | 299 (62.82) | 476 (100.00) | | |
| 45-64years | 15 (16.13) | 7 (7.53) | - | 71 (76.34) | 93 (100.00) | | |
| >=65years | 2 (12.50) | 4 (25.00) | 1 (6.25) | 9 (56.25) | 16 (100.00) | | |
| Total | 86 (13.21) | 119 (18.27) | 32 (4.90) | 414 (63.19) | 651 (100.00) | | |
| Patient type | | | | | | | |
| New | 86 (13.87) | 97 (15.65) | 25 (4.03) | 412 (66.45) | 620 (100.00) | | 0.000 |
| Relapse | - | 2 (50.0) | 2 (50.0) | - | 4 (100.0) | | |
| Failure | - | 3 (60.0) | 1 (20.0) | 1 (20.0) | 5 (100.00) | | |
| Defaulter | - | 7 (70.0) | 3 (30.0) | - | 10 (100.00) | | |
| Transfer in | - | 10 (83.33) | 1 (8.33) | 1 (8.33) | 12 (100.0) | | |
| Total | 86 (13.21) | 119 (18.27) | 32 (4.90) | 414 (63.19) | 651 (100.00) | | |

SSP= sputum smear positive, SSN= sputum smear negative, EPTB= extra pulmonary TB.

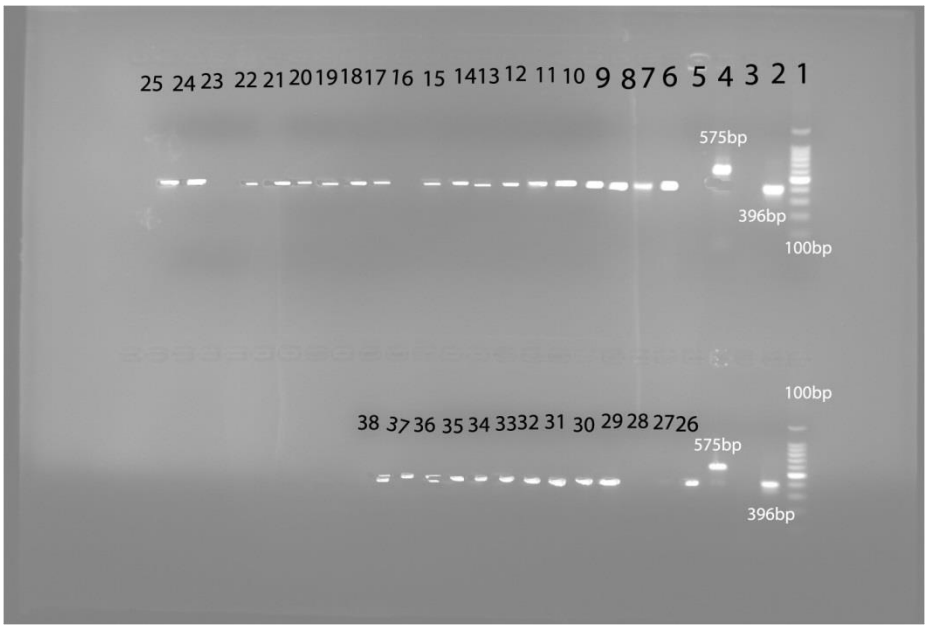


**Figure 2.** Gel electrophoresis separation of PCR products of RD9 deletion typing on mycobacteria isolates from humans. Lane 1, 100bp DNA Ladder; Lane 2, M. tuberculosis H37Rv (positive control 396 base pair band size); Lane 3, Qiagen H2O (Negative Control); Lane 4, M. bovis (positive control 575 base pair band size); Lanes 5-38) sample isolates from humans. Lane 5; 16; 23; 27 and 28 did not give RD9 signal implying there negative. (Study area is figure 1).

**Table 2.** Proportion of TB lineage and genotyping pattern.

| Characteristics | Proportion n (%) |
|---|---|
| **TB lineage** | |
| Euro-American | 13 (32.50) |
| Indo-Oceanic | 7 (17.50) |
| Unknown | 20 (50.00) |
| **Spoligotyping pattern** | |
| Non-clustered genotype | 17 (42.50) |
| **Clustered genotype** | 23 (57.50) |
| Strain type | |
| Orphan | 16(40.00) |
| Known | 24(60.00) |

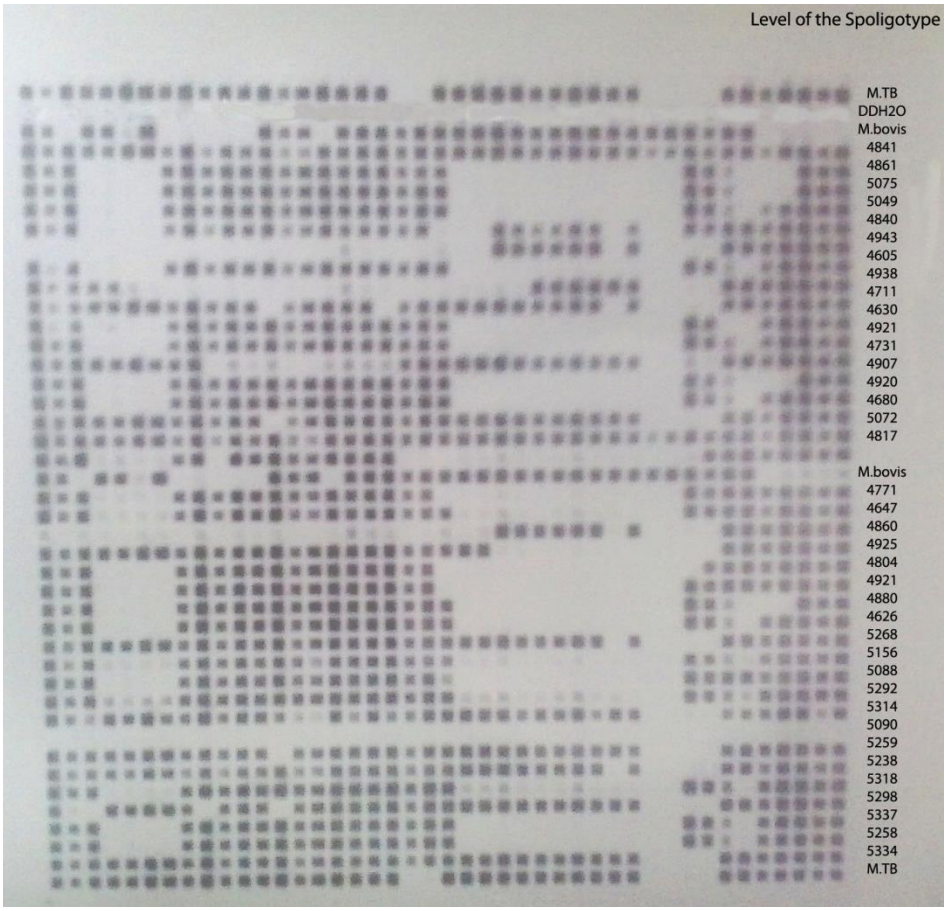


**Figure 3.** Spoligotype patterns of isolates obtained from Gambella.

town were higher as compared to those residing outside the city; this showed that urban dwellers are more prone to infection by TB. This could be due to increasing population density and high contact and social interaction, crowdedness in rapidly growing cities (Palomino et al., 2007; Rahman, 2010).

Based on the earlier established method by Shabbeer et al. *(*2012), 50% of the isolates were classified into the existing lineages while the remaining 50% of the isolates could not be grouped into the existing lineages and thus were new/unknown lineages. Further exploration is required to identify the lineages of these isolates and

**Table 3.** Proportion of spoligotyping pattern of the strains with different variables.

| Characteristics | Genotype patterns of the strains n (%) | | | |
|---|---|---|---|---|
| | Unique | Clustered | $\chi^2$/fisher exact | *P-value* |
| Sex | | | | |
| Male | 12 (48.00) | 13 (52.00) | 0.82 | 0.36 |
| Female | 5 (33.33) | 10 (66.67) | | |
| Age category | | | | |
| 15-44years | 13 (40.63) | 19 (59.38) | | 0.49 |
| 44-64years | 4 (57.14) | 3 (42.86) | | |
| ≥65years | - | 1 (100.00) | | |
| Residential area | | | | |
| Outside Gambella | 5 (35.71) | 9 (64.29) | 0.40 | 0.52 |
| Gambella Town | 12 (46.15) | 14 (53.85) | | |
| HIV status | | | | |
| Positive | 4 (40.00) | 6 (60.00) | 0.10 | 0.95 |
| Negative | 9 (45.00) | 11 (55.00) | | |
| Refused | 4 (40.00) | 6 (60.00) | | |

**Table 4.** Distribution of spoligotypes and their report status from Ethiopia.

| SIT number | Proportion | Report from Ethiopia |
|---|---|---|
| 523 | 2 (5.00) | No |
| 289 | 8 (20.00) | No |
| 25 | 1 (2.50) | Yes |
| 37 | 3 (7.50) | No |
| 21 | 1 (2.50) | No |
| 142 | 2 (5.00) | No |
| 26 | 2 (5.00) | No |
| 602 | 1 (2.50) | No |
| 50 | 2 (5.00) | No |
| 118 | 1 (2.50) | No |
| 53 | 1 (2.50) | Yes |
| Orphans | 16 (40.00) | Not reported from any country |
| Total | 40 (100.00) | |

further study is required to know if these belong to a new lineage. The predominance of Euro-American lineage finding (also known as linage 4) in this study also similar with the reports of previous studies, reported as the dominant lineage in different regions of Ethiopia (Diriba et al., 2013; Firdessa et al., 2013; Tessema et al., 2013). In a study conducted in Switzerland, the Euro-American and Indo-Oceaninc linage proportion were 72.7 and 8.3% which are higher and lower respectively compared to the present study result (Fenner et al., 2012). In another study conducted in USA, the Euro-American linage prevalence was lower than the present finding which was 13.8% while, the Indo-Oceanic prevalence in this study was lower as compared to 22.6% (Eleanor et al., 2012).

Clustered strains were less common in the present findings suggesting low frequency of transmission of TB in the area. However, there were significant numbers of unique strains in the area, which suggest reactivation of TB rather than new infection. The presence of orphans and unknown lineages in the area could suggest the localization of such strains and lineages in Gambella Region and warrants for further research. SIT289 is a common isolates from Saudi Arabia, India, Pakistan and Bangladesh (Filliol et al., 2002; Hasan et al., 2006).

In conclusion, our study shows the importance of the disease in the area that revealed the presence of unknown TB strains and lineages. Transmission is particularly well contained in the activation of previous infection rather than new infection. We identified *M. tuberculosis* was the only agent that was identified as the causative agent of human TB in Gambella Region. More attention in TB control program, strict follow up during treatment, and further study on the newly identified lineages and strains using more advanced tools is encouraged.

## Conflict of interests

The authors did not declare any conflict of interest.

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# Morphological, physiological and biochemical characterizations of some soil isolates of *Bacillus cereus* group from Algeria

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***Bacillus cereus*** **is a group of bacteria frequently found in soil, widely distributed in the environment. They are a group of ubiquitously facultative anaerobic spore-forming Gram-positive rods and are of health and economic benefits. The present study was conducted to identify, characterize about 36 *B. cereus* and examined for their potential plant growth promoting (PGP), which was tested *in vitro*. Parameters assessed were indole acetic acid (IAA) production, phosphate solubilization, starch hydrolysis, proteolytic activity and biofilm formation. Multiple *B. cereus* were isolated from several soil plots from South-Western region of Algeria and characterized by using phenotypic methods including fatty acid methyl ester. Ten bacterial isolates were examined in this study. Fatty acid profiles showed that bacterial isolates were classified into *B. cereus* group, three isolates were *B. cereus* Subgroup "A" and seven isolates were *B. cereus* Subgroup "B". Temperature effect on the maximal specific growth rate was studied in *B. cereus* between 10 and 50°C, no growth was observed in 10°C, all *B. cereus isolates* grown from 15 to 45°C and no *grown* observed in 20 to 50°C. This study demonstrates adaptation of isolates of the *B. cereus* group to different habitats. The ability to solubilize precipitated phosphate was positively exhibited by three isolates, five isolates showed ability to produce IAA. All the isolated bacterial isolates had amylolytic and proteolytic activity. All isolates did not form a biofilm in the microtiter plate assays, while all *B. cereus* in our study formed biofilm in tubes at air-liquid interfaces.**

**Key words:** *Bacillus cereus*, soil, identification, plant growth promoting rhizobacteria (PGPR), phosphate solubization, biofilm

## INTRODUCTION

In soil, microbes are especially active in the rhizosphere, which can contain more than a million microorganisms per gram of soil. Microbial community structures are variable and depend on factors such as temperature, pH,

or geographic location, but are able to tolerate environmental change. Microorganisms found in this environment use energy substrates released by roots such as and necessary to their metabolism: sugars, amino acids, organic acids, hormones. Some of these microorganisms, mainly bacteria, are able to effectively colonize the root systems and stimulating plant growth and / or protecting against infections by plant pathogens. These rhizosphere bacteria are sometimes called Plant Growth-Promoting rhizobacteria (PGPR) (Kloepper et al., 1986).

The beneficial effects of rhizobacteria on plant growth are the result of either direct or indirect mechanisms by PGPR. Indirect mechanisms occur outside of the plant, while direct mechanisms are those that occur inside of the plant and directly affect their metabolism. These mechanisms can function simultaneously or sequentially at different stages of plant growth. Many strains of PGPR can affect plant growth directly by solubilizing phosphates, nitrogen fixation and mineral nutrients, making these foods available for the plant, by producing phytohormones such as indole-3-acetic acid (IAA), and repressing of soil pathogenic microorganisms (production of hydrogen cyanide, siderophores, antibiotics, and / or competition for nutrients). In addition, PGPR can contribute to the improvement of plant resistance to biotic and abiotic stress such as salinity, drought and heavy metal toxicity (Canbolat et al., 2006).

*Bacillus cereus sensu lato (s.l.)* are Gram-positive endospore forming bacteria that are abundant in different ecological environments. The growth temperature range varies from 5 to 50°C (Guinebretiere et al., 2008).

This group of bacteria includes seven species that are genetically very similar. *B. cereus* sensu stricto (*B. cereus* s.s.) which is associated with food borne illness but is also involved in some cases of local super infections (Logan, 2012). *Bacillus thuringiensis* is an entomopathogen used with success in agriculture as a biocontrol agent against insects (De Maagd et al., 2001). *Bacillus anthracis* is the agent of anthrax (Mock and Fouet, 2001). This group (*B. cereus sensu lato (s.l.)* also species (Lechner et al., 1998). *Bacillus cytotoxicus* characterized by its high toxicity and its particular thermotolerance (Guinebretière et al., 2013), and *Bacillus mycoides* and *Bacillus pseudomycoides* species that are characterized by the formation of rhizoids colonies (Nakamura, 1998).

This study was aimed to characterized thirty six isolates of *B. cereus* from soil for a better appreciation for their potential plant growth promoting (PGP), that is, IAA production, phosphate solubilizer, starch hydrolysis, proteolytic activity and biofilm formation.

## MATERIALS AND METHODS

### Isolation of strains

Samples were isolated from several soil plots in the Naama region (South-Western region of Algeria) (Figure 1) over ten months (March till December 2014), from four different areas (about 100 $m^2$ each). 1 g of each sample of soil was added to a tube containing 9 mL of sterile distilled water. Serial dilutions were heated at 80°C for 10 min to eliminate vegetative cells and to select for spores. Each dilution (0.1 ml) was spread on Luria-Bertani (LB) agar plates. A total of thirty-six isolates of *B. cereus* group, were isolated according to the International Organization for Standardization (ISO, 1993).

### Morphological, physiological and biochemical characterizations of isolates

It is possible to differentiate *B. cereus* from other *Bacillus* species because they share similar morphology mobility, hemolytic activity, inability to utilize mannitol, and the production of an extremely active lecithinase , on Mossel culture media (MYP agar) *B. cereus* forming rough and dry colonies with a violet pink background surrounded by egg yolk precipitation. Hemolytic activity is tested using on Columbia blood agar incubated at 30°C for 24 h and is manifested by a characteristic lysis area near the bacterium (Fricker et al., 2008). To characterize the effect of temperature on the growth of *Bacillus cereus* group, isolates, were incubated on LB plates at 10, 15, 20, 30, 37, 45 and 50°C for 72 h. For long term conservation, obtained isolates were stored in Eppendorf tubes containing LB with 20% glycerol at -20ºC (Guinebretiere et al., 2008). Other biochemical characterizations of isolates are tested using the API 20E.

### Extraction and analysis of fatty acid methyl ester (FAME) profiles

We used gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA, USA) to separate FAME. We identified FAME profiles of each bacterial strain by comparing the commercial databases (TSBA 6) with the MIS software package (Sherlock 6.0 MIDI, Inc., Newark, DE, 2005).

Each samples is processed in a single test tube; a summary of the processing activities associated with each step includes:

- Harvest bacteria from third quadrant.
- Saponification: Combine water and methanol. Add NaOH pellets to the solution while stirring and stir until the pellets dissolve.
- Methylation: add acid to methanol while stirring.
- Extraction: add the methyl tert- butyl ether (MTBE) to the hexane and stir well.
- Wash: add NaOH pellets to the water while stirring and stir the pellets are dissolved.

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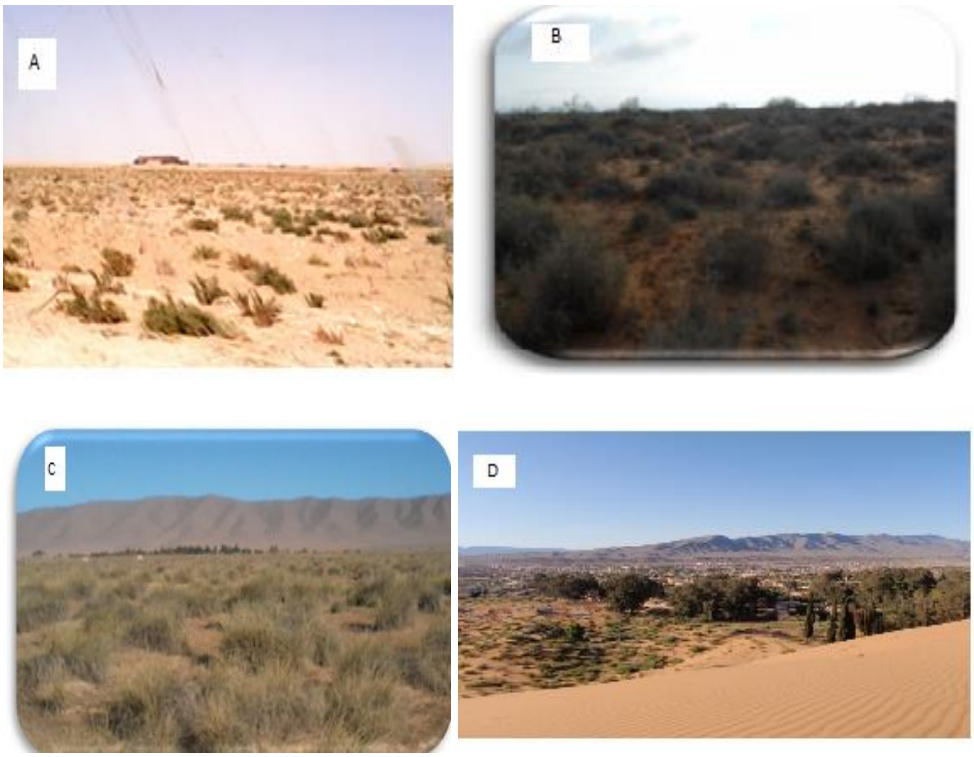


**Figure 1.** Locations of soil sampling (A) Abdelmoula (B) Mekmen Ben Amar (C) Mechria (D) Ain Sefra.

**Plant growth promotion activities for isolates**

***Production of Indole acetic acid***

40 µg/ml of L-tryptophan was added to LB medium to assure growth of our isolates. They were incubated at 30°C for 48 h. Cultures of bacteria were centrifuged for 15 min at 10000 rpm, and 1 ml of culture filtrate was mixed with 1 ml of Salkowski's reagent and the mixture incubated at room temperature for 30 min. A pink color was indicative to indole acetic acid production (Gordon and Weber, 1951).

***Phosphate solubilization***

**Prepared GYA according to Beneduzi et al. (2008):** 10 µL aliquot of each culture was plate on GYA and incubated at 28ºC for 7 days, presence of clear halos around their colonies indicate that isolate can solubilize phosphate.

***Starch hydrolysis***

This test was performed by cultivating the isolates on a nutrient agar containing 1% starch. After incubation at 30° for 48 h, cultures bacteria were covered with Lugol's solution. Hydrolysis of starch is well demonstrated by the appearance of a clear zone around the colony when placed against a negative result revealing a brown color around culture (Gupta et al., 2004).

***Proteolytic activity: Search for caseinase***

The hydrolysis of casein was studied on an agar medium containing 5% skim milk. After incubation at 30°C for 48 h, a clear halo around the streak indicated the hydrolysis of casein by against a negative result showed no hydrolysis zone around culture (Marchand et al., 2009).

**Formation of biofilm in microtiter plate**

Polystyrene microtiter plates were filled with 200 µl cultures grown in LB and inoculated. The plates were incubated at 30°C. After 24 h, we washed wells three times with 200 µl of phosphate buffered saline, and stained with 0.1% (wt/vol) crystal violet for 30 min to color biofilm. The cultures were distained twice with 200 µl sterile deionized water. The remaining crystal violet was dissolved in 200 µl 96% ethanol, and we measured at 595 nm the absorbance (Djordjevic et al., 2002).

**Biofilm formation in tube**

A loopful of isolates was inoculated in 10 mL LB medium in test tubes at 30ºC for 24 h. After incubation, tubes were decanted and washed with phosphate buffer saline and dried. Tubes were then colored with crystal violet (0.1%). Excess isolate was washed with deionized water. Tubes were dried in inverted position. Isolates which showed a visible film lined the wall of the tube were considered as positive biofilm formation (Christensen et al., 1982).

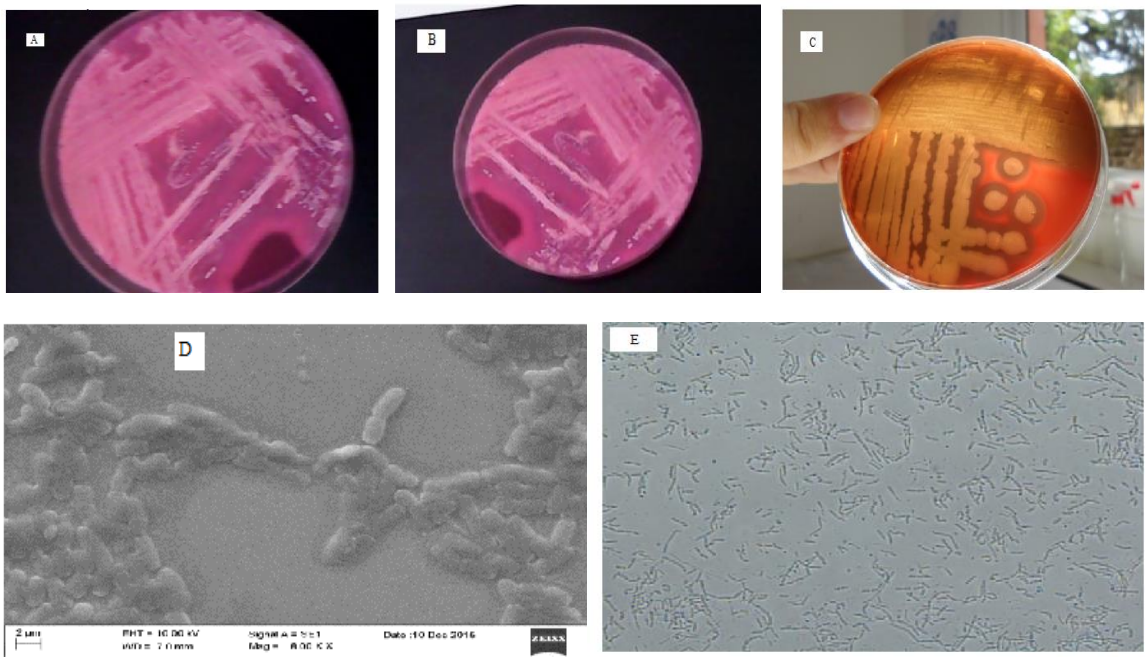


**Figure 2.** Morphological characteristics of two isolates A: *B. cereus* (Bact 2) in MYP; B: *B. cereus* (Bact 3) in MYP; C: hemolytic activity by *B. cereus* (Bact 2); D: Scanning Electron Microscopy (SAM) picture of *B. cereus* (Bact 2) E: phase contrast microscopy picture of *B. cereus* (Bact 3).

**Hydrophobicity**

The hydrophobicity of 10 *B. cereus* isolates was determined according to the microbial adhesion to solvents (MATS) (Bellon-Fontaine et al., 1996). Hydrophobicity is expressed as percentage of adhesion to hexadecane. The prepared suspension was adjusted at 595 nm of 0.6-1 ($A_0$). Samples of each suspension (2 ml) were added to 400 µL of hexadecane mixed on a vortex mixer for 10 S for 15 min. the absorbance of the aqueous phase was measured at 595 nm ($A_1$).The percentage of hydrophobicity (%h) was determined from the absorbance of the initial bacterial cell, or spore suspensions ($A_0$) and the absorbance of the aqueous phase after mixing with hexadecane ($A_1$) using the following equation: $\%h = [(A_0 - A_1)x\ 100]/ (A_0$. Spores are very hydrophilic (%h < 20%), hydrophilic (20 > %h < 40%), moderately hydrophobic (40 > %h < 60%) and highly hydrophobic (%h > 60%).

## RESULTS AND DISCUSSION

### Identification and characterization of bacterial strains

We obtained thirty six *B. cereus* group isolates from various localities in Naama south-western Algeria. All isolates showed typical colony morphology with a zone of precipitation. All of the isolates were Gram positive, rod shaped, endospore forming, catalase positive, lecithinase positive and hemolytic (Figure 2). All isolates were able to grow in the range of temperature tested 15 to 45°C (Table 1). To clarify the existing diversity of *B. cereus* group and its special ability to adapt to widely diverse habitats, Guinebretière et al. (2008) showed that the genetic structure of *B. cereus* group belong the same phylogenetic group, describe more that found. All isolates of this study affiliated to group III. This genetic group includes isolates whose growth temperature is between 15 to 45°C. Temperature is one of the most important environmental factors to which microorganisms tolerate different kinds of environmental changes. In the API 20 E, all isolates were VP, Citrate, ADH, Gelatine positive, except three isolates were ADH negative, however the results given by other tests are always negative (Table 1).

### FAME analysis

Ten bacterial isolates were examined in this study (Figure 3). They were classified into *B. cereus* groups. The characterization studies based on FAME analysis showed that total 31 different FAMEs were present in 10 bacterial isolates tested in the present study (Table 2). The data of fatty acid analysis showed that three isolates (bact3, bact4, bact7) were *B. cereus* Subgroup A, remaining isolates were *B. cereus* Subgroup B.

According to the results based on fatty acid profiles we can deduce a low diversity among the isolates. The ten samples were isolated from several soil plots, both the origin of the isolates and the pressure exerted by

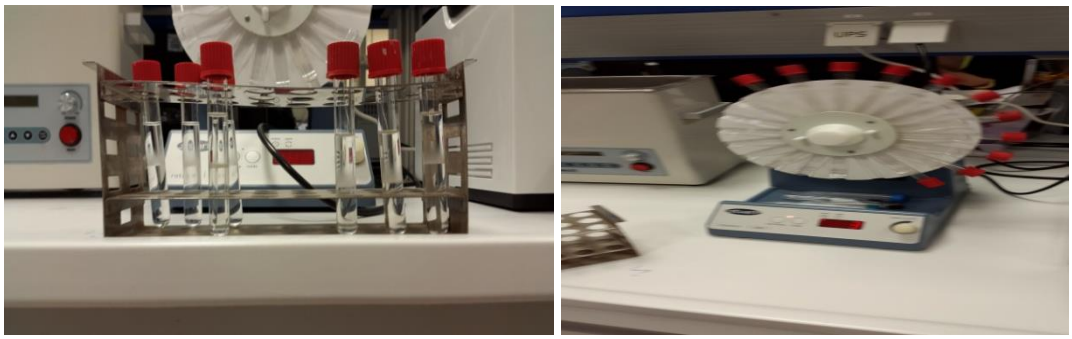

**Figure 3.** Fatty acid methyl ester (FAME) analysis.

**Table 1.** Biochemical and physiological characteristic of 36 *B. cereus* isolates.

| Number of strains | Origin | Positif biotype character | Thermic profile of growth |
|---|---|---|---|
| 5 | Abdelmoula | Biotype I (VP, Citrate, ADH, Gelatine) | Mesophilic 15 to 45°C |
| 6 | Mekmen Ben Amar | | |
| 4 | Mechria | | |
| 18 | Ain Sefra | | |
| 1 | Mechria | Biotype II (VP, Citrate, Gelatine) | |
| 2 | Ain Sefra | | |

environmental conditions in the soil can explain the low diversity identified by our results, it has been reported that temperature is assumed to select some bacterial groups (Von Stetten et al., 1999). Major environmental variables that may influence bacterial-community composition include vegetation type and temperature, plant species variables influence the composition of bacterial communities (Kuske et al., 2002), agricultural growing practice (Buckley and Schmidt 2001), temperature (Ward et al., 1998), nutrient status (Broughton and Gross, 2000), salinity (Nubel et al., 2000), and other environmental variables.

## Plant growth promotion activities for isolates

Five isolates of *B. cereus* showed IAA production (Table 3). According to Mirza et al. (2001), IAA by the production of microorganisms may vary between different species and strains of the same species. The culture conditions and substrate stage growth conditions may also cause variations in production. Also, Idris et al. (2007) showed that reduction in IAA concentration in *B. amyloliquefaciens* FZB42 was caused by inactivating of gene responsible for IAA biosynthesis consequently causing in decrease plant growth promotion activity. The level of IAA production was affected also by two genetic factors, the location of auxin biosynthesis genes in the bacterial genome and the mode of expression.

Among environmental stress factors, acidic pH, osmotic and matrix stress, and carbon limitation modulate the IAA biosynthesis in different bacteria (Spaepen et al., 2007). The ability to produce heat resistant endospores is one of the main features of *Bacillus*, tailoring it to design and marketing, since these microorganisms can be stored for a longer period and may stay longer in the soil (Kokalis-Burelle et al., 2006). The isolation efficient of bacteria depends on some factors of the interaction of plant-soil-climatic conditions (Chagas-Junior et al., 2012). There are few studies related to quantification of IAA by bacteria of the species *B. thuringiensis* and *B. cereus*. Gomes et al. (2003) demonstrated that both bacteria, *B. thuringiensis* and *B. pumilus*, isolated from the cabbage plant, increased the growth of lettuce in the greenhouse. *B. cereus* is known for its ability to produce gibberellic acid, IAA, zeatin and gibberellins (Karadeniz et al., 2006). In addition, Tilak et al. (2006) confirmed the involvement of *B. cereus* in promoting the growth of *Cajanus cajan* (L.) Mill sp., while Bullied et al. (2002) found that this promotes the growth of bacteria soybean (Glycine max). Three isolates showed capability to solubilize inorganic phosphate (Table 3). *Bacillus* species have been reported for their capacity to increase availability of phosphorus in soil (Bhattacharyya and Jha, 2012) and

**Table 2.** Fatty acid composition (%w/w) of isolates.

| Fatty acids | bact1 | Bact2 | Bact3 | Bact4 | Bact5 | Bact6 | Bact7 | Bact8 | Bact9 | Bact10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 12 :0 iso | 0.97 | 1.33 | 0.48 | 1.04 | 0.73 | 0.88 | 0.48 | 1.04 | 1.25 | 1.17 |
| 12 :0 | 0.74 | 0.80 | 0.38 | 0.81 | 0.56 | 0.66 | 0.51 | 0.57 | 0.71 | 0.70 |
| 13 :0 iso | 8.16 | 7.71 | 6.57 | 9.49 | 7.61 | 7.46 | 8.78 | 8.93 | 11 .32 | 8.96 |
| 13 :0 anteiso | 0.94 | 1.45 | 0.79 | 1.17 | 1.42 | 1.62 | 0.65 | 1.04 | 2.16 | 1.24 |
| 13 :0 | - | 0.31 | - | - | - | 0.33 | - | 0.17 | - | 0.36 |
| 14 :0 iso | 5.99 | 7.15 | 4.01 | 5.10 | 5.52 | 5.08 | 3.71 | 6.58 | 5.21 | 6.34 |
| 14 :1 w5c | -- | - | - | - | - | - | 0.22 | - | - | - |
| 14 :0 | 5.06 | 6.21 | 3.29 | 5.84 | 5.50 | 5.02 | 4.67 | 4.69 | 4.43 | 5.36 |
| 15 :0 iso | 27.49 | 24.08 | 30.99 | 30.98 | 29.89 | 29.16 | 31.91 | 28.15 | 25.28 | 29.44 |
| 15 :0 anteiso | 4.29 | 5.14 | 4.21 | 4.05 | 6.85 | 6.73 | 3.36 | 4.63 | 6.35 | 4.75 |
| 15 :1 w5c | - | 0.27 | - | 0.28 | - | 0.27 | - | 0.27 | - | 0.26 |
| 15 :0 | - | - | - | - | - | - | - | - | - | - |
| 16 :1 w7c alcohol | 0.44 | 0.55 | 0.59 | 0.44 | 0.50 | 0.70 | 0.44 | 0.49 | 0.91 | 0.95 |
| 16 :0 iso | 9.59 | 10.72 | 9.76 | 7.33 | 7.89 | 8.12 | 6.05 | 9.77 | 7.39 | 9.00 |
| 16:1 w11c | 0.36 | 0.34 | 0.43 | 0.28 | 0.40 | 0.45 | 0.22 | 0.29 | 1.97 | 0.54 |
| 16 :1 w5c | - | 0.26 | - | 0.27 | 0.43 | 0.37 | 1.00 | - | - | - |
| 16 :0 | 11.01 | 10.03 | 8.12 | 8.30 | 9.13 | 5.90 | 5.77 | 10.27 | 10.51 | 8.41 |
| 15 :0 2OH | - | - | 0.30 | - | - | 0.25 | 0.34 | - | - | - |
| 17 :1 iso w10c | 0.94 | 1.15 | 1.96 | 1.29 | 1.43 | 1.31 | - | 0.90 | 1.93 | 1.84 |
| 17 :1 iso w5c | 1.67 | 1.53 | 2.59 | 2.32 | 2.03 | 3.16 | 2.90 | 1.71 | 1.19 | 1.47 |
| 17 :1 anteisoA | 0.48 | 0.54 | 0.81 | 0.54 | 0.70 | 1.39 | 0.70 | 0.52 | 0.49 | 0.48 |
| 17 :0 iso | 9.19 | 7.69 | 11.71 | 8.62 | 7.09 | 5.84 | 8.54 | 8.30 | 7.91 | 8.81 |
| 17 :0 anteiso | 1.87 | 2.12 | 2.78 | 1.52 | 2.39 | 2.36 | 1.40 | 1.84 | 2.57 | 1.84 |
| 17 :1 w6c | - | - | - | - | - | 0.24 | 0.36 | - | - | - |
| 17 :0 | 0.40 | 0.41 | 0.29 | 0 .27 | - | 0.24 | - | 0.32 | - | 0.36 |
| 18 :0 iso | 0.26 | 0.37 | - | - | - | - | - | 0.20 | - | - |
| 18 :1 iso H | - | -- | - | - | - | - | 0.29 | - | - | - |
| 18:1 w9c | 1.62 | 0.76 | 1.28 | 0.31 | 0.40 | 0 .23 | 0.35 | 0.31 | 0.79 | 0.46 |
| 18 :1 w5c | - | -- | - | - | - | -- | 0.17 | - | - | - |
| 18 :0 | 0.69 | 0.43 | 0.59 | 0.34 | 0.30 | 0.19 | 0.23 | 0.46 | 0.49 | 0.33 |
| 18 :1 2OH | - | - | - | 0.29 | 0.29 | - | - | 0.19 | - | 0.31 |

many environmental factors such as pH and soil type can affect highly the phenomena of fixation and precipitation of P in soil. Thus, according to Jones et al. (1991) in alkaline soils it is fixed by calcium, causing a low efficiency of soluble P fertilizers.

From the present study, pH of soil isolates was alkaline (8.5), Hwangbo et al. (2003) reported that the inverse relationship between pH and soluble phosphate. However, P-solubilizing bacteria (PSB) are widely distributed in soil; environmental stress factors can affect establishment and performances of these bacteria (Ahemad and Khan, 2012).

Furthermore, obtained results showed that all *Bacillus cereus* in our study were starch hydrolysis and proteolytic activity (Figure 4A and 4B). There are many reports (Sellami-Kamoun et al., 2008) indicating that *Bacillus sp.* were able to produce a large variety of extracellular enzymes, such as amylases and proteases are the most significant industrial. It has been confirmed that variation of amylases and proteases not only in type but also in pH and optimum temperature (Gupta et al., 2003).

## Role of hydrophobicity of spores in attachment

The hydrophobicity of *B. cereus* spores was determined and expressed a percentage and illustrated in Table 4. Surface hydrophobicity of untreated *B. cereus* spores(ranged between 18.5 and 57%). In this study, it is interesting to note that hydrophobicity of spores varied among the analyzed *B. cereus* soil isolates which displayed either a hydrophilic or moderate hydrophobic character. Three isolates were moderately hydrophobic, adherence to hexadecane range between 43, 37 and

**Table 3.** IAA production, phosphor solubilization, amylolytic and proteolytic activity and biofilm-forming capacities in tube and microtiter plate of the thirty six *B. cereus* strains from four different sites. (A): Abdelmoula; (B): Mekmen Ben Amar; (C): Mechria; (D): Ain Sefra.

| Strains | IAA production[a] | Phosphor Solubilization[b] | Amylolytic activity[c] | Proteolytic activity[c] | Microtiter plate biofilm formation[d] | Biofilm-forming capacities in tube[d] | Sites |
|---|---|---|---|---|---|---|---|
| Bact1 | - | - | + | + | - | + | B |
| Bact2 | + | - | + | + | - | + | D |
| Bact3 | + | + | + | + | - | + | D |
| Bact4 | + | + | + | + | - | + | D |
| Bact5 | - | - | + | + | - | + | A |
| Bact6 | - | - | + | + | - | + | D |
| Bact7 | + | - | + | + | - | + | D |
| Bact8 | - | - | + | + | - | + | B |
| Bact9 | + | + | + | + | - | + | C |
| Bact10 | - | - | + | + | - | + | D |
| Bact11 | - | - | + | + | - | + | D |
| Bact12 | - | - | + | + | - | + | D |
| Bact13 | - | - | + | + | - | + | C |
| Bact14 | - | - | + | + | - | + | C |
| Bact15 | - | - | + | + | - | + | C |
| Bact16 | - | - | + | + | - | + | A |
| Bact17 | - | - | + | + | - | + | A |
| Bact18 | - | - | + | + | - | + | D |
| Bact19 | - | - | + | + | - | + | D |
| Bact20 | - | - | + | + | - | + | D |
| Bact21 | - | - | + | + | - | + | D |
| Bact22 | - | - | + | + | - | + | D |
| Bact23 | - | - | + | + | - | + | C |
| Bact24 | - | - | + | + | - | + | A |
| Bact25 | - | - | + | + | - | + | A |
| Bact26 | - | - | + | + | - | + | B |
| Bact27 | - | - | + | + | - | + | D |
| Bact28 | - | - | + | + | - | + | D |
| Bact29 | - | - | + | + | - | + | D |
| Bact30 | - | - | + | + | - | + | B |
| Bact31 | - | - | + | + | - | + | B |
| Bact32 | - | - | + | + | - | + | D |
| Bact33 | - | - | + | + | - | + | D |
| Bact34 | - | - | + | + | - | + | D |
| Bact35 | - | - | + | + | - | + | B |
| Bact36 | - | - | + | + | - | + | D |

([a]): +: able to produce IAA, -: not able to produce IAA; ( [b] ): +: able able to solubilize Phosphor, - : not able to solubilize; ([c]): +: amylolytic and proteolytic activity; ( [d] ): +: forms a biofilm, - : does not form a biofilm.

57%. Remaining isolates were hydrophilic character, with hydrophobicity values between 18.5% and 36%.

According to the literature, spores of *B. cereus* groups generally considered hydrophobic. On the other hand, the hydrophilic character of the soil isolates of *B. cereus* could also be related to an adaptation to alkaline pH conditions. This notice is an accordance with those mentioned by Seale et al. (2008). They stated that hydrophobicity increased at acidic pH while it decreased at alkaline pH (Giotis et al., 2009). Decreased hydrophobicity was observed for the spores of a strain of *B. cereus* alkali-tolerant (Bernardes et al., 2010). These data are confirmed by the results of Hamadi et al. (2004) who found that the hydrophilicity of strains of *S. aureus*

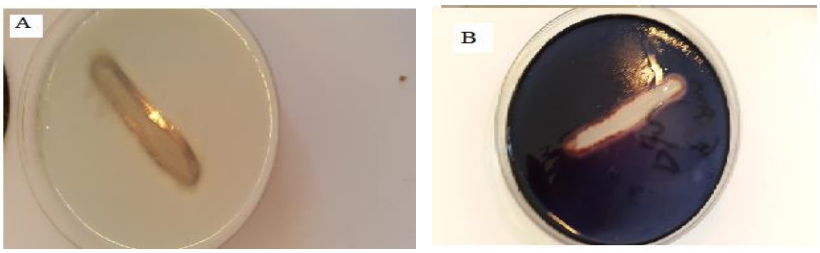


**Figure 4.** Starch hydrolysis, proteolytic activity by *B cereus* (Bact2). A: proteolytic activity B: starch hydrolysis.

**Table 4.** Hydrophobicity percentage of the ten *Bacillus cereus* selected isolates.

| Isolates | Hydrophobicity (%) |
|---|---|
| Bact1 | 19 |
| Bact2 | 36 |
| Bact3 | 44.15 |
| Bact4 | 18.5 |
| Bact5 | 26 |
| Bact6 | 35.13 |
| Bact7 | 32.55 |
| Bact8 | 57 |
| Bact9 | 43.37 |
| Bact10 | 20 |


**Figure 5.** Biofilms formed by *B. cereus* on LB at 30°C for 48 h at air-liquid interfaces. *B. cereus* Bact2 (lane 1), *B. cereus* Bact3 (lane 2).

and *E. coli* is low apparent for neutral to alkaline pH (> 5 to 11) so that the hydrophobicity is more apparent at acidic pH (2-3). In our results, pH of soil isolates was alkaline between (8 to 9) and hydrophobicity associated with very moderate hydrophobicity is an argument for adaptation at pH alkaline conditions.

## Biofilm formation

In our study, we use a tube method and microtiter plate assay to test the capacity of thirty six isolates to form biofilms. The tube test showed significantly different results from the results obtained by the standard microtiter-plate test. All isolates did not form a biofilm in the microtiter plate assays, while in the Tube assays, formation of biofilm took place preferentially at the point of the interface between the liquid and air for all *B. cereus* (Figure 5). According (Deighton and Balkau, 1990) the adherence capability of Staphylococci was influenced by many factors include different mechanisms of adherence for plastic and glass surfaces. Number of spores attached to the polystyrene surface was dramatically affected by hydrophobicity. Auger et al. (2006) assured same observations and reported that *B. cereus* ATCC14579 did not produce biofilm in the microtiter plate.

The dynamic condition had negative effect not only on attachement of *B. cereus* cells to polystyrene surface but also on biofilm development, where all tested isolates were no biofilm producer. According to Carpentier and Cerf (2011), the conditions for growth are not always favorable for the adhesion of bacteria. The choice of material in which these microtiter are produced, also remains imperative to avoid negative interference with the adhesive properties of the tested microorganisms. This illustrates the need for reliable and reproducible techniques to culture and biofilm development. It is recognized that biofilms are formed in response to environmental signals which differ from one species to another. These signal nutrient availability and quorum-sensing involved in the regulation of maturation of the biofilm (Stanley and Lazazzera, 2004). Factors which influence structure and development of biofilm, and the composition of the microbial community include nutrient availability and type of sugar provided (Stoodley et al., 2001). Finally, the results indicate that the bacteria tested in this study may be promising in promoting plant growth.

## Conflict of Interests

The authors have not declared any conflict of interest.

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*Full Length Research Paper*

# Isolation of *Salmonella* species and some other gram negative bacteria from rats cohabitating with poultry in Ibadan, Oyo State, Nigeria

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**The aim of this study was to isolate *Salmonella* species and other enteric bacteria in rats cohabiting with poultry; in order to consider the potential role of rats in their transmissions to poultry and humans. Four hundred samples comprising 200 oral swabs and 200 anal swabs were collected from rats cohabiting with poultry from 5 local government areas in Ibadan. The samples were subjected to standard bacteriological analysis. A total of 228 Gram negative bacteria including 21 different species of both Lactose fermenters and non-Lactose fermenters were isolated. The identified organisms include: *Salmonella* subspecies *1, Salmonella* Arizonae, *Escherichia coli*, *Escherichia coli inactive, Pseudomonas aeruginosa, Pseudomonas* fluorescens, *Enterobacter cloacae, Enterobacter hormaechei, Enterobacter agglomerans, Enterobacter dissolvens, Enterobacter gergoviae,* and *Aeromonas hydrophila, Proteus mirabilis, Proteus vulgaris, Hafnia alvei*, *Morganella morganii biogp 1,Morganella morganii subspecies Siboni 1*, *Citrobacter diversus, Serratia liquefaciens, Pragia fontium and Providencia alcalifaciens.* The organisms were identified using Oxoid Microbact GNB 24E® (MB24E) and accompanying computer software package (Oxoid Microbact®) 2000 version 2.03. Some of the isolated bacteria from rats' cohabitating with poultry have been associated with diseases in poultry and humans. The findings therefore serves to create fresh awareness among poultry farmers and other stakeholders in the industry from the studied area, that rats do not only constitute physical threat in terms of destruction of infrastructures and feeding on poultry feeds, they also pose a great risk in terms of transmission of bacterial infection to poultry and men associated with poultry production. Possible measures to control rats' infestations within poultry houses are highlighted.**

**Key words:** Rats, poultry, co-habitating, Gram negative Bacteria.

## INTRODUCTION

Rodents are known to be so numerous and very diverse in nature; and they are well acknowledged as a major contributors to human disease, right from the middle age (Meerburg et al., 2009; Webster, 1996). Black rats for example were associated with the transmission of plague long time ago; in the same way, more than 24 different infectious agents have been directly or indirectly reported as been transmitted by rodents to humans (Meerburg et

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al., 2009; Webster,1996). Wild rodents serve as reservoirs and vectors for some pathogenic organisms like: *Leptospira* spp., *Salmonella* spp., *Campylobacter* spp., *Trichinella* spp., *Toxoplasma* spp. which are well known disease causing agents of food animals and humans (Gratz, 1994, Hiett et al., 2002). For instance, it had been noted that, rat population at a poultry farms can be a major reservoir of pathogenic bacteria and they are capable of transmitting the pathogens to the environment, food and animals (Rose et al*.,* 2000). Henzler et al. (1998) demonstrated the direct association between the presence of rats and poultry diseases because they documented that effective control measure of rodents in the poultry house resulted in reduced bacterial infection of the associated poultry. It has also been shown that rodent control measures can effectively decrease *Salmonella* species in poultry houses (Rodenburg et al*.,* 2004). Rats are common commensal pest (Meerburg et al*.,* 2009), which can damage the food products, buildings, stored products, and also serve as potential vector of pathogenic diseases in human and animals. Rats may transmit the bacteria through feces, urine, and hair remnants (Meerburg et al*.,* 2009).

Rodents are capable of perpetrating long existence of pathogens in the animal house and population. They can acquire infection through contact with faeces of infected livestock on the farm (Oosterom, 1991), from other wild animals such as wild birds or from their own family members as rodents. And since they tend to live close to each other, it encourages the infections to remain resident in the population for a long time (Singh et al*.,* 1980; Hilton et al*.,* 2002). Notably among the group of bacteria organisms carried and transmitted by rodents are enteric bacteria belonging to the Family enterobacteriaceae, of which *Salmonella* species; an important animal and human pathogen belongs.

*Salmonella* species are Gram-negative, non-spore forming rod-shaped bacteria, a member of the family Enterobacteriaceae (Jay et al., 2003). They are generally known to be actively motile with the exception of *Salmonella* Pullorum and Gallinarum; they grow at optimum temperature of 35 to 37°C and they are facultative anaerobic organisms (Jay et al., 2003). They are non-capsulated except *Salmonella typhi* (Cheesbrough, 2002). Persistence of *Salmonella* on farms is about two times higher for a farm where rodents are resident (Rose et al., 2000). According to Mehmood et al. (2011), bandicoot rat served as reservoir of bacterial infection of *Escherichia coli, Klebsiella* species, *Proteus* species and *Salmonella* species at poultry farms at Rawalpindi/Islamabad, Pakistan.

The Family Enterobacteriacea to which *Salmonella* belong are a large family of Gram-negative bacteria that includes many of the more familiar pathogens, such as, *E. coli*, *Yersinia pestis*, *Klebsiella* and *Shigella*, *Proteus*, *Enterobacter*, *Serratia* and *Citrobacter (*Engelkirk, 2007*)*. The family belongs to the order Enterobacteriales, the class Gammaproteobacteria and phylum Proteobacteria (Brenner et al., 2005). Members of the Enterobacteriaceae are rod-shaped, typically 0.6 to 6 µm in length and appear as small grey colonies on blood agar *(*Engelkirk, 2007*)*. They are facultative anaerobes, they ferments sugars to produce lactic acid and various other end product (Ewing, 1986), most also reduce nitrate to nitrite (Mac Faddin, 2000). Bacteriological screening of rodents in close association with poultry houses for their carriage of pathogenic bacteria could be helpful to determine their role in bacterial disease transmission in poultry. The role of rodents, rat inclusive in destruction of poultry infrastructures is well appreciated among poultry farmers in Ibadan, Nigeria. There is however limited information on the types of bacteria carried by the rats, despite their often close interactions with poultry / poultry feeds and water sources. We therefore screened the oral and anal region of rats cohabitating with poultry from some poultry farms in Ibadan, Oyo State Nigeria to isolate and identify the kind of pathogen they carry as a mean of accessing their possible roles in disease transmission to poultry and human.

## MATERIALS AND METHODS

### Sample location

Purposive samples were collected from poultry farms(comprising of both commercial and breeder flocks) in Suburbs of Ibadan, Oyo State, South-Western Nigeria (Latitude 7° 23' N and Longitude 3° 56' E) with known problems of rat infestation. The south-western States including; Oyo, Osun, Ogun, Ondo and Lagos are the main centers of poultry industry in Nigeria and Ibadan is a major and largest central city in the south-western States playing strategic roles in the national poultry production. Samples were collected from 5/11 local government areas located in Ibadan suburbs namely: Akinyele, Ido, Ona ara, Lagelu and Egbeda, where most of the poultry farms are located. One farm each was chosen from each LGA for sample collection in rats cohabiting with chicken flocks.

### Sample collection

Two hundred rats were captured alive using adhesive glue boards. They were subsequently retrained with hands fortified with hand gloves for oral and anal sample collections. The oral and anal regions were sterilized with methylated spirit for aseptic collection of samples and to reduce contamination. A sterile swab stick was then inserted into the anus and mouth and rolled gently for sample collection. A total of four hundred samples were collected including; two hundred oral swab and two hundred anal swabs from rats cohabiting with poultry. The samples were transported to the laboratory and bacteriologically processed under 24 h of collection to reduce contamination and to ensure viability of organisms.

### Bacteriological analysis

The samples were processed for bacteria isolation and identification using standard morphological, biochemical screening and serological procedures (Barrow and Felthams, 1993; Garcia and Isenberg, 2007; Cheesbrough, 2002). The oral and anal samples

were streaked on MacConkey agar plates and inoculated into Selenite F Broth and subsequently incubated at 37°C for 24 h for primary isolation. Lactose fermenters were sub-cultured to Eosine Methylene Blue agar and incubated at 37°C for 24 h. Organisms that produced metallic sheen colonies on EMB were stored for further biochemical identification. Discrete colonies of Lactose and non- Lactose fermenters were also sub cultured from MacConkey agar on to Tryptose agar slope for further identification. Isolates that showed brick red colouration on Selenite F Broth were sub-cultured onto Deoxycholate Citrate Agar and incubated at 37°C for 24 h. All the Gram-negative isolates were identified with Oxoid Microbact GNB 24E® (MB24E) and accompanying computer software package (Oxoid Microbact®) 2000 version 2.03 according to the manufacturers procedures. The list of the reagents included in the kits are: Lysine, Ornithine, $H_2S$, Glucose, Mannitol, Xylose, ONPG, Indole, Urease, Voges Proskeur, Citrate, Gelatine, TDA (Tryptophan deaminase), Malonate, Inositol, Sorbitol, Rhamnose, Sucrose, Lactose, Arabinose, Adonitol, Raffinose, Salicin and Arginine.

#### Serology

All the suspected *Salmonella* species were subjected to slide agglutination test by standard method using Polyvalent Salmonella Antisera (Difco$^m$ Salmonella O Antiserum Poly A-I and V1) (Cheesbrough, 2002; Andrews et al*.,* 2005).The *Salmonella* polyvalent antisera was reconstituted with 3mls of sterile 0.85% NaCl solution according to the manufacturer's instructions. From test culture on a non selective media (Mueller Hilton), a discrete colony of the suspected *Salmonella* species organism was emulsified in a drop of sterile saline on a glass slide. A drop of the antiserum was then mixed with the emulsified culture on the slide, rotated for one minute to observe for agglutination. Aggutination indicated a positive result while absence of agglutination indicated a negative result.

## RESULTS

### Colonial morphology

One hundred and nineteen lactose fermenters were isolated on MacConkey agar; colonies were circular, 0.5 to 5 mm, pink coloured, with sweet odour and glistening surface. Forty-one non-lactose fermenting organisms were isolated from primary isolation; the colonies were light yellow, circular, translucent with entire edges (some had serrated edges), they were low convex, 0.5 to 2 mm with bad odour and with glistening surface. Sixty four isolates had characteristic metallic sheen on EMB. One hundred and thirty three isolates exhibited brick red coloration in selenite F Broth, 68 isolates grew on Deoxycholate Citrate Agar, out of which 32 isolates appeared as cream transparent to translucent circular, convex colonies with black centers, whereas 36 isolates appeared as white opaque convex colonies without black centers.

### Biochemical test

A total of 10 genera of Enterobacteriaceae family namely: *Citrobacter, Escherichia, Enterobacter, Hafnia, Morganella, Pragia, Proteus, Providencia, Salmonella and Serratia* were identified to species level based on Microbact$^R$ identification procedure. The other non-lactose fermenters identified include *Pseudomonas species* and *Aeromonas hydrophilia* (Table 1). Table 2 shows the various biochemical reactions of each of the isolated organism to the 24 biochemical tests included in the Microbact$^R$ kit. Table 1 shows the details of the numbers and respective percentages of the identified organisms. *Enterobacter* species had the highest percentage (22.7%) while *A. hydrophila* had the lowest (0.25%). Only 7(1.75%) *Salmonella* organisms belonging to two serovars: namely, *Salmonella* subspecies 1, 4(1%) and *Salmonella* Arizonae 3(0.75) were isolated and identified.

### Serology

The identities of the 7 suspected *Salmonella* isolates were confirmed by slide agglutination with the Polyvalent Salmonella Antisera (Difco$^m$ Salmonella O Antiserum Poly A-I and V1),

## DISCUSSION

Members of the family Enterobacteriacea have been associated with diseases in poultry and humans (Yusop et al*.,* 1991; Tsolis et al., 2008; Velge et al*.,* 2012). In this current study only 2 groups of *Salmonella* was identified namely *Salmonella* subspecies 1 with an isolation rate of 1% and *Salmonella* Arizonae with an isolation rate of 0.75% giving a total isolation rate of 1.75%. These two subspecies of *Salmonella* have been implicated in poultry diseases and human infection. Brenner, 1998 stated that *Salmonella enterica* subspecies I are the predominant pathogens associated with birds and mammals; Velge et al. (2012) also reported that strains of *S. enterica* subspecies 1 causes 99% of Salmonella infections in humans and other warm blooded animals. It produces cytotoxin and causes inflammatory diarrhea due to their ability to invade colonic epithelium, and they produce fever in humans (Tsolis et al., 2008; Guerrant and Steiner, 2005). On the other hand, *Salmonella* Arizonae subspecies have been associated with diseases in Turkey and Sheep (Grimont and Weill, 2007), although they are mostly isolated from reptiles and rarely isolated from man (Weiss et al., 1986). It was believed to be the cause of septiceamia, pneumonia, otitis media, brain abscess, meningitis, osteomyelitis, gastroenteritis and hepatic abscess in humans in many studies (Mclntyre et al*.,* 1982, Petru and Richman, 1981 and Yusop et al*.,* 1991).

The other 9 organisms belonging to the Enterobacterecea family were isolated and identified. Five *Enterobacter* species with highest isolation rate of

**Table 1.** Types, number and the percentage of bacteria isolated from the mouth and anus of rats co-habituating with poultry.

| Organism | No. | Percentage (%) |
|---|---|---|
| *Aeromonas hydrophila* | 1 | 0.25 |
| *Citrobacter diversus* | 3 | 0.75 |
| *Escherichia coli* | 64 | 16 |
| *E. coli inactive* | 2 | 0.5 |
| *Enterobacter agglomerans* | 2 | 0.5 |
| *Enterobacter cloacae* | 73 | 18.25 |
| *Enterobacter dissolvens* | 3 | 0.75 |
| *Enterobacter gergoviae* | 2 | 0.5 |
| *Enterobacter hormaechei* | 11 | 2.75 |
| *Hafnia alvei* | 2 | 0.5 |
| *Morganella morganii* biogroup 1 | 15 | 3.75 |
| *M. morganii* subsp. *Siboni* | 5 | 1.25 |
| *Pragia fontium* | 1 | 0.25 |
| *Proteus mirabilis* | 12 | 3 |
| *Proteus vulgaris* | 6 | 1.5 |
| *Pseudomonas aeruginosa* | 15 | 3.75 |
| *Pseudomonas fluorescens* | 1 | 0.25 |
| *Providencia alcalifaciens* | 1 | 1.25 |
| *Salmonella subspecies 1* | 4 | 1 |
| *Salmonella* Arizonae | 3 | 0.75 |
| *Serratia liquefaciens* | 2 | 0.5 |

**Table 2.** Biochemical reactions of the bacteria isolated from rats co-habitating with poultry.

| Bacteria | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. hydrophila* | + | - | - | + | + | - | + | - | + | + | + | + | + | + | - | - | - | + | - | + | - | - | - | + |
| *C. diversus* | + | + | - | + | + | + | + | + | + | + | + | - | - | + | - | - | + | + | - | + | + | - | - | + |
| *E. coli* | + | - | - | + | + | + | + | + | - | - | - | - | - | - | - | - | + | - | + | + | - | - | - | + |
| *E. coli inactive* | + | - | - | + | + | + | + | + | + | - | + | - | - | + | - | - | - | - | - | - | - | - | - | + |
| *E. agglomerans* | + | - | + | + | + | + | + | + | + | + | + | + | + | + | - | + | - | + | - | + | - | + | - | + |
| *E. cloacae* | + | + | - | + | + | + | + | - | + | + | + | - | - | + | - | + | - | + | - | + | - | + | - | + |
| *E. dissolvens* | + | + | - | + | + | + | + | - | + | + | + | - | - | + | - | + | + | + | - | + | - | + | + | + |
| *E. gergoviae* | + | + | - | - | + | - | + | - | + | + | + | - | - | + | - | - | - | + | - | + | - | + | - | + |
| *E. hormaechei* | + | + | - | + | + | + | + | - | + | + | + | - | - | + | - | - | + | + | - | + | - | - | - | + |

**Table 2.** Contd.

| | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Hafnia alvei* | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | + | - | + | - | - | - | + |
| *Morganella morganii* biogroup 1 | + | + | + | + | - | - | - | + | + | - | - | + | - | + | - | - | - | - | - | - | - | - | - | + |
| *M. morganii* subsp *Sibon*i | + | - | - | + | - | - | - | - | + | - | + | + | - | - | - | - | - | - | - | - | - | - | - | + |
| *Pragia fontium* | + | - | + | + | - | - | - | - | + | - | + | + | - | - | - | - | - | - | - | - | - | - | + | - |
| *Proteus mirabilis* | + | + | + | + | - | - | - | - | + | - | + | + | + | - | - | - | - | - | - | - | - | - | - | + |
| *Proteus vulgaris* | + | - | + | + | + | + | + | + | + | - | + | + | + | + | - | - | - | + | + | - | - | - | - | + |
| *P. aeruginosa* | + | - | + | - | - | + | - | - | + | - | + | + | + | + | - | - | - | - | - | - | - | - | - | + |
| *P. fluorescens* | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| *Providencia alcalifaciens* | + | - | - | + | - | - | + | + | - | + | + | + | - | - | - | - | - | - | - | - | + | - | - | + |
| *Samonella* subsp. 1 | + | + | + | + | + | - | - | - | - | - | + | - | - | - | - | + | - | - | - | + | - | - | - | + |
| *Samonella* Arizonae | + | + | + | + | + | + | + | + | + | - | + | + | + | + | - | + | - | - | - | - | - | - | - | + |
| *Serratia liquefaciens* | + | - | - | + | + | + | + | - | - | + | + | - | - | - | - | + | - | + | - | + | - | - | - | + |

1, Lysine; 2, Ornithine; 3, $H_2S$; 4, Glucose; 5, Mannitol; 6, Xylose; 7, ONPG; 8, Indole; 9, Urease;10, Voges Proskeur; 11, Citrate; 12, TDA;13, Gelatin;14, Malonate;15, Inositol;16, Sorbitol;17, Rhamnose; 18, Sucrose; 19, Lactose; 20, Arabinose; 21, Adonitol 22, Raffinose;23, Salicin; 24, Arginine; *P. aeruginosa= Pseudomonas aeruginosa; P. fluorescens= Pseudomonasfluorescens; E. hormaechei = Enterobacter hormaechei; E. gergoviae = Enterobacter gergoviae; E. agglomeran = Enterobacter agglomerans; E. cloacae = Enterobacter cloacae; E. dissolven s= Enterobacter dissolven; A.hydrophila = Aeromonas hydrophila; C. diversu = Citrobacter diversus; E col i= Escherichia coli; E coli inactive = Escherichia coli inactive.*

22.75% included, *Enterobacter agglomerans* (0.5%), *Enterobacter cloacae* (18.25%), *Enterobacter dissolvens* (0.75%), *Enterobacter gergoviae* (0.5%) and *Enterobacter hormaechei* (2.75%). This was followed by *E. coli* with an isolation rate of 16.5%. *Morganella* species isolation rate was 5% comprising: *Morganella morganii biogroup* 1(3.75%) and *M. morganii* subspecie*s* Siboni (1.25%). *Proteus* species (4.5%) identified are; *Proteus mirabilis* (3%) and *Proteus vulgaris* (1.5%). The other bacteria isolates are *Citrobacter diversus* with isolation rate of 0.75%, *Serratia liquefaciens* and *Hafnia alvei* had the same isolation rate of 0.5% while *Providentia alcalifaciens* and *Pragia fontium* had the lowest isolation rate of 0.25% respectively.

Most of these organisms have been reported to be pathogenic to poultry and humans. For example, *E. coli* according to Todar (2007), can cause urinary tract infection (UTI), neonatal meningitis and intestinal disease (gastroenteritis) in humans. It is also the aetiology of avian colibacillosis which is considered as one of the principal causes of morbidity and mortality, associated with heavy economic losses to the poultry industry (Calnek et al., 1997). A group of *E coli* called *E coli* inactive was also isolated and identified, this was discovered to be non- lactose fermenters and also non-motile. This is in agreement with Le Minor and Richard (1993) and Holt et al. (1994) who discovered that a lactose-negative, non- motile organism thought to be *Shigella* species were inactive *E. coli* when identified serologically. *M. morganii* had been implicated in outbreaks of septicemia and bacteremia in humans and animals (Barragan Casas et al., 1999; Heard et al., 1988; Rowen and Lopez, 1998). *Serratia liquefaciens* had been implicated in the contamination of chicken carcasses (Lahellec et al., 1975) and it is also occasionally isolated from clinical specimens, although its pathogenic role has not been established (Grimont and Grimont, 1984). The presence of *Proteus mirabilis* in poultry meat had also been documented (Kim et al., 2005, Wong et al., 2013), and the presence of *Proteus species* in chicken droppings had been acknowledged as one of the route of its transmission as a zoonotic pathogens to vulnerable workers while handing infected chicken directly (Tonkic et al., 2010) or through fecal-contaminated poultry products as other Enteric bacteria (Lima-Filho et al., 2013). A more current report also associated poultry-borne *Proteus* species with zoonotic urinary tract infection (Armbruster et al., 2014).It was also associated with rheumatoid arthritis that is common in developing countries (Ebringer and

Rashid, 2014).

*Hafnia* species has been recovered from mammals as well as from poultry where it has been identified as infectious agent (Farmer, 2003; Proietti et al., 2004). Additionally, it has been reported as agent of nosocomial infections and community acquired infections in humans (Günthard and Pennekamp, 1996), of urinary tract infection and sepsis in infants (Laupland et al., 2006). Likewise, *Enterobacter species* have been reported to cause disease in poultry (Nandi et al., 2013), it has also been isolated form egg shell of poultry together with other pathogenic Enterobacterecea (Musgrove et al., 2008). Some species of *Enterobacter* have also been isolated from patients' cerebrospinal fluid (Parodi et al., 2003).

*Psedomonas* species and *Aeromonas hydrophila* which do not belong to the Enterobacteriacea family were also isolated. Two species of *Pseudomonas* (4%) namely: *Pseudomonas aeruginosa* (3.75%) and *Pseudomonas fluorescens* (0.25%) were isolated and identified. The percentage isolation rate of 0.25% was obtained for *Aeromonas hydrophila*. These two organisms have been implicated in poultry diseases and are also of zoonotic importance. It has been reported that *Pseudomonas* can be highly virulent causing 50 to 100% mortality in experimentally inoculated 4-week-old chickens (Saif et al., 2003). Fekadu (2010) also reported high mortality in newly hatched chickens and death at later stage of embryos in poultry farms as a result of *Pseudomonas* infection. On the other hand, *A. hydrophila* has been isolated from organs of clinically sick commercial chickens in Jos, Nigeria (Dashe et al., 2014). According to Okpokwasili and Ogbulie (2001), disease caused by *A. hydrophila* is endemic in Nigeria and is the most important zoonotic disease of fish. It can be deduced from this study that rats cohabiting with poultry habour pathogens of poultry and humans, these rats should be put into consideration whenever the sources of bacterial diseases aetiologic agents in poultry and humans is been traced. From this study, rat cohabitating with poultry poses public health threat to poultry and humans as possible agents of disease transmission.

Preventive measures to reduce rats' population in contact with poultry are highly recommended. This becomes imperative, since rat do not just constitute physical threat through their activities in destruction of poultry house infrastructures, they are also of great risk in the transmission of bacterial infection to poultry and man. Therefore adequate measures should be taken to prevent and eradicate rats from poultry houses and human abodes. Some of the recommended steps that could be taken to control rats population in and around poultry facilities include: Good sanitation; all feed bins must be kept in good repairs, and outside debris such as old equipments must be eliminated and grain of feed spillage which occurs during grain delivery must be cleaned immediately. Bushes around poultry houses should also be cleared regularly, as this act as breeding sites for rodents.

Rodent-proof construction should be encouraged: A lasting form of rodent control is to keep them out of poultry houses by eliminating all openings through which rodents can enter a poultry houses, this is often cost prohibitive but it is an effective measure. Likewise, grain storage houses should be made rodent proof.

Farmers should be educated and enlightened on the role of rodents in the transmission of bacterial disease to poultry, this will enable them pay more attention to rodent control.

## Conflict of interests

The authors have not declare any conflict of interest.

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# Mycorrhiazal association in relation to soil properties and plant species density in Sidama of Southern Ethiopia

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**In Sidama, agroforestry represents land-use systems with deliberate management of multipurpose trees and shrubs that grow in intimate association with annual and perennial agricultural crops and/or livestock. The interaction of microbiota with the trees, shrubs and crops make the system fertile, productive and sustainable. One of the beneficial microbiota which has symbiotic association with most of the plants in agroforestry is arbuscular mycorrhizal fungi (AMF). In November and December of 2012, root and rhizosphere soil samples of 21 plant species from nine peasant associations (PAs) (villages within districts where 300 to 500 families live) were collected from the agroforestry practices in Sidama of Southern Ethiopia for the determination of diversity of AMF based on selected soil parameters and plant species density. Findings on the diversity of AMF based on soil properties showed that at moderate to low P and N concentrations the rate of root colonization and spore density was high in comparison with the rhizosphere soils with the highest P and N concentration. The highest percentage of total colonization was recorded for shade trees *Millettia ferruginea* (84%) *and Erythrina brucei* (80%) followed by intercropped perennial crops *Ensete ventricosum* (86%), *Catha edulis* (85%) and *Coffea arabica* (80%) and the lowest percentage colonization was recorded for *Rhamnus prinoides* (53%) and *Colocasia esculenta* (52%). Though found in almost all homegarden agroforestry practices and with broad coverage in Sidama agroforestry, some crops and vegetables such *Brassica integrifolia* and *Cucurbita pepo*, grown intercropped were found to be non-mycorrrhizal as none of the AMF structures were recorded. The highest number of AM spore population was recorded in rhizosphere soils of *Croton macrostachyus* (1066±19.33) and *Catha edulis* (1054±53.12) and the lowest spore density was recorded for *Dioscorea alata* (100.00±2.89) spore per 100 g of dry soil. The percentage fungal colonization in any individual plant species and spore population in the rhizosphere soils of that species did not correlate to each other and percentage root colonization and spore density of all plants in the agroforestry of Sidama were found significantly different at P<0.05 level.**

**Key words:** Arbuscular mycorrhizal fungi (AMF), dependency, rhizosphere, parameters, colonization, density.

## INTRODUCTION

Forests are cleared for agriculture and settlement in different parts of Ethiopia. Agroforestry is an age old traditional practice which gradually replaced forests. It is a sustainable management system for land that

combines agricultural crops, trees, forest plants and/or animals simultaneously or sequentially, and applies management practices that are compatible with the cultural patterns of the local population (Raintree, 1997). It is practiced on Homegarden (Millat-e-Mustafa, 1997; Zebene, 2003), cropland (Roy, 1997), forestlands (Emiru et al., 2010; Wang et al., 2004). Although the role of agroforestry in conserving biological diversity is being appreciated in many parts, the sustainability of these practices is a major concern in Ethiopia.

Low soil fertility, acidity and deficiency in major nutrients such as phosphorus and nitrogen are some of the problems related to successful agroforestry systems. The conversion of forest to different agricultural systems causes loss of plant species that expose the soil to water and wind erosion. Besides, the removal of plant species from forests and agroforests may be accounted for the loss of biodiversity, not only of plants, but also the microbiota important for the soil health as a whole. Therefore, maintenance of floristic diversity in agroforestry is number one crucial condition for sustainable productivity of the agroforests. One of such situations is planting of mycorrhizal seedlings that can be a good measure to increase the yield of the plants for the sustainable management for land that will increase the overall production.

Arbuscular mycorrhizal fungi are widespread and symbiotic copious members of the soil biota, a generally acknowledged key factor in agricultural ecosystem functioning and sustainability (Verbruggen and Kiers, 2010). These symbiotically associated fungi play an important role to the plants in agroforestry practices by enhancing establishment and growth, increasing nutrient and water uptake, maintaining diversity by accelerating the ability of host plants to compete for resources, contributing to efficient recycling of nutrients and thus to long term stability and stabilization of the soil (Smith and Read, 1997; Jasper, 1992). AMF are widespread in tropical soils and associate with a wide range of plant species, including most commercial crops (Sieverding, 1991) and trees (da Silva Sousa et al., 2013). They are keystone organisms and form an interface between soils and plant roots, and are sensitive to changes in soil physicochemical properties and plant conditions (Power and Mills, 1995).

Under the insufficient concentration of nutrients in the root zone of plants, exploring further in the soil with its extended extra radical hyphae, AMF supply phosphorus, macro and micro soil nutrients to roots which has symbiosis with AMF. In these symbiotic associations both partners benefit from each other under certain conditions (Demir, 2008; Rhodes, 1980; Bolan et al., 1987; Li et al., 1991). Fungi take some organic matter and carbohydrates from the plant. In return, AMF supply nutrients such as phosphorus, nitrogen (N), calcium (Ca), copper (Cu), manganese (Mn), sulphur (S) and zinc (Zn) (Sieverding, 1991; Ortas, 2002). But when the soil is rich in necessary nutrients, plants may not waste more energy for the association with AMF and go for the cheapest source of energy, and individual plant species and plant communities in natural and farming systems affect the distribution and diversity of AMF species (Dalpe et al., 2000; Jefwa et al., 2006).

Therefore, according to the previous investigators, AMF spore density, diversity and rates of root colonization are dependent on soil physicochemical properties (Mahmud et al., 1999) and plant species density and richness (Allsopp and Stock, 1992). Though there are some research reports (Zebene, 2003) on mycorrhizal association of agroforestry trees, information is scarce on spore density and diversity of AMF based on soil parameters and type of plant species density and richness in Sidama agroforestry. Therefore, the objective of the present investigation is to study diversity of AMF based on selected soil parameters and plant species density in selected sites of Sidama agroforestry practices.

## MATERIALS AND METHODS

### Sampling site

This study was conducted in Sidama Administrative region of Southern Ethiopia during dry season in November and December of 2012. It lies between 06°45´33" and 06$^0$54´713" N and 038°27´432" and 038$^0$31´788"E and 1740 to 2135 m above the sea level. The study area is characterized by a moist to sub-humid warm sub-tropical climate with annual rainfall of 1000 to 1800 mm and mean annual temperature of 15 to 20°C. The main agricultural system in the region is the Tree-enset-coffee based home-garden agroforestry systems commonly practiced in Southern Ethiopia. The study was undertaken in 36 home gardens located in 9 peasant association (PAs, smallest Ethiopian administrative unit, where 300 to 500 families live) distributed over two woredas or district. Each district has 40 to 60 PAs where these agroforestry systems are practiced. In this study, we selected only 9 PAs (Table 2) because these PAs are located at closest proximity to the original forest relics from where the agroforestry practices were emerged. From each PAs, 4 home gardens were selected randomly and soil and root samples were collected from 23 commonly occurring plant species in selected agroforestry practice (Table 1).

### Soil sampling and analysis

Rhizosphere soils from under the canopy of the 23 plant species

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**Table 1.** Plant species present in Sidama agroforestry systems the time of sampling.

| Latin names | Family | Amharic | Sidamigna |
|---|---|---|---|
| *Ensete ventricosum (Welw.) Cheesman)* | *Musaceae* | Enset | Wesse |
| *Catha edulis (vahl.)Forssk.ex Endl* | *Celastraceae* | Khat | Khat |
| *Millettia ferruginea Hochst* | *Fabaceae* | Birbira | Hengedicho |
| *Erythrina brucei Schweinf* | *Fabaceae* | Korch | Wellako |
| *Cordia africana Lam.* | *Boraginaceae* | Wanza | Wadicho |
| *Croton macrostachyus (HochstExDel.)* | *Euphorbaceae* | Bisana | Mesincho |
| *Prunus africana (Hook.f.)Kalkm.* | *Rosaceae* | Tikur Inchet | Garbicho |
| *Vernonia amygdalina Del.* | *Asteraceae* | Grawa | Hecho |
| *Persea americana Mill.* | *Lauraceae* | Avokado | Abukato |
| *Mengifera indica L.* | *Anacardiaceae* | Mango | Mango |
| *Saccharum officinarum L.* | *Poaceae* | Shenkorageda | Shenkora |
| *Ricinus communis L.* | *Euphorbiaceae* | Castor | Qenboo |
| *Coffea arabica L.* | *Rubiaceae* | Coffee | Buna |
| *Zea mays L.* | *Poaceae* | Bokolo | Bedela |
| *Phaseolus vulgaris L.* | *Fabaceae* | Common bean | Wahe |
| *Ipomoea batatas (L.) Lam* | *Convolvulaceae* | Sikuar dinich | Metatessa |
| *Solanum tuberosum L.* | *Solanaceae* | Potato | Dinich |
| *Cucurbita pepo L.* | *Cucurbitaceae* | Pumpkin | Baqula |
| *Brassica integrifolia* (West) O.E. schulz | *Brassicaceae* | Kale | Shana |
| *Cappisicum annuum L.* | *Solanaceae* | *Chilly* | *Mitmita* |
| *Rhamnus prinoides L' herit* | *Rhamnaceae* | Gesho | Taddo |
| *Dioscorea alata L.* | *Dioscoraceae* | Boyna | Bohe |
| *Colocasia esculenta (L.)Schoot.* | *Araceae* | Godere | Qolchoma |

**Table 2.** Geographical location and altitudinal ranges of the Peasant Associations (PA's)

| Study site(PAs) | Latitude | Longitude | Altitude |
|---|---|---|---|
| Ferro I | 06°46'30"N | 038°29'00"E | 1795-1896 |
| Ferro II | 060°49'602"N | 038°29'135"E | 1860-1940 |
| Halekena | 06°45'333"N | 038°27'432"E | 1740-1825 |
| Taramesa | 06°086'055"N | 038°45'605"E | 1745-1868 |
| Tellamo | 06°083'629"N | 038°48'489"E | 1825-1906 |
| Gonowa goda | 06°084'609"N | 038°50'916"E | 1855-1990 |
| Abela Lida | 06°54'993"N | 038°29'317"E | 1945-1960 |
| Galakohireye | 06°54'864"N | 038°30'315"E | 2006-2011 |
| Arossa | 06°54'713"N | 038°31'788"E | 2111-2135 |

which included trees, crops and vegetable species were collected during dry season (November-December) in year 2012 from each sampling site (PAs). Soil sample collection was carried out at four 10m X 10m quadrats from each PAs. The soil was sampled to a depth of 0 to 15 cm in 828 sampling points using a soil auger. A composite soil sample of 500 g was collected from each plant species. In total (23x9x4) samples were collected for analysis from all the 9 PAs (sampling sites), that is, 23x4 samples from each sampling site. The soil samples were air-dried at room temperature for two weeks and preserved at 4°C for analysis of soil physicochemical properties, AMF spore abundance and AMF diversity. About 0.5 mg of fine root samples from each plant were also collected, washed with tap water, preserved in 50% ethanol, and stored at 4°C for percentage root colonization analysis.

Soil analysis were undertaken at the Southern Nations, Nationalities and Peoples Region (SNNPR) Soil Laboratory and Debrezeite Agricultural Research Center following standard procedures and methods: Soil textural fractions were analyzed following the hydrometric method after removing organic matter using $H_2O_2$ and dispersing the soils with sodium hexameta-phosphate (Black et al., 1965). Soil pH was determined by potentiometric methods using 1:2.5 soil: water ratio. Soil organic

carbon (SOC) was determined by the Walkley-Black oxidation method (Schnitzer, 1982). Total nitrogen (TN) was determined using the Kjeldahl distillation method (Bremner, 1982), and available phosphorous (AP) was determined using Olsen's extraction method (UV/visible Spectrometer, Lambda EZ 201) (Olsen et al., 1965). Available potassium (Av. K) was determined by Sodium Acetate method (Jones, 2001).

#### Root colonization

The root pieces collected and preserved were stained according to Phillip and Hayman (1970) with some modifications. The root pieces were cleared in 10% KOH solution for 15 to 20 min at 90°C in a boiling water bath. Then, they were rinsed in water several times and acidified with 1% HCl solution. Pigmented roots were bleached in $H_2O_2$ for about 20 min and again washed with water. Finally the root segments were stained for 10 min in 0.05% trypan blue at 90°C for about 10 min and subsequently distained at room temperature in acidic glycerol.

The stained root segments were mounted in acidic glycerol on slides. The roots were then observed under the compound microscope (200x objective magnification). The presence of colonization in a root segment was recorded if hyphae (only), vesicle or arbuscule were found. About 100 to 150 intersections were examined for each sample. Total root colonization was calculated using the following formula: % Colonization = Total number of positive segments/Total number of segments studied × 100.

#### Spore population

From each soil sample 100 g soil was mixed in a 2 L capacity beaker containing 1.5 L of water. The soil in water was agitated by stirring vigorously by hand and left to settle down for about five minutes. The suspension was then sequentially (480, 100, 50 and 38 µm) sieved following the wet sieving and decanting method (Gerdemann and Nicolson, 1963). The last pellet (38 µm) was suspended in 60% sucrose solution and thoroughly mixed and centrifuged at 2000 rpm for 1 min and the spores were rinsed carefully with tap water and transferred into plastic petri-dishes.

The AMF spores and sporocarps of each sample were counted under 4x stereomicroscope. The spore densities were expressed as the numbers of spores and sporocarps per 100 $g^{-1}$ of dry soil. Representative Morphospecies were mounted on slides with polyvinyl-lactic acid-glycerol (PVLG) or PVLG mixed with Melzer's reagent (1:1 v/v). Taxonomic differentiation was made based on the descriptions of the International Culture Collection of Vesicular/Arbuscular Mycorrhizal Fungi (http://invam.caf.wvu.edu; 2005), and following descriptions by Schenck and Pěrez (1990).

#### Statistical analysis

Data on spore abundance and root colonization was log(x) and arcsine (the inverse sine of the square root of the proportion) transformed using PAST3 (ver. 1.0.0.0) and SPSS software package (version 20.0) respectively, prior to analysis to meet assumptions of ANOVA such as normality and homogeneity of variance.

Significance of differences in AM fungal spore abundance and percentage of root colonization between the samples was tested using Duncan's Multiple Range Test at $p < 0.05$ after one-way analysis of variance (ANOVA) with the SPSS software package (version 20.0) (Fisher et al., 1970). Pearson's correlation analysis was used to study the relationship between AMF and soil parameters.

## RESULTS

### Soil physicochemical properties under different plant species

The physicochemical properties of soil samples of each plant are shown in Table 3. The soil in the study area was slightly acidic with mean pH of 5.87; mean organic carbon, total nitrogen, available phosphorus and potassium being 2.73, 0.30, 12.39 and 1.10 respectively. When individual plant species were considered, all plant species studied significantly affected the rhizosphere soil chemical properties (Table 3). They showed variation in soil pH ($H_2O$) from 5.0 (acidic) - 7.0 (neutral), whereas soil nitrogen content was in between 0.19 (medium) - 0.36 % (high).

Similarly, phosphorus (Olson) content varied from the lowest 6.75 mg P/kg (*Solanum tuberosum*) to the highest 18.25 mg P/kg (*Persea americana*). Potassium was highest in the rhizosphere soil of *Ipomoea batatas* (2.18cmol (+)/kg) and the lowest (0.55 cmol (+)/Kg) was recorded from *Prunus africana* and *Ricinus communis.* Organic carbon (OC) content of soil sample fall in between 2.04 (high) to 3.45 (very high).

### AMF root colonization and spore density

The AMF root colonization on the different agroforestry plant species is presented in Table 4. The highest percentage root length colonization (RLC) was recorded in *Ensete ventricosum* (86%) and the lowest in *Rhamnus prinoides* (53%) and *Colosica esculenta* (52%) respectively (Table 5). All plants were infected with AMF arbuscules and vesicles except *Brassica integrifolia* and *Cucurbita pepo* species (data not shown) (Table 4).

In case of vesicle formation, the highest colonization was found in *Ensete ventricosum* and *Coffea arabica* each 20.33% followed by *Zea mays* 18.67% and *Catha edulis* (17%) and the lowest in *Persea americana* (9.67%), *Mangifera indica* (9.33) and *Discorea alata* (9%). Considering arbuscule formation, the highest percentage was observed in *Ricinus communis* (18%), *Persea americana* (18%) followed by *Ensete ventricosum* (15%) and *Solanum tuberosum* (15.33%). The lowest arbuscular colonization was recorded for *Colocasia esculenta* (5%). In most of the records arbuscular and vesicular colonization are not significantly different at $P<0.05$ level.

The highest number of spore population/100 g dry soil was recorded in the rhizosphere soil of *Croton*

**Table 3.** Rhizosphere soil chemical properties of plants.

| Plant species | pH($H_2O$) | OC% | TN% | AP(Olson) (mg/kg) | Kcmol (+)/kg |
|---|---|---|---|---|---|
| *Ensete ventricosum (Welw.) Cheesman)* | 6.49±.12$^{b}$ | 2.41±.23$^{a}$ | 0.26 ±0.02$^{c}$ | 10.20±1.3$^{e}$ | 0.61±0.33$^{a}$ |
| *Catha edulis (vahl.)Forssk.ex Endl* | 5.02±0.00$^{a}$ | 2.04±0.02$^{a}$ | 0.23±0.00 $^{b}$ | 7.12±0.01$^{b}$ | 0.88±0.01$^{a}$ |
| *Millettia ferruginea Hochst* | 6.530.03$^{b}$ | 2.46±0.01$^{a}$ | 0.36 ±0.01$^{d}$ | 9.13±0.01$^{d}$ | 1.01±0.00$^{b}$ |
| *Erythrina bruce Schweinf* | 6.11±0.01$^{b}$ | 2.48±0.03$^{a}$ | 0.31 ±0.00$^{d}$ | 9.10±0.05$^{d}$ | 1.16±0.01$^{b}$ |
| *Cordia africana Lam.* | 7.12±0.01$^{c}$ | 3.45±0.03$^{a}$ | 0.36 ±0.01$^{d}$ | 15.33±0.02$^{g}$ | 1.22±0.01$^{b}$ |
| *Croton macrostachyus (HochstExDel)* | 6.50±0.06$^{b}$ | 2.50±0.06$^{a}$ | 0.33 ±0.02$^{d}$ | 8.15±0.01$^{c}$ | 0.98±0.01$^{ab}$ |
| *Prunus africana (Hook.f.)Kalkm.* | 5.71±0.32$^{a}$ | 2.64±0.01$^{ab}$ | 0.29±0.00 $^{d}$ | 16.12±0.01$^{h}$ | 0.55±0.01$^{a}$ |
| *Vernonia amygdalina Del.* | 5.60±0.08$^{a}$ | 3.15±0.00$^{c}$ | 0.34±0.01$^{d}$ | 17.07±0.06i | 1.19±0.05$^{b}$ |
| *Persea americana Mill.* | 6.40±0.12$^{b}$ | 2.63±0.00$^{ab}$ | 0.29 ±0.01$^{d}$ | 18.25±0.14$^{j}$ | 0.88±0.01$^{a}$ |
| *Mengifera indica L.* | 5.20±0.23$^{a}$ | 2.74±0.01$^{ab}$ | 0.36±0.02 $^{d}$ | 16.50±0.06$^{h}$ | 0.75±0.02$^{a}$ |
| *Saccharum officinarum L.* | 5.35±0.02$^{a}$ | 2.31±0.00$^{a}$ | 0.29±0.01$^{d}$ | 9.23±0.03$^{c}$ | 1.35±0.0$^{b}$ |
| *Ricinus communis L.* | 5.38±0.03$^{a}$ | 2.63±0.04$^{ab}$ | 0.29 ±0.00$^{d}$ | 8.21±0.01$^{c}$ | 0.55±0.06$^{a}$ |
| *Coffea arabica L.* | 6.50±0.06$^{b}$ | 3.21±0.01$^{c}$ | 0.32 ±0.01$^{d}$ | 7.68±0.01$^{b}$ | 1.21±0.02$^{b}$ |
| *Zea mays L.* | 5.63±0.04$^{a}$ | 2.51±0.29$^{a}$ | 0.30 ±0.01$^{d}$ | 8.93±0.04$^{c}$ | 0.71±0.02$^{a}$ |
| *Phaseolus vulgaris L.* | 5.51±0.29$^{a}$ | 3.14±0.02$^{c}$ | 0.33 ±0.01$^{d}$ | 7.68±0.28$^{b}$ | 1.14±0.01$^{b}$ |
| *Ipomoea batatas (L.) Lam* | 5.14±0.09$^{a}$ | 2.53±0.00$^{a}$ | 0.30 ±0.01$^{d}$ | 7.35±0.03$^{b}$ | 2.18±0.01$^{c}$ |
| *Solanum tuberosum L.* | 5.33±0.09$^{a}$ | 2.71±0.02$^{ab}$ | 0.35 ±0.00$^{d}$ | 6.75±0.03$^{a}$ | 1.15±0.01$^{b}$ |
| *Cappsicum annuum L.* | 6.05±0.48$^{b}$ | 2.47±0.02$^{a}$ | 0.30 ±0.01$^{d}$ | 14.52±0.25$^{f}$ | 0.95±0.03$^{ab}$ |
| *Rhamnus prinoides L' herit* | 5.67±0.22$^{a}$ | 2.65±0.00$^{ab}$ | 0.34 ±0.01$^{d}$ | 16.01±1.16$^{h}$ | 1.20±0.02$^{b}$ |
| *Dioscorea alata L.* | 5.93±0.08$^{ab}$ | 2.37±0.07$^{a}$ | 0.19 ±0.01$^{a}$ | 17.09±0.03i | 1.34±0.01$^{b}$ |
| *Colocasia esculenta (L.)Schoot.* | 5.23±0.09$^{a}$ | 2.78±0.01$^{ab}$ | 0.27 ±0.01$^{c}$ | 16.60±0.12$^{h}$ | 2.22±0.01$^{c}$ |
| **Mean Total** | 5.87±0.00$^{ab}$ | 2.73±0.05$^{ab}$ | 0.30 ±0.01$^{d}$ | 12.39±0.54$^{ef}$ | 1.10±0.05$^{a}$ |

OC, Organic carbon; TN, Total nitrogen; AP, Available phosphors, K, Potassium. Similar letters in columns show not significant difference between plant species at p<0.05.

*macrostachyus (*1066) followed by *Catha edulis* (1054) and *Coffea arabica (*995) and the lowest density was recorded in *Dioscorea alata* (100 spores/100 g soil) (Table 4). From the data (Table 4), it is also observed that the percentage root colonization and spore population do not correlate to each other. Current result in this study reveals that under highest P and N concentrations spore density decreased in soils from rhizospheres of some plant species, although the opposite is true for the other species. For soils from the rhizospheres of *P. americana* (P, 19.25; N 0.29), *Vernonia amygdalina* (P, 17.07; N 0.34), *M. indica* (P, 16.50; N, 0.36), and *D. alata* (P, 17.09; N, 0.19) was recorded, 550 and 80, 600 and 78, 580 and 80 and 100 and 66 spores per 100 g soil and percentage root colonization respectively.

On the other hand for agroforestry plant species with low to medium P concentration such as *Catha edulis* (P, 7.12; N, 0.23) *Croton macrostachyus* (P, 8.15; N, 0.33, *Coffea arabica* (p,7.68; N, 0.32) was recorded 1054 and 85, 1066 and 64 and 995 and 80 spores per 100 g soil and percentage root colonization respectively. This result indicates that different plant species respond to AMF differently under low, medium and high concentrations of nutrients in their rhizospheres.

Pearson's correlation coefficient (Table 4) showed that spore density was not significantly correlated with soil pH, OC, total nitrogen and potassium and significant negative correlation with available phosphorus (r = -0.346, p = 0.005) and electron conductivity (r = -0.441, p =0.00) at P<0.01 level (2-tailed). Spore density was also negatively correlated with total nitrogen, but not significantly different.

Pearson's correlation coefficient between root colonization and soil parameters showed that it is negatively correlated to available phosphorus (r = -0.324, p = 0.010) and potassium (r = -0.301, p = 0.017) and significantly different at P<0.01 and P<0.05 level (2-tailed) respectively. It was positive with pH and total nitrogen but not significantly different and was negative but significantly different between root colonization and organic carbon, and negative but not significantly different in between EC and root colonization.

## AMF spore diversity

In general 8 different AMF genera were identified (Table

**Table 4.** Root colonization and spore density of AMF in agroforestry plant species.

| Plant species | AMF structural colonization (%) and spore density | | | |
|---|---|---|---|---|
| | AC | VC | RLC | SD/100 $g^{-1}$ dry soil |
| *Ensete ventricosum* (Welw.) Cheesman) | 15.00±2.08$^{ab}$ | 20.33±0.33$^{a}$ | 85.73±3.73$^{a}$ | 630.00±2.90$^{d}$ |
| *Catha edulis* (vahl.) Forssk.ex Endl | 16.67±2.33$^{a}$ | 17.00±1.15$^{b}$ | 84.74±1.24$^{a}$ | 1054.00±53.12$^{a}$ |
| *Millettia ferruginea* Hochst | 11.67±0.88$^{b}$ | 16.67±1.76$^{b}$ | 83.80±2.22$^{ab}$ | 686.00±2.31$^{d}$ |
| *Erythrina brucei* Schweinf | 13.67±1.67$^{abc}$ | 14.67±1.33$^{bc}$ | 80.23±1.04$^{b}$ | 768.00±39.26$^{cd}$ |
| *Cordia africana* Lam. | 12.00±2.31$^{c}$ | 14.00±3.00$^{bc}$ | 72.33±1.57$^{c}$ | 880.00±49.07$^{c}$ |
| *Croton macrostachyus* (HochstExDel.) | 10.00±0.58$^{c}$ | 14.33±0.33$^{c}$ | 64.11±3.20$^{d}$ | 1066.00±19.63$^{a}$ |
| *Prunus africana* (Hook.f.)Kalkm. | 11.67±0.33$^{b}$ | 9.67±0.88$^{e}$ | 57.67±2.69$^{d}$ | 675.00±14.43$^{d}$ |
| *Vernonia amygdalina* Del. | 7.00±1.53$^{c}$ | 13.67±2.40$^{cbc}$ | 77.78±1.91$^{b}$ | 600.00±46.19$^{d}$ |
| *Persea americana* Mill. | 18.33±3.84$^{a}$ | 15.33±3.18$^{bc}$ | 80.32±2.47$^{ab}$ | 550.00±21.94$^{e}$ |
| *Mengifera indica* L. | 10.33±1.20$^{c}$ | 9.33±0.88$^{de}$ | 79.97±0.77$^{b}$ | 580.00±69.28$^{d}$ |
| *Saccharum officinarum* L. | 13.67±4.18$^{abc}$ | 16.00±4.04$^{ab}$ | 68.87±2.25$^{b}$ | 625.00±14.43$^{d}$ |
| *Ricinus communis* L. | 18.00±5.69$^{ab}$ | 14.00±4.1$^{bcd}$ | 74.25±4.31$^{bc}$ | 800.00±86.60$^{c}$ |
| *Coffea arabica* L. | 11.33±1.45$^{c}$ | 20.33±2.19$^{a}$ | 80.14±1.89$^{b}$ | 995.00±2.89$^{b}$ |
| *Zea mays* L. | 12.67±0.33$^{b}$ | 18.67±1.76$^{ab}$ | 80.59±1.82$^{b}$ | 700.00±46.19$^{c}$ |
| *Phaseolus vulgaris* L. | 11.33±1.67$^{c}$ | 15.00±0.58$^{b}$ | 73.27±1.10$^{c}$ | 495.00±83.72$^{d}$ |
| *Ipomoea batatas (L.) Lam* | 12.00±0.58$^{b}$ | 15.00±0.58$^{b}$ | 70.62±1.96$^{c}$ | 665.00±39.84$^{d}$ |
| *Solanum tuberosum L.* | 15.33±5.55$^{ab}$ | 12.00±2.52$^{c}$ | 73.04±1.63$^{c}$ | 520.00±11.55$^{e}$ |
| *Cappisicum annuum L.* | 12.33±1.45$^{b}$ | 10.00±3.00$^{dcd}$ | 72.67±1.76$^{c}$ | 632.00±27.71$^{d}$ |
| *Rhamnus prinoides L' herit* | 9.67±1.20$^{c}$ | 9.67±0.88$^{de}$ | 53.33±5.02$^{d}$ | 751.00±5.20$^{c}$ |
| *Dioscorea alata L.* | 11.33±1.45$^{c}$ | 9.00±1.53$^{de}$ | 60.52±4.67$^{d}$ | 100.00±2.89$^{f}$ |
| *Colocasia esculenta (L.)Schoot* | 5.00±1.0$^{d}$ | 14.33±7.17$^{c}$ | 51.50±7.17$^{d}$ | 660.00±92.38$^{cd}$ |

AC, Arbuscular colonization; VC, vesicular colonization; RLC, Total root length colonization; SD, Spore Density. Similar letters in columns show not significant difference between groups at p<0.05.

**Table 5.** Pearson's correlation coefficient in between AMF parameters and soil chemical properties.

| Parameter | SD | RLC | pH | OC | T.N | Av.P | Ec | K |
|---|---|---|---|---|---|---|---|---|
| Root colonization | 0.243$^{ns}$ | 1 | | | | | | |
| pH | 0.101$^{ns}$ | 0.129$^{ns}$ | 1 | | | | | |
| Organic carbon | 0.120$^{ns}$ | -0.010$^{ns}$ | 0.281* | 1 | | | | |
| Total nitrogen | -0.002$^{ns}$ | 0.121$^{ns}$ | 0.006$^{ns}$ | 0.236$^{ns}$ | 1 | | | |
| Available P | -0.346** | -0.324** | 0.088$^{ns}$ | 0.227$^{ns}$ | 0.142$^{ns}$ | 1 | | |
| Electron conductivity(Ec) | -0.441** | -0.105$^{ns}$ | 0.085$^{ns}$ | 0.163$^{ns}$ | 0.384** | 0.182$^{ns}$ | 1 | |
| Potassium(K) | -0.130$^{ns}$ | -0.301* | -0.198$^{ns}$ | 0.137$^{ns}$ | 0.043$^{ns}$ | 0.057$^{ns}$ | 0.138$^{ns}$ | 1 |

significant at the 0.05 level (2-tailed); ns, Not significantly different.

6): *Acaulospora, Claroidioglomus, Funneliformis, Glomus, Gigaspora, Rhizophugus, Septoglomus, and Scutellospora of which Glomus* and *Acaulospora* were the dominant genera found in all species. The highest percentage of *Glomus* was recorded in *C. arabica* (15.27%) and *E. ventricosum* (12.68%) the lowest being recorded in *Cappisicum annuum and C. esculenta* (0.29%). The highest percentage of *Acaulospora* was recorded in three species; *E. ventricosum, C. arabica, and Zea mays* with 14.93, 10.85 and 11.76% respectively. The highest percentage was recorded for *Claroideoglomus* (24.18%) *in Croton macrostachyus, Funneliformis* (33.9%) in *E. ventricosum, Gigaspora* (22.94%) in *Catha edulis, Rhizophugus* (28.67%) in *E. ventricosum, Septoglomus* (20%) in *Saccharum officinarum,* and *Scutellospora* (16.67%) in *E. ventricosum.* In this study, observed Plant species density (Figure 1) in the agroforestry does not induced consistent spore formation and percentage root colonization values in all plant species. *C. esculenta* with its relative abundance value of 0.1% (982) showed

**Table 6.** Percentage AMF diversity in the 21 plant species in Sidama agroforestry.

| Plant species | Different Genera of AMF (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ***Ac.*** | ***Clar.*** | ***Fun.*** | ***Glo.*** | ***Giga.*** | ***Rhi.*** | ***Sep.*** | ***Scut.*** | ***Sp.No*** |
| *Ensete ventricosum (Welw.) Cheesman)* | 14.93 | 14.38 | 33.9 | 12.68 | 17.65 | 28.67 | 13.33 | 16.67 | 8 |
| *Catha edulis (vahl.)Forssk.ex Endl* | 3.18 | - | 10.17 | 9.8 | 22.94 | 16 | - | 2.38 | 6 |
| *Millettia ferruginea Hochst* | 4.07 | 14.38 | 11.86 | 11.82 | 2.35 | 12.67 | 6.67 | 5.95 | 8 |
| *Erythrina brucei Schweinf* | 4.07 | 5.23 | 16.95 | 2.31 | 3.53 | 3.33 | - | 1.19 | 7 |
| *Cordia africana Lam.* | 3.62 | 5.23 | 5.08 | 0.86 | 3.53 | 0.67 | - | 2.38 | 7 |
| *Croton macrostachyus (HochstExDel)* | 9.95 | 24.18 | 6.78 | 9.51 | 1.76 | 7.33 | 13.33 | 8.33 | 8 |
| *Prunus africana (Hook.f.)Kalkm.* | 3.17 | 1.31 | - | 1.73 | 1.18 | 6 | - | 4.76 | 6 |
| *Vernonia amygdalina Del.* | 2.71 | 2.61 | 3.39 | 4.9 | 1.18 | 6 | 13.33 | 4.76 | 8 |
| *Persea americana Mill.* | 4.07 | - | - | 1.73 | 1.18 | 2.67 | - | 3.57 | 5 |
| *Mengifera indica L.* | 1.36 | 1.31 | - | 3.17 | - | 2 | - | 3.57 | 5 |
| *Saccharum officinarum L.* | 3.62 | 2.61 | - | 3.46 | 0.58 | 3.33 | 20 | 7.14 | 7 |
| *Ricinus communis L.* | 3.18 | - | 3.39 | 1.44 | 2.53 | 0.67 | - | 3.57 | 6 |
| *Coffea arabica L.* | 10.85 | 17.64 | - | 15.27 | 17.65 | 2 | - | 13.1 | 6 |
| *Zea mays L.* | 11.76 | - | - | 12.1 | 14.1 | 0.67 | 13.33 | 4.76 | 6 |
| *Phaseolus vulgaris L.* | 4.07 | 1.31 | 3.39 | 1.44 | 2.53 | 3.33 | - | 4.76 | 7 |
| *Ipomoea batatas (L.) Lam* | 2.26 | 3.92 | - | 2.59 | 2.94 | 2 | - | 3.57 | 6 |
| *Solanum tuberosum L.* | 1.8 | - | 3.39 | 1.73 | 1.76 | - | 13.33 | 5.95 | 6 |
| *Cappisicum annuum L.* | 3.18 | 1.31 | - | 0.29 | - | - | - | - | 3 |
| *Rhanmnus prinoides L'herit* | 2.26 | 1.96 | 1.69 | 0.58 | - | - | - | - | 4 |
| *Dioscorea alata L.* | 1.36 | 1.31 | - | 0.86 | 1.18 | - | 6.67 | 1.19 | 6 |
| *Colocasia esculenta (L.)School.* | 0.45 | 1.31 | - | 0.29 | 1.18 | - | - | - | 4 |
| *Mean* | 9.91 | 7.29 | 2.814 | 16.29 | 8.05 | 6.95 | 0.71 | 3.90 | |

*Ac., Acaulospora; Claro., Claroidioglomus; Fun., Funneliformis; Glo., Glomus; Giga., Gigaspora; Rhi., Rhizophugus; Sep., Septoglomus; Scut., Scutellospora*.

percentage colonization of 52%, spore density of 660 and for *D. alata* with its relative abundance value of 0.05% (511) was recorded root colonization of 61%, more than *C. esculenta* (Figure 1) and the least spore density of 100 spore/100 g soil.

On the other hand for species with the highest value of percentage abundance 0.22% (2107) in *E. ventricosum*, was recorded the highest percentage colonization (86%) and medium number of spore density (630 spores/100 g dry soil).

For *C. macrostachyus* with percentage abundance value of about 0.008% (75), was recorded 1066 spores/100 g soil and 64.11% root colonization.The next highest spore density (1054 spores/1001 g soil) and root colonization (85%) was recorded for perennial crop *Catha edulis* with its percentage abundance value of about 0.02% (188), followed by *C. arabica,* 995 spores 100-1 g soil and 80% colonization with its abundance value of 0.12% (1147) respectively. This indicates that in some plant species (e.g. *E. ventricosum*) density decreased the spore production but in some other plant species such as *C. arabica* plant species density has increased spore production.

## DISCUSSION

The effects of plant species density and its associated soil chemical properties in Sidama agroforestry homegardens were studied to determine their influence on AMF diversity, spore density and root colonization. The arbuscular mycorrhizal fungi spore population and root colonization pattern varied in different plant species in Sidama homgarden agroforestry systems. The study on diversity of AMF in relation to soil parameters and plant species density did not show significant differences in AMF diversity indices (Shannon-Weaver diversity index, Simpson's Index of Diversity (data not shown)) due to plant species density and soil physical and chemical properties. However, there were differences in AMF spore density and root colonization between plant species. Shifts in AMF species composition with increasing or decreasing plant species density may be explained in part by differential responses of individual AMF species to individual host plant species and edaphic factors in the rhizospheres of each plant species.

According to Koide and Dickie (2002), three mechanisms have been proposed to account for plant

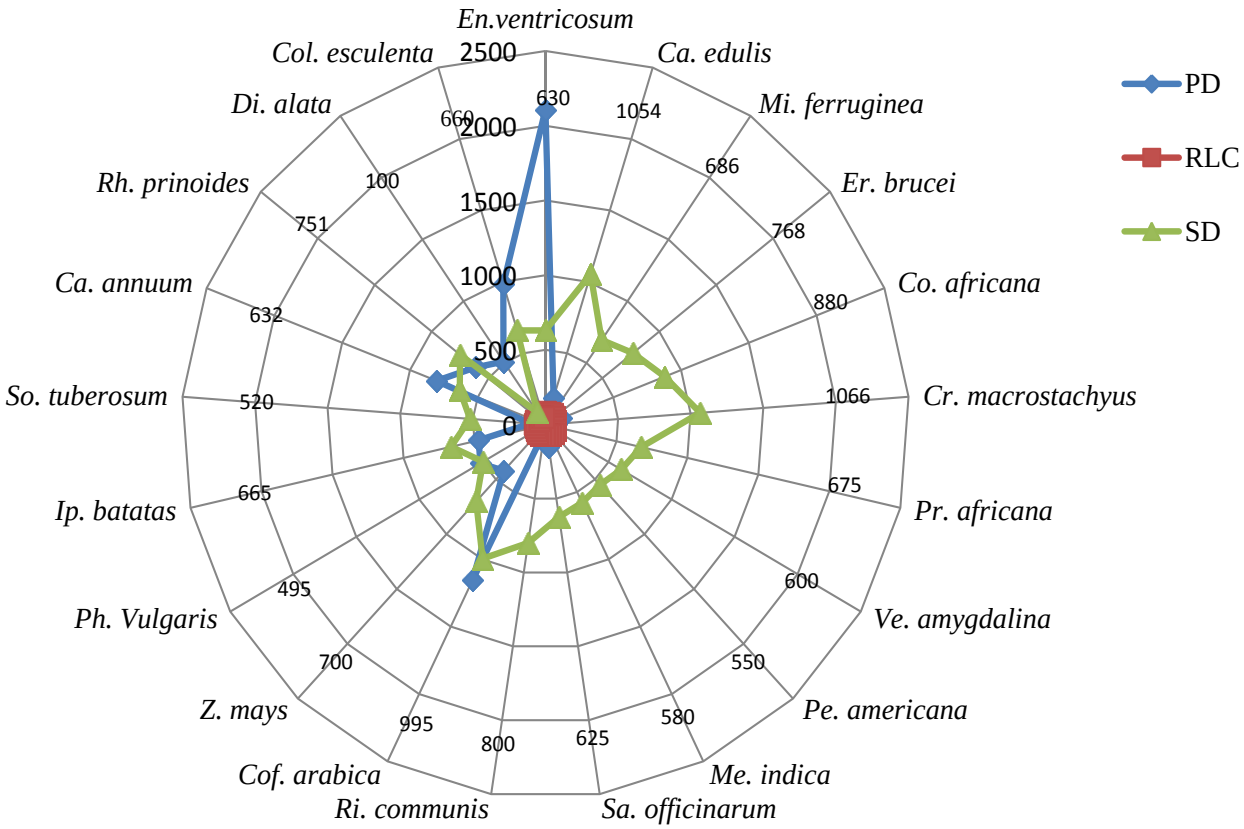


**Figure 1.** Effect of plant species density on root colonization and spore density. PD, plant density; RLC, total root length colonization; SD, Spore density, *En, Enset; Ca, Catha; Mi, Millettia; Er, Erythrina; Co, Cordia; Cr, Croton; Pr, prunus; Ve, Vernonia; Pe, Persea; Me, Mengifera; Sa, Saccharum; Ri, Ricinus; Cof, Coffee; Z, Zea; Ph, Phaseolus; Ip, Ipomoea; So, solanum; Ca, Cappisicum; Rh, Rhamnus; Di, Discorea; Col, Colocasia*

density-dependent regulation of mycorrhizal colonization. According to their report, higher plant together with higher root density causes, (a) an increase in overlap of phosphorus depletion zone, (b) a competition for light results in less carbohydrate available for mycorrhizal fungi and c) an increase in cost benefit ratio of mycorrhizal colonization. As plant density increase, competition for light increases and photosynthesis per plant declines and plants become limited more by carbon than by P reducing the root colonization. However, in some cases, increasing plant density has been associated with increasing AMF colonization (Eissenstat and Newman, 1990; Allsopp and Stock, 1992).

Most of the plants included in this study are multiple purpose trees and perennial crops with course root systems. The variation in the percentage of colonization in the roots and the AM population in the rhizosphere soils of different plants recorded in the present study, therefore might explain the generally held view that plants with coarse roots gain more AMF (Mahmud et al., 1999) compared to those with fine roots. These differences might be due to the presence of diverse type of AMF in the rhizosphere soils of individual plant species or might be a manifestation of greater host susceptibility to AMF (Mehrotra, 1998).

In this study 8 different AMF genera were identified: *Acaulospora, Claroidioglomus, Funneliformis, Glomus, Gigaspora, Rhizophugus, Septoglomus,* and *Scutellospora* of which *Glomus* and *Acaulospora* were the dominant genera found in all species. This is not surprising since it was previously shown that *Glomus* and *acaulospora* species are most abundant among the glomeromycotan genera in tropical areas (Gerdemann and Trappe, 1974; Blaszkowski, 1989; Talukdar and Germida, 1993; Zerihun Belay et al., 2014). *Glomus* species is widely distributed regardless of the type and intensity of disturbance in the different ecosystems, whereas *Acaulospora* is dominant in least disturbed agro ecosystems (Snoeck et al., 2010).

In our study, the total spore density, in general, did not correlate with mycorrhizal colonization, possibly because of the presence of a diverse population of AM fungal species or according to Biermann and Linderman (1983), it could be due to the fact that there are AMF species that

rely more on extensive formation of hyphal networks instead of survival through spore formation as primary infective propagules. The relationship between spore numbers and mycorrhizal colonization has been found positive by several workers (Giovannetti and Nicolson, 1983) but negative by others (Louis and Lim, 1987). Some workers have also found no relationship between mycorrhizal colonization and spore density of AMF (Diaz and Honrubia, 1994). The factors like edaphic or climatic condition; host fungus compatibility, root properties and soil microorganisms might influence the abundance of spore population and mycorrhizal associations with a particular tree species.

It is noticeable in the present study that the wide spread presence of *Glomus* and *Acaulospora* in the rhizospheres of selected plants, makes them favorable fungi for mass multiplication as well as seedling inoculation for their better establishment.

The present survey of AM fungi on Sidama agroforestry plant species reveals that AM fungi are common, occurring in 21 of the 23 plant species examined. However, when establishing new agroforestry practices the tree species must possess adequate amounts of mycorrhizal colonization at the planting stage in order to survive better and perform well in adverse agroforestry sites. More studies are needed to select the suitable indigenous AM fungal strains for the production of quality plant and expanding the awareness of the role of AMF in agroforestry systems to the small holder peasants for better organic and sustainable production should be initiated.

## Conclusion

The investigation reveals that AM fungi are common, occurring in 21 out of 23 plant species examined. The study also demonstrated the influence of different plant densities on the diversity of arbuscular mycorrhizal fungi. For some plant species such as *E. ventricosum* was recorded up to 8 AMF species compared to the least (3 species) for *Cappisicum annum*. As to the dependency of AMF on soil parameters, plants exhibited variable patterns; some with high concentration of P showed lower spore density and root colonization, while others shows the opposite phenomena. *Glomus* and *Acaulospora were* dominant in this study. They could be used for mass multiplication as well as seedling inoculation for better establishment of major trees and perennial crops which can also serve as inoculums sources. Mycorrhization of agroforestry plants has gained considerable attention over the last few years because of their role as bio-fertilizers for improving host growth. More studies are needed to select the suitable indigenous AM fungal strains for the production of quality plant and expand the awareness of the role of mycorrhiza in agroforestry systems to the small holder farmers.

## Conflict of Interests

The authors have not declared any conflict of interests.

## ACKNOWLEDGMENT

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*Full Length Research Paper*

# Inhibitory activity of *Trichoderma viride* against *Phytophthora infestans* that affects the Spunta potato (*Solanum tuberosum* L.) variety

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**The potato (*Solanum tuberosum L*.) is a tuberous herbaceous plant that belongs to the Solanaceae family. Several biotic and abiotic factors affecting its production include fungi such as mildew. In this present work, we proposed to evaluate the antagonistic power of *Trichoderma viride* against parasite *Phytophthora infestans* of potato tubers. We conducted two tests; for the first, in confrontation *in-vitro*, the direct confrontation was performed on an agar PDA medium to demonstrate the inhibitory capacity of *T. viride* on *P. infestans*. For the second test, *in- vivo* confrontation, we realized two methods; the first was carried out using a mycelia disk on the antagonist and the second was done by injecting the spore solution of *T. viride* into the potato tubers after this pathogen was applied. The results of the *in-vitro* test revealed that the confrontation between *T. viride* and the *P. infestans* showed the inhibitory capacity of *T. viride*, be it directly (68%) or remotely (58%) on the growth medium. Interesting results were also obtained *in- vivo*. The injection of the tubercles with a *T. viride* spore solution reduced the development of *P. infestans* with an average penetration of 3.28 mm for the *T. viride* and of 1.65 mm for the *P. infestans*. The findings of the mycelia discs method were similar to the injection method with a penetration average of 2.62 mm for *T. viride* and 1.81 mm for *P. infestans*. The test results *in -vitro* showed the efficiency of *T. viride* against *P. infestans*; while for *in-vivo,* it was proved that this antagonist possesses a very significant inhibitory effect that suppresses the spread of the pathogen.**

**Keywords:** Biological control, pathogen, confrontation, symptoms, *in vivo*, *in vitro.*

## INTRODUCTION

The potato (*Solanum tuberosum L*.) is a herbaceous tuber plant, originally from Latin America, that belongs to the Solanaceae family (Sonnewald and Sonnewald, 2014). It is a species that is vegetatively propagated and cultivated for its tubers, storage and multiplication organs rich in nutrients, mostly carbohydrate (starch). Three types of production are distinguished: potato for consumption, potato starch, and potato seeds or plants (Bohl and Johnson, 2010). Several biotic and abiotic factors affect its production and these include fungi such

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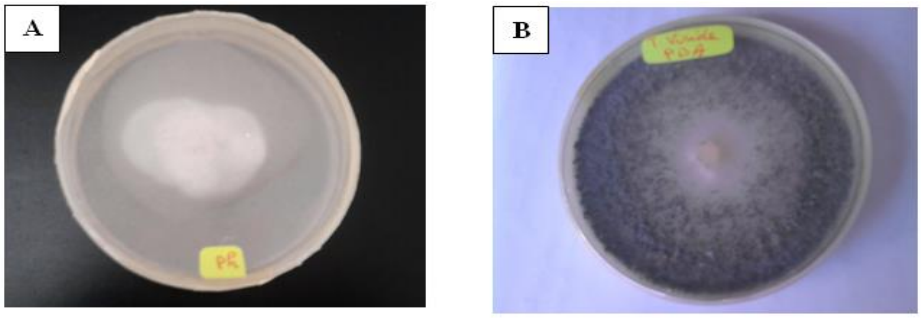


**Figure 1.** The colonies of the pathogen and the antagonist (A: *P. infestans*, B: T. *viride*).

as mildew. Mildew is a dreadful disease caused by Oomycete Phythophtora infestans and a heterothallic species with two mating mates A1 and A2. It can affect all the plant organs and cause considerable yield losses (Singh and Islam, 2010). To face these losses, it requires fighting this disease such as the use of living micro-organisms (biological control) as *Trichoderma viride*. This fungus has an inhibiting activity on the plant Phyto-pathogenic, which typically occurs either by competition, hyperparasitism or by antibiosis (Struik, 2007). The aim of this research was to study *in–vitro* and *in-vivo* antagonist power of *T. viride* against *Phytophthora infestans* pest of potato tubers.

## MATERIALS AND METHODS

### Biological materials

#### *Pathogenic agent*

In this work, we have used as target micro-organisms *P. infestans* isolated from the potato tubers and identifies in Mohammed Sadik Ben Yahiya Laboratory of Applied Microbiology in Jijel (Figure 1A).

### Antagonist agent

The antagonist agent used to fight against the *P. infestans* is the *T. viride*, which comes from the applied mycology laboratory at Mentouri University 1 Constantine (Figure 1B).

### Vegetative materials

The potato cultivar: the potato variety tested in the present study was gotten from pesticide sellers in Jijel (Taher).

### Antagonist activity *in-vitro*

Direct confrontation of *Trichoderma* on an agar medium was studied by Chet (1990).This technique consists of placing in the same Petri dishes PDA medium (20 g of agar, 20 g of glucose, 200 g of potato, 1000 g) ml of distilled water; and two agar pellets (6 mm in diameter), one carrying the *T. viride* and the other carrying the *P. infestans*. The two pellets were positioned along a diametrical axis of 3 cm and equidistantly from the center of the box. Transplanting is performed simultaneously (Benhamou and Chet, 1996). The incubation was carried out at 30°C for 7 days.

A notation regarding the diametric growth inhibition of the *P. infestans* and invasion by the mycelium of *T. viride* was conducted every two days. Also, microscopic observations on the direct effect of the antagonist agent on the *P. infestans* mycelia were made. The witness sample consists of subculturing of the pathogen at the box center.

Evaluation of the inhibition exerted by *T. viride* is estimated by calculating the percent inhibition of mycelial growth using the following formula:

$$I (\%) = (1- Cn / Co) \times 100$$

Where: Cn is the mean diameter of the colonies in the presence of the antagonist and Co the average diameter of control colonies.

### Antagonistic activity *in-vivo* of *T. viride*

An in-vivo antagonism test for *T. viride* and *P. infestans* was performed by applying both methods to demonstrate the inhibitory capacity of *T. viride.*

### Method of injection

The tubers were superficially disinfected with sodium hypochlorite solution (10%) for 5 min, and then rinsed thoroughly with distilled sterile water. Inoculation sites on the tubercles with the dimension of 6 mm width and depth was prepared. *T. viride* was applied by the injection of 100 µL on the sites of inoculation for 24 h before the application of the pathogen. The witness tubercles were treated similarly with distilled sterile water. The vaccination involves deposing an agar plate (6 mm diameter) colonized by the pathogen in the injuries that were sustained. Incubation of tubers was carried out at 25°C for 15 days.

After the incubation period, the potato tubers were longitudinally cut through for inoculation spots. The induced penetration parameters of the maximum width (w) and depth (d)) were noted. Penetration of the pathogenic and antagonist agents in tubers was calculated according to the formula of Elad and et al. (1994).

### Method of mycelial disks

Potato tubers (*spunta*) were washed in running water and then disinfected by soaking them for 5 min in a hypochlorite sodium solution (10%). Thereafter, they were rinsed three times

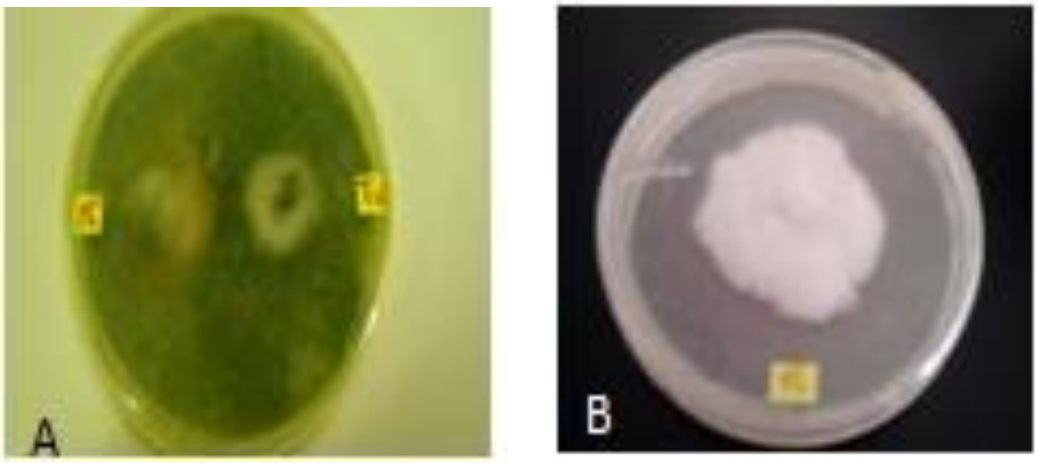


**Figure 2.** Inhibitory effect by direct confrontation of *T. viride* on mycelia growth of *P. infestans* after six days of incubation at 30°C (A: Testing, B: control).

**Table 1.** The average diameter of *P. infestan*s and *T. viride* compared to the control and the percentage inhibition after 4 and 6 days of incubation at 30°C.

| Incubation period at 30°C | Average diameter of *P. infestans* face (mm) | Average diameter of control (mm) | Average diameter of *T. viride* (mm) | Percent inhibition (%) |
|---|---|---|---|---|
| After 4 days | ±11 | ±15 | ±75 | ±26 |
| After 6 days | ±15 | ±48 | ±85 | ±68 |

successively in distilled sterile water.

Injuries of 6 mm in width and depth in the tubers using a sterile punch were made. Furthermore, a disc of 6mm in diameter carrying the phytophthora infestans pathogenic agents to be dropped at each injury was prepared. The witness tubers were inoculated by 6mm in diameter of agar explants. Incubation was performed at 25°C for 24 h. As a result, we made the treatment by the use of discs of 6 mm containing *T. viride* and incubation for 15 days under the same conditions.

After the incubation period, the potato tubers were cut longitudinally over the infection spots. The induced penetration parameters of maximum width (w) and depth (d) were noted. Penetration of the agent and the antagonist in the tubers was calculated according to the formula of Elad et al. (1994).

## RESULTS AND DISCUSSION

### Antagonist activity *in- vitro*

There was a direct confrontation on a culture medium between *T. viride* and *P. infestans*. Before the implementation on a culture medium between *T. viride* and Phytopathogenic fungi using organic products, it is necessary to know the antagonist behavior and interactions with the pathogen, which is why an antagonistic activity test was directed by *T. viride* through the confrontation between *P. infestans* (Figure 2). Simultaneous planting of *T. viride* and *P. infestans* showed faster growth of *T. viride* than the isolate of *P. infestans*. After four days of incubation, the box was almost completely invaded by the antagonist agent, while the isolate of *P. infestans* occupies only a surface of 11mm in diameter, which corresponds to an inhibition of mycelial growth of more than 26% (Table 1).

The *P. infestans* witness grown alone occupies an area of about 15 mm in diameter. Beyond this period, and after six days, *T. viride* invaded the *P. infestans* colony and even sporulates on them revealing its highly microparasitic power which corresponds to an inhibition of mycelial growth equal to 68% (Figure 3).

In the same sense, Benhamou and Chet (1996) reported an alteration of mycelium *Sclerotium rolfsii* caused by *T. harzianum*, resulting in aggregation, a retraction and a vacuolization of the cytoplasm which illustrates the highly micro-parasitic power that the *T. harzianum* possesses.

Similar results were obtained with *T.lignorum* which is capable of warping about the mycelium of *Rhizoctonia solani* causing the pathogenic cytoplasm to dissolve (Howell, 2003; Timothy and Widmer, 2014).

The microscopic observations realized at the contact area between *T. viride* and *P. infestans* showed that there was a profound change at the mycelia pathogen level. It was marked by recognition of the hyphae in strips and a winding start of the mycelium of the *T. viride* on

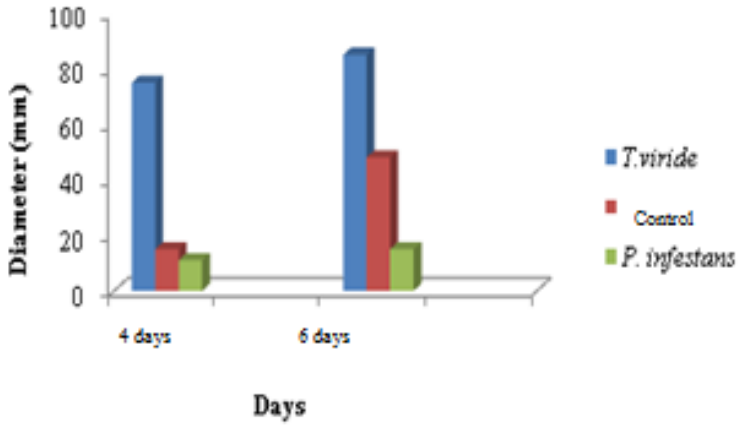


**Figure 3.** The average diameter of *P. infestans* and *T. viride* compared to the control after four and six days of incubation at 30°C.

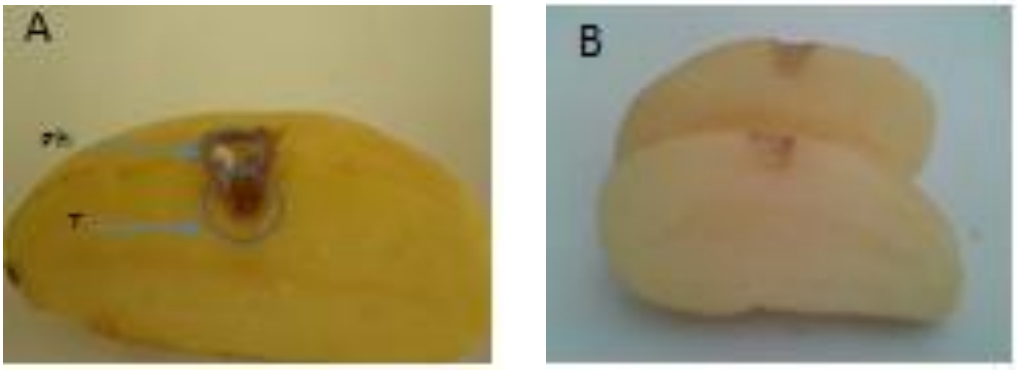


**Figure 4.** Effect of *T.viride* on the development of *P. infestans* compared to control after 15 days of incubation at 25°C (A: Testing, B: control), ( Ph: *Phytophthora*, T: *Trichoderma).*

that of *P. infestans*.

**Antagonist activity *in -vivo***

***Injection method***

The results obtained showed a colonization of the incubation site by the antagonist agent after 15 days of incubation, with the appearance of small whitish spots representing the pathogenic agent on the superficial parts of the injuries (Figure 4).

This explains that *T. viride* exerts a competition mechanism, taking place before the arrival of the pathogen, and therefore hinders the development of mildew. The average antagonist penetration is estimated to be 3.28mm, while the average pathogen penetration is 1.65 mm (Table 2).

This phenomenon (competition) was not observed in the witness sample treated with distilled sterile water. It has been seen that the metabolites produced by the antagonist agent have a direct impact on the development of *P. infestans* penetration. This result explains that the antagonist agent has a great inhibitory capacity of the *P. infestans* as it is installed before the pathogen without damaging the plant tissues (Figure 5). Moayedi et al. (2010) opined that *Trichoderma sp*. demonstrated an antagonistic effect against *Phytophthora* root of potato rot, particularly *in vitro* and *in vivo*.

**Discs method**

The results obtained displayed appearances of different colors on the injuries. The white color represents *P.infestans* and the green color represents *T. viride* (Figure 6).

The average pathogen penetration is 1.81mm and the average antagonist penetration was estimated to be 2.62 mm (Table 3). This explains that *T. viride* exerts different

**Table 2.** The average penetration of *P. infestans* and *T. viride* after 15 days incubation at 25°C.

| Bacteria | Repetition | W (mm) | D (mm) | Penetration (mm) | Average penetration (mm) |
|---|---|---|---|---|---|
| *T. viride* | R1 | 7.5 | 9 | 3.37 | ±3.28 |
| | R2 | 8 | 8 | 3 | |
| | R3 | 8 | 9 | 3.5 | |
| | R4 | 9 | 8 | 3.25 | |
| *P. infestans* | R1 | 7.5 | 4.5 | 1.12 | ±1.65 |
| | R2 | 8 | 4 | 1 | |
| | R3 | 8 | 7 | 2.5 | |
| | R4 | 8 | 6 | 2 | |

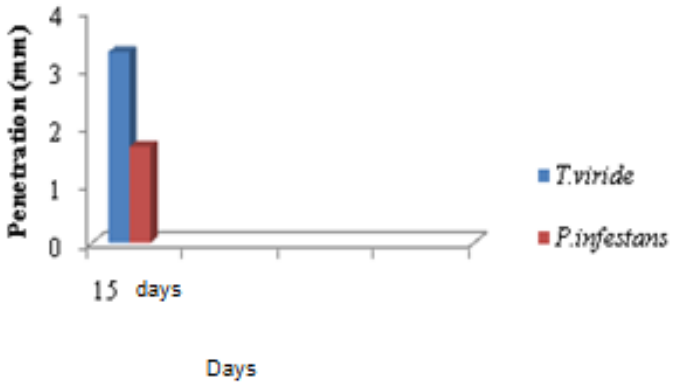


**Figure 5.** The average penetration of *P. infestans* and *T. viride* after 15 days of incubation at 25°C.

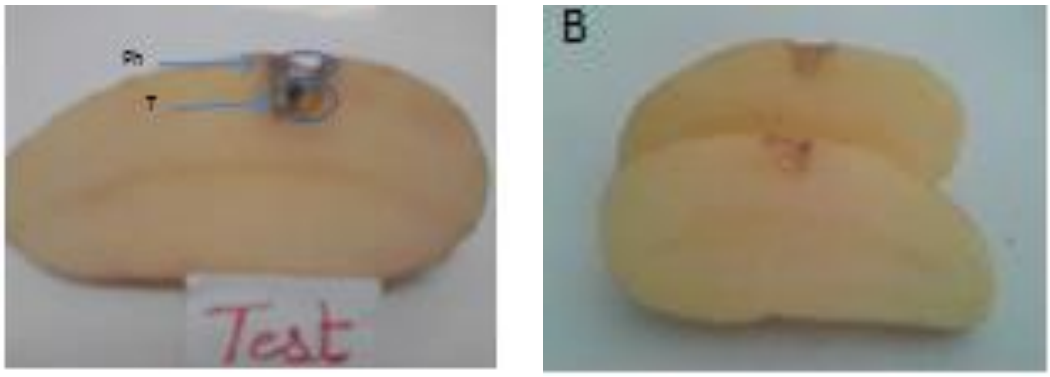


**Figure 6.** Variety of potato tubers (*Spunta*) treated with *T. viride* compared to control after 15 days of incubation at 25°C (A: Testing, B: control), (Ph: *Phytophthora*, T: *Trichoderma*).

antagonistic mechanisms to inhibit the development of *P. infestans* (Figure 7). The results agree with the results of Yang et al. (2013). The studies of Kerroum et al. (2015) demonstrated the antagonist activity of *Trichoderma sp*. on *P. infestans* on potato tubers and tomato variety, and the antagonistic activities of *Trichoderma* species, including the competition and colonization against *P. infestans*.

## Conclusion

This study has clearly demonstrated the antagonist effect of *T. viride* in relation to *P. infestans* which is the

**Table 3.** The average penetration of *P. infestans* and *T. viride* after 15 days of incubation at 25°C.

| Bacteria | Repetition | W (mm) | D (mm) | Penetration (mm) | Average penetration (mm) |
|---|---|---|---|---|---|
| *T. viride* | R1 | 11 | 5 | 2.25 | ±2.62 |
| | R2 | 10 | 8 | 3.5 | |
| | R3 | 11 | 5 | 2.25 | |
| | R4 | 10 | 6 | 2.5 | |
| *P. infestans* | R1 | 11 | 3 | 1.25 | ±1.81 |
| | R2 | 10 | 5.5 | 2.25 | |
| | R3 | 11 | 4 | 1.75 | |
| | R4 | 10 | 5 | 2 | |


**Figure 7.** The average penetration of *P.infestans* and *T.viride* after 15 days of incubation at 25°C.

responsible agent of mildew of potato tubercles (*Spunta*). In effect, the confrontation attempts between *P. infestans* and *T. viride* showed that *T. viride* invaded the *P. infestans* colony with an inhibition percentage equal to 68% in six days.

In the case of the remote confrontation and despite the lack of direct contact between the two fungi, a reduction of the *P. infestans* colony diameter has been observed compared to the untreated witness sample with an inhibition percentage equal to 68% in six days. This demonstrated that in addition to the microparasitic power of the antagonist agent, *T.viride* may act by the secretion of volatile substances which are able to distantly stop the development of the pathogenic agent.

Consequently, the in-vivo test showed the ability of the *T. viride* to reduce or inhibit the penetration of the pathogen in tubers with an average penetration of the pathogenic agent equal to 1.65 mm (by injection), whereas the treatment by discs gives an average penetration equal to 1.81 mm. It can be concluded that the mechanisms placed by *T. viride in-vitro* will be the same as that demonstrated *in-vivo*, namely, mycoparasitic secretion of volatiles, antibiosis, and competition for space.

## Conflict of interest

The authors have not declared any conflict of interest

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