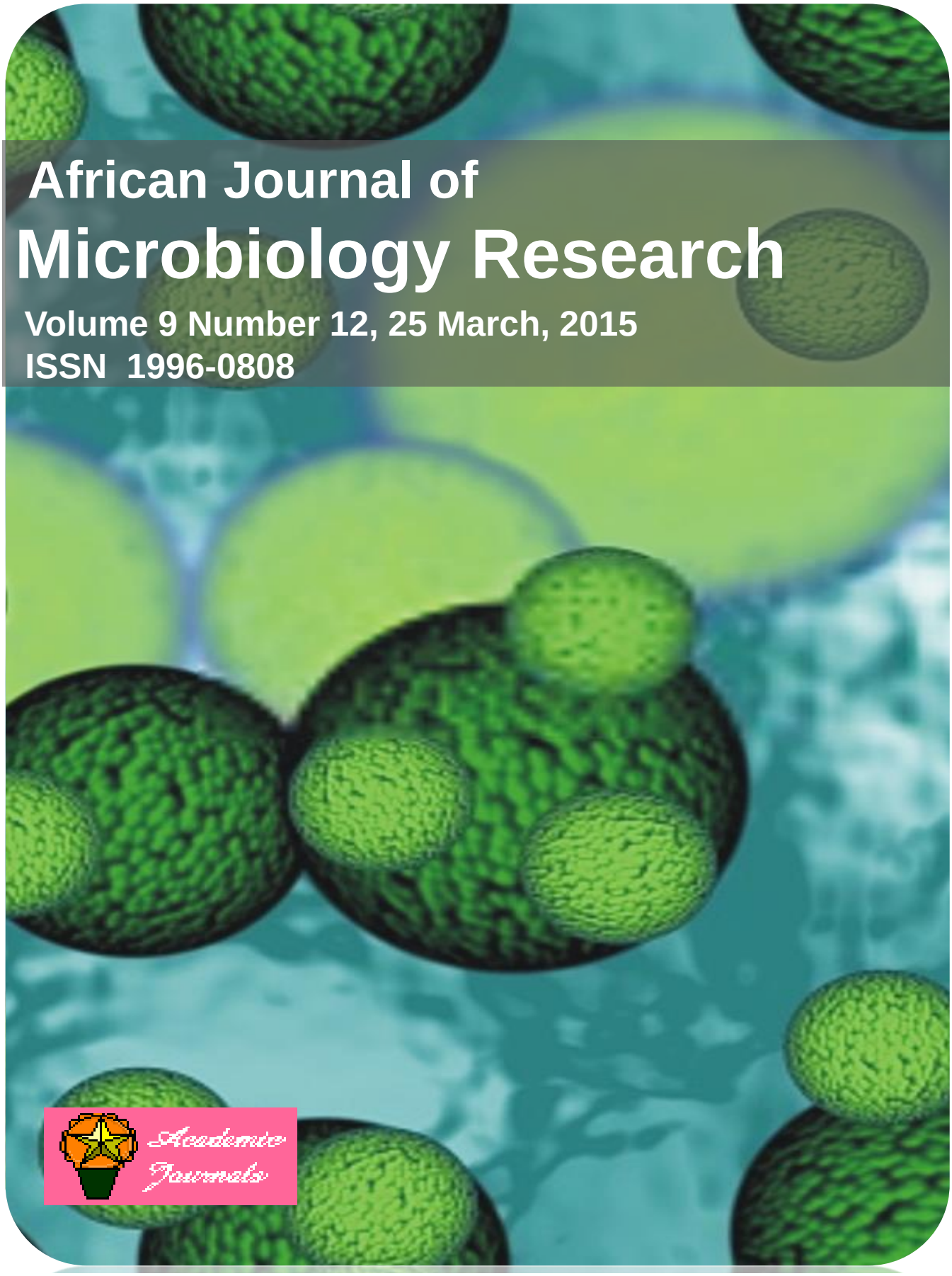

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Review

Alternaria epidemic of apple in Kashmir

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During the months of July-August 2013, apple orchards of the valley were suddenly struck with *Alternaria* disease which was so far considered a disease of minor importance as compared to apple scab. The disease spread like wild fire leading to very high severity and often resulted in extensive defoliation and fruit fall in almost all the orchards through the valley, resulting into a widespread epidemic. The various factors which lead to this epidemic were a possible effect of climate change leading to untimely prolonged rains following high temperatures. The situation was worsened due to extensive presence of susceptible delicious cultivars, non adherence of practice of orchard sanitation, use of inappropriate or spurious fungicides and absence of disease forecasting system in the valley.

Key words: *Alternaria,* apple, kashmir, epidemic.

INTRODUCTION

The Valley of Kashmir is the leading producer of apple (*Malus x domestica* Borkh.) in India which contributes a major portion of about 65% of total apple production in India which ranks 7th with an annual production of 2163400 MT of apple fruit (FAO, 2012). Apple production has attained status of industry in the state of Jammu and Kashmir. Like other crops apple is also attacked by a number of diseases like apple scab, Alternaria leaf blotch, Marsonena, sooty blotch, fly speck and a number of post-harvest diseases. After the heavy outbreak of scab disease in 1970s decade major thrust were given on its management and Alternaria was considered a disease of less importance in comparison to apple scab. Like in all apple growing areas of the world, this disease is prevalent in almost all districts and all apple orchards of Kashmir (Sofi et al., 2013a; Shahzad, 2003). The occurrence of the disease (*Alternaria mali*) was reported by Shahzad et al. (2002) in Kashmir valley of Jammu and Kashmir state.

During the summer of 2013 starting in the month of July due to consistent Rainfall coupled with high temperature, the environment set a most favorable stage for the Alternaria and within no time Alternaria leaf blotch cached apple growers of Kashmir by a sudden surprise. The leaves rapidly started blotching leading to heavy leaf blight rendering less photosynthetic area for the plant which badly effected development of apple fruit by influencing it at its critical fruit developmental stage. Although exact official figures have not been estimated yet but it is estimated that in the districts of Baramula and Bandipore the disease spread like wild fire infecting more than 70% of delicious cultivars; lower belt of Bandipora and District Handwara was hit most. Similarly, Zainageer belt of of Baramulla was worst hit followed by Rafiabad

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Figure 1. Areas which reported breakout of Alternaria disease in apple in Kashmir during summer 2013.

(Annonymous, 2013a). Areas of Bandipore Viz., Sumbal, Hajin, Safapora and Bandipora reported heavy intensity of the disease (Annonymous, 2013b). In district Budgam the lower belts of Chadura and Magam like Bugam, Kralpora, Wanabal, Chewdara, Berwah etc, were worst hit as compared to upper belts of Khag and Khan Sahab (Figure 1). Certain reports based on growers estimates have reported losses of 40-60% and the disease has considerably reduced the market value of apple by reducing it from grade “A” to grade “C”. (Annonymous, 2013c).

The infection of the disease was almost uniform though the valley had moderate to severe intensity. A moderate intensity was however observed at remote upper reaches of the valley with comparatively much lower temperature but at other places the onslaught of disease was so intense that apple trees defoliated and at a number of places along with leaf fall there was a heavy fruit fall also, leading to a direct set back to the apple production. Blighting however reduced the production indirectly by reducing photosynthetic area and hence less accumulation of photosynthetic products into the developing fruits which negatively affected the total apple production of the entire valley. This article deals with the objectives of the description of the disease, main causes of its epidemic, and lessons learnt in some detail.

ALTERNARIA LEAF SPOT

Lesions of *Alternaria* disease in apple first appear on leaves in late spring or early summer as small, round, purplish or blackish spots, gradually enlarging in diameter, with a brownish purple border. Lesions may coalesce or undergo secondary enlargement and become irregular and much darker, acquiring a "frog-eye “appearance. When lesions occur on petioles, the leaves turn yellow and 50% or more defoliation may occur (Plates 1 and 2). Severe defoliation (Plates 3 and 4) leads to premature fruit drop (Plate 4).

The pathogen

Alternaria disease of apple is caused by a fungal pathogen *Alternaria mali* belonging to phylum ascomycota and family Pleosporaceae. A full description of the fungus is given by Roberts (1924). Hyphal segments are short, mostly unbranched and without constrictions at their septa and 3-8 µm wide. Conidia are produced in chains of 3-9 and average 28 x 12 µm (maximum 29 x 13 µm). They are similar to those of *A. gaisen* but smaller. They are typically 3-septate, with transverse and longitudinal septa, with constrictions at the septa, especially when old. Conidiophores are usually fasciculate on apple leaves and are of variable length and show a dark-coloured scar at the point of attachment of the conidium. The fungus was first identified in United States in 1924 but was not considered a serious pathogen (Roberts, 1924). However, a disease outbreak occurred in Japan in 1956 (Sawamura, 1972). The disease was also observed in north Carolina in 1987 where it caused widespread damage to apple crop (Filajdic and Sutton, 1991). The fungus appear over winter as mycelium on dead leaves

Plate 1. Symtoms of Alternaria leaf spot on apple and yellowing of leaf due to petiole infection (photo taken during Alternaria disease breakout in Kasmir during summer 2013).

Plate 2. Enlargind and coalescing lesions of Alternaria disease on apple during epidemic of 2013 in Kashmir valley.

Plate 3. Defoliation in apple leaving behind developing fruit due to Alternaria disease breakout in district Budgam of Kashmir valley during 2013.

Plate 4. Whole orchard defoliated along with fruit drop due to Alternaria disease in Kashmir valley during 2013.

on ground, in mechanical injuries, in twigs or in dormant buds. Primary infection usually occurs around one month after petal fall (Sawamura, 1990). All pathogenic species of fungus attacks the susceptible cultivars using chemical toxin (Logrieco, et al., 2003; Otani et al., 1995). Existence of considerable variation in cultural, morphological, pathogenic and molecular characters of *A. mali* isolates prevalent in Kashmir valley have already been reported (Sofi et al., 2013b) besides *A. mali* isolates from valley of Kashmir which exhibited considerable variation in their virulence. The wide variation of isolates indicated that the fungus has a high potential to adapt to resistant cultivars or fungicides (Sofi et al., 2013a). European red mite (Panonychus ulmi Koch.) significantly increases the incidence of Alternaria Leaf Blotch and premature leaf fall in Apple. A significant positive correlation was found between Alternaria Leaf blotches intensity and number of mites per leaf (Shahzad, 2007).

In Kashmir valley the disease outbreak occurred in late summer due to secondary infection of this polycyclic pathogen which might be due to already existing pathogen density available from previous cycles. Since the pathogen mostly survives in fallen leaves and non adherence to orchard sanitation practice in Valley, will always keep such pathogens available for such breakouts in case of favorable environments for the disease developments.

Alternaria leaf blotch is most likely to occur on 'delicious' strains of apple. The disease assumed alarming threat to the crop owing to premature defoliation in North Carolina and has potential of becoming threat especially in those apple producing regions where susceptible cultivars/strains of Delicious are grown which also included North Carolina (Filajdic and Sutton, 1991). By 1993, growers in nine counties in southern and central Virginia reported seeing this problem, some with as much as 50 to 60% (Yoder and Biggs, 1998). Reportedly, it can infect up to 85% of leaves on susceptible cultivars, compared with less than 1% on resistant cultivars (Yoon and Lee, 1987). Apple cultivars can be ranked in order of increasing resistance (Sawamura, 1990) as follows: Indo, Red Gold, Raritan, Delicious, Fuji, Golden Delicious, Ralls, Toko, Tsugaru, Mutsu, Jonagold, Jonathan. Yellow Newtown, American Summer Pearmain, McIntosh, Ben Davis and Stayman Winesap are other resistant cultivars, to which Shin et al. (1986) would add Gala, Honey Gold and Mollie's Delicious. Resistant cultivars are homozygous for the recessive gene *alt alt*. Certain *Malus* spp. are highly resistant, for example *M. asiatica*, *M. baccata* and *M. robusta*, but resistance in these species is controlled by a single dominant gene, epistatic to the dominant gene controlling susceptibility (Saito and Niizeki, 1988).

In Kashmir valley one of the most favorable situations for the Alternaria disease breakout was a uniform cultivation of susceptible cultivars of “delicious” verities. During the curse of the epidemic it was observed where ever resistant varieties like American Aprigoue, (Plate 5) Gala, Ambri Maharaji and other varieties which are traditionally local verities where seen defending the disease even if such trees were surrounded by heavily infected Delicious verities.

The favorable environment

Through the early part of the fruit production season the pathogen stays relatively inactive, causing only small lesions and often not being observed at all. The disease develops explosively following heavy summer rainfall events and high humidity. Trees that have mite infestations are predisposed to rapid disease development. Secondary spread of the disease occurs where spores (conidia) that develop on lesions are splashed by wind-blown rain. This dispersal is relatively rapid, and entire orchard blocks are quickly infected (Annonymous, 2013d). Primary infection takes place about one month after petal fall. The disease advances rapidly in the optimum temperature range of 77 to 86 F (25-30°C) and wet weather. At optimum temperatures, infection occurs with 5.5 h of wetting, and lesions can appear in the orchard two days after infection, causing a serious outbreak. The fungus produces a chemical toxin which increases the severity of the disease on susceptible cultivars (Yoder and Biggs, 1998).

In valley of Kashmir, the summer of 2013 reported excessive hot temperatures and in the month of July there were heavy and consistent rains which prolonged for long periods coupled consistently with high temperatures. Such conditions favored the disease and within no time due to continuing rain and favorable temperatures for a period of several weeks, the disease spread rapidly. It is unusual for the valley of Kashmir to have such a combination of high temperature and a sudden and prolonged rainfall during this period of year, however probably it might be due to the effects of climate change scenario leading to erratic rainfall behavior and rise in temperature which resulted in this minor disease causing major losses in apple in Kashmir.

Control measures

Chemical control of *A. mali* can be achieved through use of fungicides such as iprodione, mancozeb and captan (Osanai et al., 1987; Asari and Takahashi, 1988). However, later it was reported by Filajdac and Sutton (1992) that fungicides like captan, mencozeb, and mixtures with benomyl are unable to control Alternaria disease in apple and iprodione gives best control of the disease. In South Korea and Japan best chemical control and suppression of *A. mali* was achived by Polyoxin, captafal, and iprodione (Lee and Lee, 1972; Filajdac and Sutton, 1992). Yoder and Biggs, 1998, reported that

Plate 5. A resistant cultivar “American” showing no disease symptoms surrounded by susceptible and disease Delicous trees during Alternaria disease outbreak in Kashmir.

currently registered fungicides do not provide satisfactory control under severe disease pressure and some strobilurin fungicides are registered for management of Alternaria blotch in the U.S. Moshe and Dimitri (2002) studied the activity of azoxystrobin, difenoconazole, Polyoxin B and trifloxystrobin on one or more stages of the life cycle of *A. alternata* and on decay development in fruits and suggested that these compounds potentially could provide control of moldy-core disease in apple. They further reported chopping leaves with a mower or removing them from the orchard will help reduce the inoculum level for the following season. Since defoliation from the disease is more severe if high mite populations are present, mites population should be maintained at or below the established IPM thresholds.

Under the conditions of Kashmir valley where orchard sanitation and other good horticultural practices to manage diseases are absent, the management is solely carried out by chemicals. During the course of this epidemic there were complaints from a good number of growers that chemicals they used failed to check the disease. In all the cases it was assumed that chemicals used are spurious (Annonymous, 2013a; Annonymous, 2013c) however, since it is a fact from above reports that most of the conventional fungicides used in apple management fail to control this disease (Lee and Lee, 1972; Filajdac and Sutton, 1992) and weather fungicides were spurious or they were not the right ones to control this disease or both is a matter demanding study. Under the changing environment there is a good possibility that this disease might be a problem to apple industry in future as well, therefore, it is imperative that researchers should work out right chemicals that could check this disease effectively under severe disease stress.

Disease forecasting

Modern agriculture being cost oriented requires greater vigilance than before to ensure stable yield and for reducing expenditure on chemicals for disease management. This is possible only if reliable disease forecasting systems are developed at least, for some of the destructive diseases of major crops (Bedi, 1986). A model of the relationship between the temperature threshold for infection and the influence of rainfall has been developed to predict the severity of Alternaria disease in apple (Kim et al., 1986). This predictive model developed in the Korea Republic has been evaluated in the USA (Filajdic and Sutton, 1992b). Disease simulation models helpful in disease forecasting, have been developed for diseases caused by *Alternaria spp.* like

EPIDEM and TOM-CAST (forecasting early blight of tomato and potato). Since apple is an important industry for the state of Jammu and Kashmir and the state being a major producer of apple in India coupled with the facts that majority of orchards have susceptible delicious plantations, non adherence of orchard sanitation and changing weather conditions there is and “always was” a need for disease and pest forecasting systems to avert or minimize the losses due to diseases or pests at least in this important crop of the Valley. Hence this is the time that researchers should also consider disease forecasting seriously for disease management in near future.

CONCLUSION

During the late summer months of year 2013 in Kashmir valley, due to possible effects of climate change, there was a consistent period of rainfall couple with high temperature which resulted an epidemic of *Alternaria* disease in apple which was otherwise considered a disease of less importance. Other major factors which became helpful tools for wide spread destruction due to this disease were availability of highly susceptible cultivars of “delicious” variety of apple, little or non-practicing of orchard sanitation and availability of less effective and spurious fungicides or both. Hence, the need for an effective forecasting system for this disease and other diseases of major and minor importance of this important horticultural crop is warranted coupled with introduction and use of effective chemicals, and effective integrated disease management strategy.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Occurrence and abundance of arbuscular mycorrhizal fungi (AMF) in agroforestry systems of Rubavu and Bugesera Districts in Rwanda

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Arbuscular mycorrhizal fungi (AMF) help to facilitate mobilization of nutrients from soil to plant. The study was carried out in humid Rubavu and semi-arid Bugesera districts in Rwanda. We hypothesized that the presence of tree species in farming systems enhances mycorrhizal fungal density. The occurrence and abundance of AMF in the soil around main agroforestry tree species in these regions was studied. Tree species in Rubavu included *Alnus acuminata*, *Markhamia lutea, Grevillea robusta* and *Eucalyptus* sp. and in Bugesera *Acacia polyacantha, Senna spectabilis, Grevillea robusta* and *Eucalyptus* sp. AMF spores were isolated from soil samples collected under and outside the trees canopies. Results show significant differences in spore density between species. The density of AMF spores was highest under *A. acuminata* and *A. polyacantha* and lowest in *Eucalyptus* sp. and *G. robusta* in Rubavu and Bugesera, respectively. Generally, the mean spore abundance (spores/g of soil) was significantly higher in Bugesera (3.1-6.6) than Rubavu (1.6-4.4). Spores abundance was also affected by distance from the tree trunk and tree size. The present work is the first attempt to study the AMF communities associated with tree species in agroforestry systems in Rwanda. We propose further studies relating mycorrhizal diversity in the agroforestry systems to performance and yields of crops.

Key words: Arbuscular mycorrhizal fungi, spore abundance, agroforestry system.

INTRODUCTION

Crop productivity is declining in Rwanda mainly due to declining soil fertility and other challenges like high population density, water scarcity, land degradation, land fragmentation and deforestation notwithstanding. Most

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options to improve productivity involve the use of expensive inputs that inherently increase risks that farmers are often unwilling or unable to bear. The government of Rwanda is interested in eco-efficient means to raise land productivity that farmers can afford to adopt like scaling up the adoption of farm trees. This study targets the semi-arid and humid districts in Rwanda, sites of an ongoing Australian funded project investigating the effects of trees on water, nutrients and crops performance and hence food security. The study focused on investigating the occurrence and abundance of arbuscular mycorrhizal fungi (AMF) in the rhizosphere of the key agroforestry tree species found in these two contrasting districts.

AMF are root-inhabiting soil fungi which form obligate symbiotic associations with over 80% of terrestrial plant families (Smith and Read, 2008; Van Hoewyk et al., 2001; Harley and Smith, 1983). They are ubiquitous in almost all plant communities in both natural and managed ecosystems, but the number has decreased due to tillage, fertilization, removal of topsoil, erosion, fumigation and over-fertilization (Raja and Tang, 2005). They are widespread in tropical soils and associated with a wide variety of plant species, including most commercial crops (Sieverding, 1991) and trees (Atayese et al., 1993; Adjoud-Sadadou and Halli-Hargas, 2000). These very important organisms form an interface between soils and plant roots (Ingleby, 2007; Power and Mills, 1995) increasing the absorptive surfaces of the roots (Manjunath and Habte, 1988). Extra-radical hyphae of the AMF extend beyond the root and act as extensions of the root system in acquiring nutrients from the soil (Rhodes and Gerdemann, 1975). AMF can therefore absorb mineral nutrients from soil through their extended intricate hyphal network and deliver them to their host plants in exchange for carbo-hydrates (Oehll et al., 2003). AMF can also enhance tolerance of abiotic stresses such as drought and metal toxicity (Meharg and Cairney, 2000).

AMF are not host-specific (Ingleby, 2007). Because of this, the same fungi can associate with tree and crop species and therefore have the potential to enhance both tree and crop growth in agroforestry systems. In this situation, the tree species can act as a ‘reservoir’ of AM fungi, from which roots of germinating crop seedlings can quickly form mycorrhizal associations (Ingleby, 2007).

Arbuscular mycorrhizal associations are characterized by structures called arbuscules and vesicles which are produced inside the host plant root cells together with asexual spores which they produce in the soil (Ingleby, 2007). Though hyphal networks, dead root fragments and other organic material occupied by fungal structures are important, AMF spores have traditionally been considered to be the most important propagules of AMF (Chandrasekara et al., 2005; Brundrett and Abbott, 1994). Therefore, analysis of spore populations in soils is currently the most used method to assess the species density and diversity of AM fungal communities (Chandrasekara et al., 2005). However, the interpretation of these results remains conditional as isolates of AMF vary greatly in spore production; some isolates produce spores copiously, while others rarely or never sporulate (Chandrasekara et al., 2005).

All the soils harbor AMF spores despite the different structural and chemical differences of the cropping fields (Don-Rodrgue et al., 2013). However, the major factors affecting their diversity, abundance and distribution in agro-ecosystems are soil pH, availability of phosphorous (P), nitrogen (N), organic matter and water. These factors could also affect the crop production in different agro-ecosystems (Porras-Soriano et al., 2009).

The relationship between plants and AMF species abundance and diversity is not completely understood for most natural ecosystems (Bainard et al., 2011). Even though species richness of mycorrhizal fungal communities has been correlated with the species richness of plant communities in temperate grasslands and tropical agro-ecosystems (Nancy and David, 1997; Eom et al., 2000), agricultural soils have a low density and diversity of AMF as compared to natural ecosystems. When a soil is put to agricultural use, it undergoes a series of physical changes, like tillage and fertilizer use, which can negatively affect microorganism population (Bellgard, 1994).

Declining of soil fertility and crop production are challenges to food security in Rwanda. Past studies in Rwanda have concentrated on water and nutrient cycling and nothing has been done on the role of AMF in productivity systems. Trees are being incorporated in the agricultural lands to provide ecosystem services and products for example, firewood, fruits and furniture. Therefore, there is a need to document the AMF around trees in these systems as a preparatory phase to understand their contribution in these systems as they provide several free ecosystem services. Research has shown that there is greater soil biota in agroforestry systems than in agriculture systems (without trees) with greater biodiversity generally reported near the trees but the effect varies with tree species (Barrios et al., 2010). In our study, the agroforestry systems of Rubavu and Bugesera districts were taken to represent the humid and semi-arid agroecological zones of Rwanda, respectively.

MATERIALS AND METHODS

Site description

Bugesera district is located in eastern province of Rwanda. Its altitude varies between 1300 and 1667 m with soft slopes. Its relief mainly constituted of a succession of low plateau, dry valleys and swamps. The annual precipitation ranges between 700-900 mm with the mean atmospheric temperature between 21 and 29°C. Soils in the region are sandy-loam of moderate fertility (JICA, 2006; MINITERE, 2003). Dominant crops and trees observed are relatively homogenous across Bugesera district - crops: banana, maize, beans and cassava; and trees: *Acacia*, *Senna spectabilis*, *Grevillea robusta*, *Eucalyptus* (Kiptot et al., 2013; CRA, 2005; http://www.bugesera.gov.rw/).

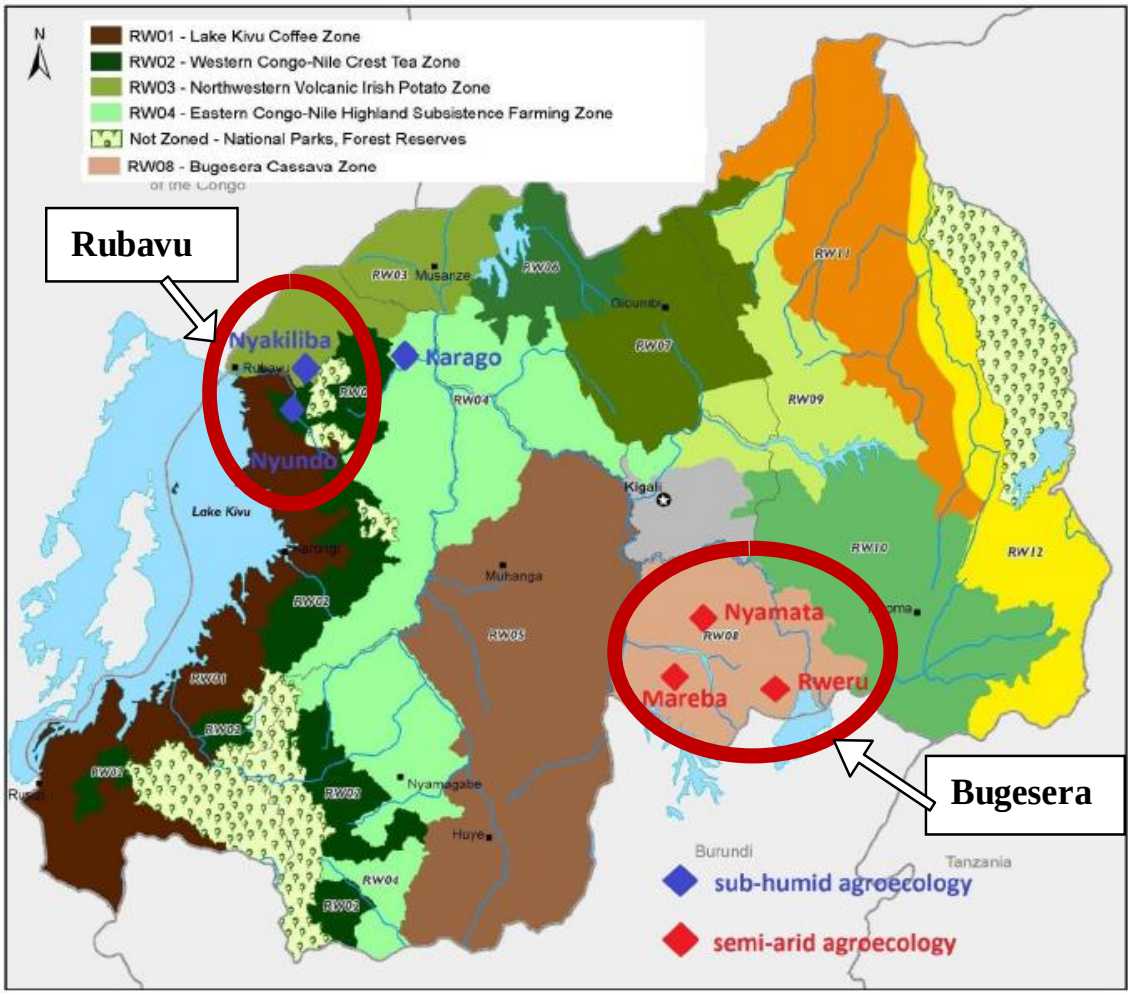

Figure 1. Agroecological Map of Rwanda with selected sites (Districts/sectors).

Rubavu is one of the western province districts of Rwanda. The region is characterized by an elevation ranging between 2000 and 3000 m and higher slopes (the mean slope is 35%). Temperatures are generally cool with an average of 10°C. Its mean annual rainfall is 1800 mm (Nyandwi and Mukashema, 2011). Major crops in the region include maize, climbing beans, irish potatoes, wheat and vegetables such as carrots and cabbages along with tea plantations on valley bottoms. The dominant trees observed are *Alnus acuminata* along the contours, *Markhamia lutea* on farm, *Eucalyptus* woodlots, *Grevillea robusta*, bamboo, avocado and some indigenous trees such as ficus (Kiptot et al., 2013).

Sampling

The rhizosphere soil was sampled from Nyundo and Rweru sectors of Rubavu and Bugesera districts, respectively (Figure 1). These two sites represent areas where there are complimentary ongoing project activities on tree-crop interactions with a wide range of participatory trials by the farmers.

Soil samples were collected using a soil auger to a depth of 0-10 cm. This was done around each tree of the four most common tree species found in the agroforestry systems: *Alnus acuminata*, *Markhamia lutea, Grevillea robusta* and *Eucalyptus* sp. (in Rubavu) and *Acacia polyacantha, Senna spectabilis, Grevillea robusta* and *Eucalyptus* sp. (in Bugesera).

Three replications for each tree species were sampled and this is on the same day. Soil samples were taken at three different positions from the tree trunk, that is, 1) 0.5 m from the tree trunk, 2) the edge of the tree canopy and, 3) 3 m from the edge of the tree canopy. At every position, the soil was sampled in the east and west of the tree and samples from the same position were pooled. A total of 72 soil samples were collected (Table 1) and stored in sealed plastic bags kept at 4°C until spores were extracted, counted and analyzed.

Additional information about sampled tree replicates, sizes of the trees, geographic coordinates of the sites and some soil chemical characteristics are provided in Tables 2 and 3.

Extraction of AMF spores

Soil samples were air-dried before extraction and counting of AM fungal spores. AMF spores extraction was done using the method adapted from Ingleby (2007). A 50 g sample of air-dried soil was mixed with water to obtain a 1 L suspension, and the suspension was strongly agitated to disperse the soil aggregates and release AMF spores. The liquid was then poured onto a nest of two sieves:

Table 1. Summary of soil samples collected from the field

Agroforestry system	Number of tree species	Number of replicates per species	Sampled sites around a tree	Total soil samples
Rubavu	4	3	3	4X3X3=36
Bugesera	4	3	3	4X3X3=36
Grand total				72

Table 2. Geographical coordinates and critical chemical characteristics of rhizospheres of the sampled trees (Rubavu agroforestry system).

Tree species	Sampled tree replicate	Geographic coordinates of the sites			Important chemical characteristics of the site*	
		Latitude S	Longitude E	Elevation (m)	Mean pH	Mean P (ppm)
Alnus acuminata	1	01°44'33"	029°20'54"	2025	4.9	10.32
	2	01°44'28"	029°20'59"	1998	4.9	10.32
	3	01°44'27"	029°21'01"	2006	4.9	10.32
Markhamia lutea	1	01°44'50"	029°21'00"	2114	4.9	10.32
	2	01°44'04"	029°21'14"	1967	4.9	10.32
	3	01°44'01"	029°21'16"	1959	4.9	10.32
Grevillea robusta	1	01°44'28"	029°20'57"	2007	4.9	10.32
	2	01°44'51"	029°21'01"	2126	5.1	10.07
	3	01°44'50"	029°21'00"	2119	5.8	19.73
Eucalyptus sp.	1	01°44'50"	029°21'00"	2116	5.8	19.73
	2	01°44'43"	029°20'47"	2089	5.1	10.07
	3	01°44'52"	029°21'01"	2144	5.1	10.07

*Chemical characteristics of the sampled sites provided by Rwanda Agriculture Board (RAB), 2014.

Table 3. Geographical coordinates and critical chemical characteristics of rhizospheres of the sampled trees (Bugesera agroforestry system).

Tree species	Sampled tree replicate	Geographic coordinates of the sites			Important chemical characteristics of the site*	
		Latitude S	Longitude E	Elevation (m)	Mean pH	Mean P (ppm)
Senna spectabilis	Tree No 1	02°17'30"	030°15'23"	1336	5	24.98
	Tree No 2	02°17'39"	030°15'14"	1352	5	24.98
	Tree No 3	02°17'40"	030°15'13"	1349	5.1	8.8
Acacia polyacantha	Tree No 1	02°17'28"	030°15'05"	1329	6.1	58.4
	Tree No 2	02°17'30"	030°15'04"	1324	5.1	8.8
	Tree No 3	02°17'28"	030°15'02"	1329	6.1	58.4
Grevillea robusta	Tree No 1	02°17'32"	030°15'22"	1342	5	24.98
	Tree No 2	02°17'33"	030°15'22"	1339	5	24.98
	Tree No 3	02°17'28"	030°15'24"	1334	5	24.98
Eucalyptus sp.	Tree No 1	02°17'38"	030°15'14"	1353	5	24.98
	Tree No 2	02°17'38"	030°15'14"	1344	5.1	8.8
	Tree No 3	02°17'37"	030°15'12"	1342	5	24.98

*Chemical characteristics of the sampled sites provided by Rwanda Agriculture Board (RAB), 2014.

Table 4. Abundance of AMF spores around four most common tree species in the agroforestry system of Rubavu district.

Tree species	Sampled tree replicate	Tree size		AMF spores abundance (Number of spores/g of soil)		
		Height (m)	Diameter at breast height (m)	0.5m from tree	End of tree crown	3m from tree crown
Alnus acuminata	1	15.5	0.22	6.6	5.8	4.2
	2	14.0	0.20	3.5	5.1	3
	3	11.5	0.18	3.1	2	1.6
	Mean and SE*			4.4(±1.9)	4.3(±2.0)	2.9(±1.3)
Markhamia lutea	1	10.5	0.14	1.1	2.2	1.7
	2	13	0.17	1.8	2.3	1.6
	3	14.5	0.18	2.9	2.8	2.5
	Mean and SE*			1.9(±0.9)	2.4(±0.3)	1.9(±0.5)
Grevillea robusta	1	22	0.27	1.7	2.8	3
	2	21.5	0.26	1.6	3	2.8
	3	19.5	0.24	2.5	1.5	1.8
	Mean and SE*			2.0(±0.5)	2.4(±0.8)	2.5(±0.6)
Eucalyptus sp.	1	17.5	0.25	1.8	2.1	1
	2	19	0.27	1.3	3	2.2
	3	16.8	0.22	2	2.2	1.6
	Mean and SE*			1.7(±0.3)	2.4(±0.5)	1.6(±0.6)

*Mean values are the mean of n; SE = Standard Error.

200 µ size on top to allow passage of spores but retain large soil and organic matter particles, and 45 µ on the bottom to retain AMF spores yet allow passage of the finest soil particles. The collected residue in the smallest sieve was washed and transferred into 50 ml centrifuge tubes and centrifuged with water for 5 min at 1,800 rpm. The supernatant was then discarded and the pellet re-suspended in 48% (w/v) sucrose and centrifuged again for 1 min at 1,800 rpm. The supernatant (with spores) was poured onto 45 µ sieve and rinsed with water to remove the sugar. The remaining debris on the sieve were transferred to a Petri dish for initial observation and collection of AMF spores under dissecting microscope with 40x magnification. Spore abundance was expressed as the number of AMF spores per gram of soil.

Statistical analysis

The analysis of variance (ANOVA) was used to assess the data. Comparison among tree species and between the two agroforestry systems was carried out at p=0.05 significant level.

RESULTS

The distribution of AMF on the basis of spore density showed difference among the experimental tree species. Spore abundance in the rhizosphere of different tree species from the agroforestry systems of Rubavu district is shown in Table 4. The mean spore count varied from 1.7 to 4.4 spores g^{-1} soil at 0.5 m from tree trunk, 2.4 to 4.3 spores g^{-1} soil at the end of tree canopy and 1.6 to 2.9 spores g^{-1} soil at 3 m from tree canopy. *Alnus acuminata* had the highest AMF spores across all the three positions from the tree trunk, that is, 4.4 spores g^{-1} soil at 0.5 m from tree trunk, 4.3 spores g^{-1} soil at the edge of the canopy and 2.9 spores g^{-1} soil at 3 m from the canopy. The tree species showed a significantly greater density of AMF spores than *Markhamia lutea* (p=0.001), *Grevillea robusta* (p=0.02) and *Eucalyptus* sp. (p=0.01) which among themselves do not show any significant difference. Within trees of the same species, there was generally a positive correlation between AMF spores density and size of the sampled trees. Around individual tree species, AMF spores abundance varied with the distance from the tree trunk, but not consistently. *A. acuminata* showed a decrease of AMF spore density with the distance from the tree, *M. lutea* and *Eucalyptus* sp. showed higher density of AMF spores at the end of the tree canopy and *G. robusta* gave AMF spore density increasing with the distance from the tree.

Table 5 shows the abundance of AMF spores around four most common tree species in the agroforestry system of Bugesera. The mean spore count varied from 3.1 to 6.6 spores g^{-1} soil. *Acacia polyacantha* and *Senna spectabilis*, with no significant difference between the spores count associated with their rhizospheres, showed the highest AMF spores with an average of 6.1 and 6.0 spores g^{-1} soil, respectively. *Senna spectabilis* showed a greater density of AMF spores than *G. robusta* and *Eucalyptus* sp. (p=0.0002 and 0.030, respectively). *A. polyacantha* with no significant difference with *G. robusta*, showed a greater density of AMF spores than *Eucalyptus*

Table 5. Abundance of AMF spores around four most common tree species in the agroforestry system of Bugesera.

Tree species	Sampled tree replicate	Tree size		AMF spores abundance (Number of spores/g of soil)		
		Height (m)	Diameter at breast height (m)	0.5m from tree	End of tree crown	3m from tree crown
Senna spectabilis	1	7.5	0.14	9.9	8.2	7.5
	2	6.0	0.13	6.4	5	3.6
	3	5.5	0.11	3.4	4.4	6.1
	Mean and SE*			6.5(±3.2)	5.8(±2.0)	5.7(±1.9)
Acacia polyacantha	1	7.0	0.17	2.9	5	5.4
	2	7.6	0.19	4.8	4.9	5.9
	3	8.2	0.22	8.7	9.2	8.5
	Mean and SE*			5.4(±2.9)	6.3(±2.4)	6.6(±1.6)
Grevillea robusta	1	12.0	0.17	3.2	3.4	4.9
	2	11.5	0.16	3.4	2.6	1.8
	3	10.5	0.14	3.6	3.4	3
	Mean and SE*			3.4(±0.2)	3.1(±0.4)	3.2(±1.5)
Eucalyptus sp.	1	11.0	0.14	2.9	4.1	4.6
	2	13.5	0.21	2	5.8	6.5
	3	11.5	0.16	5.3	4.9	4.3
	Mean and SE*			3.4(±1.7)	4.9(±0.8)	5.1(±1.2)

*Mean values are the mean of n; SE = Standard Error.

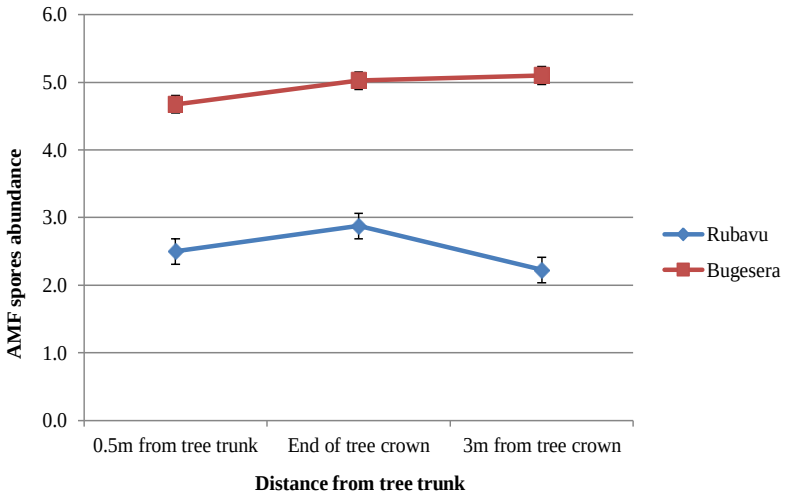

Figure 2. Variation of AMF spore abundance (number of spores/g of soil) with position from tree trunk in Rubavu and Bugesera agroforestry systems.

sp. (p=0.022). Within each tree species, AMF spore density was positively correlated with tree size. Around individual tree species, lack of constancy in variation of AMF spore density was also noticed. Around *Senna spectabilis* and *G. robusta,* a decrease of AMF spores density with the distance from tree trunk was generally noticed while the density increased as moving away from the tree around *Acacia polyacantha* and *Eucalyptus* sp.

The difference in AMF spore density between the two agroforestry systems was also noticed (Figure 2). The spore count varied from 3.1 to 6.6 spores per gram of soil in Bugesera and 1.6 to 4.4 spores per gram of soil in Rubavu. The mean AMF spore density in Bugesera agroforestry system was significantly higher with p=0.036 than in Rubavu.

DISCUSSION

Various studies were previously conducted to determine AMF abundance in different rhizospheres. Abundance of

775 to 1240 spores 100 g^{-1} soil were found in *A. albida* Del. in Senegal (Diop et al., 1994); 500 to 1500 spores 100 g^{-1} soil in *A. farnesiana* and *A. planifrons* in moderately fertile alkaline soils in India (Udaiyan et al., 1996); 110 to 2600 spores 100 g^{-1} soil in tropical forest and pasture (Picone, 2000) and 5 to 6400 spores 100 g^{-1} soil in a valley savanna of the dry tropics (Tao et al., 2004). By contrast, low spore densities of 11 to 32 spores 100 g^{-1} soil were detected in dry deciduous woodlands of Northern Ethiopia associated with different *Acacia* species (Birhane et al., 2010). Low AMF spore numbers were also recorded in a survey of *Acacia* tree species (49 to 67 spores 100 g^{-1} soil) in India (Lakshman et al., 2001) and in *Acacia* and *Prosopis* tree species (8 to 51 spores 100 g^{-1} soil) in Senegal (Ingleby et al., 1997).

In comparison with the above findings, the abundance of AMF spores got in the agroforestry systems of Rubavu and Bugesera agroforestry systems is generally moderate, that is, 1.6 to 4.4 spores/g of soil and 3.1 to 6.6 spores/g of soil in Rubavu and Bugesera, respectively.

The moderate level of AMF spores density found in the current study agrees with research findings of Picone (2000) who found a total of 110-770 AMF spores 100 g^{-1} in forest and 830-2600 spores 100 g^{-1} in pasture. Conversion from natural habitats to agricultural lands has been identified as one of the leading causes for loss of biodiversity worldwide. As shown by previous researchers, some modern agricultural practices such as continuous monoculture, non host crop in rotation and tillage can impact on the AMF association, both directly, by damaging or killing AMF and indirectly, by creating conditions unfavorable to AMF. These practices especially tillage can also cause soil erosion, reduce soil fertility and disrupt biodiversity in general including the previous crops. In cultivated lands, therefore, AMF population, species composition and diversity are often decreased as compared to natural ecosystems (Helagson et al., 1998; Mathimaran et al., 2007).

The abundance of AMF is also influenced by many factors including soil P and pH. However, in this study, the influence of P was not recorded. In their study, Zerihun et al. (2013) showed a significant negative correlation between AMF spore density and available P. These findings were similar to some reports from India and Northern Europe (Udaiyan et al., 1996; Kahiluoto et al., 2001). In contrast to this, Muleta et al. (2007) observed a positive relationship between spore number and available P in soil samples from natural coffee forest in Ethiopia. They suggested that available P level in their study sites was not high enough to inhibit mycorrhizal development. Similarly to this view of Muleta et al. (2007), the available P in our sampled sites may not be at a level of inhibiting AMF spores development.

Another important soil factor on AMF development is pH and this is positively correlated with AMF abundance. Low soil pH negatively affects AMF species richness (Don-Rodrgue et al., 2013). Therefore, the low pH in our sampled sites (Mean pH = 4.9-5.8 in Rubavu and 5.0-6.1 in Bugesera) may also be one of the causes of the moderate abundance of AMF spores in the regions, that is, 1.6 to 4.4 spores/g of soil and 3.1 to 6.6 spores/g of soil in Rubavu and Bugesera, respectively.

Results obtained in this study are supported by various findings from previous studies. Various researches showed that rhizosphere in close proximity to trees has a greater spore densities relative to the soil beyond the tree canopies (Mutabaruka et al., 2002; Pande and Tarafdar, 2004; Prasad and Mertia, 2005). In addition, studies in agroforestry coffee (*Coffea arabica L*.) systems observed higher spore densities in the rhizosphere of coffee plants under shade trees as compared to monocultural coffee systems (Muleta et al., 2007; Muleta et al., 2008). The explanation was given by Tadesse and Fassil (2013) that greater numbers of spores under the tree canopies in agroforestry systems may be due to greater amount of roots at this specific site. In contrast, other studies have shown no effect or in some cases a negative effect of trees on AMF (Boddington and Dodd, 2000; Leal et al., 2009). Spores of AMF may also occur in clumped distributions in the field, not correlated with root distribution (Douds and Millner, 1999).

The difference in spore density between the two agroforestry systems could be explained partly by a certain number of reasons. Most of Bugesera parts are made of valleys and a succession of low plateaux. This relief implies soft and middle slopes and smooth flow of rainwater that do not much transport away AMF spores, and then resulting in the accumulation of AMF spores in the soil around the host plants. This is not the case at Rubavu district as the region is characterized by a higher elevation and higher slopes causing strong flows of rainwater and erosion transporting away both soil and AMF spores. This could explain partly the higher density of AMF spores at Bugesera zone than Rubavu. This is in agreement with Chandrasekara et al. (2005) who concluded that there is a decreasing trend in spore density and spore diversity with increasing elevation. The researchers concluded that higher density of spores at lower elevations could be explained by the accumulation of spores, which are coming down with rainwater.

Another possible explanation for the higher density of spores at Bugesera zone than Rubavu may be the fact that during the period the soil samples were collected, Bugesera zone was experiencing a dry period and most of possible host crops were out of season. The AMF may have remained as spores without germinating. This was not the case at Rubavu as it was the period of much rain, and the soil samples were collected in agricultural lands with growing maize where very many AMF spores might have germinated to colonize the maize roots. This is supported by the view of Janos (1980) who pointed out that the variation in spore population could be attributed to many factors in a given site. The researcher argues that AMF spores always need live root contacts for

germination since they are obligate fungi; they may persists as spores in the absence of suitable hosts.

Environmental differences are also important factors in determining spore production by AMF. It is known that high temperature can increase AMF sporulation (Guadarrama and Álvarez-Sánches, 1999). These characteristics could also be used to explain the reason for the higher abundance of AMF spores in Bugesera characterized by a climate quite hot with the annual temperatures averaging between 21 and 29°C (http://www.bugesera.gov.rw/) as compared to Rubavu with an average temperature of 10°C (Nyandwi and Mukeshimana, 2011).

In conclusion, this work represented one of the first attempts to study the native AMF communities associated with some common tree species of agroforestry system in Rwanda. We recommend that the study be extended by characterization of the fungi as well as analyzing their effect on some common crops cultivated in the country with the aim of selecting and developing well performing and adapted inoculum to enhance tree growth and crop production.

Conflict of interest

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

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Isolation and classical identification of potent extracellular alkaline protease producing alkalophilic *Bacillus* sp from coastal regions of Tamil Nadu

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Microbial proteases are vastly demanded industrial enzyme with broad commercial application in various sectors. Naturally, the bacterial strains are well known for their ability to excrete enzymes into the environment. The current work was undertaken to isolate and characterize an effective extracellular alkaline protease producing *Bacillus* sp. A total of twenty nine bacterial strains were isolated from coastal regions of rhizosphere soil around the Cuddalore District Tamil Nadu, India. Out of twenty nine isolates, seven strains namely, IAS01, IAS02, IAS03, IAS04, IAS05, IAS06 and IAS07 show 45% clearance of hydrolyzing zone on skim milk agar plate. The positive isolates were further assay on small scale laboratory fermentation media for their protease productivity when compared with standard culture, *Bacillus subtilis* (MTCC-1789). As a result, the highest enzyme productivity that appeared in the strain IAS01 was found to be 332.13±1.31 U/ml at alkaline condition. Therefore, the selected isolate IAS01 was a promising strain for alkaline protease producer and identified as *B. subtilis* based on its morphological, physiological, biochemical characters and analysis of the 16S rRNA gene sequencing study. The partial 16S rRNA sequence was submitted to Genbank, with the accession number KF761633.

Key words: *Bacillus subtilis*, alkaline protease production, SPSS statistic analysis.

INTRODUCTION

Enzymes are well known as biocatalysts and are used for various commercial purposes in industries. Generally, bacterial proteases are the most preferred group of industrial enzymes as compared to plant, animal and fungal proteases, because of their ability to grow in simple culture medium with minimum space requirement and to obtain higher yield within short period. The *Bacillus* sp. produce various type of proteases, interestingly the alkaline proteases have extensive applications like laundry detergents, pharmaceutical, food

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industry, leather processing and waste bioremediation (Bayoudh et al., 2000; Jellouli et al., 2009). There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial process (Gupta et al., 2005; Habib et al., 2012). Proteases represents one of the three largest groups of industrial enzymes and have traditionally held the predominant share of the industrial enzyme market accounting for about 60% of total sale of enzymes (Beg et al., 2003; Deng et al., 2010)

Protease production is an inherent capacity of some microorganisms and large numbers of bacterial species are known to produce alkaline proteases. Considering the richness of microbial diversity, there is always a chance of searching new organisms to produce enzyme with better properties and suitability for commercial exploitation. Microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals. Mukhtar and Ikram-ul-Haq (2008) reported the production of alkaline protease by *Bacillus subtilis* and its application as a depilating agent. The aim of the present study was to isolate and characterize an effective alkaline protease producing *Bacillus* sp. from rhizosphere soil which might have useful industrial applications.

MATERIALS AND METHODS

Collection of soil sample and isolation of microorganisms

Samples of rhizosphere soil were collected from five different locations in the coastal regions of Cuddalore District, Tamil Nadu. Plant roots with adherent soil were taken from healthy plants, placed in sterile bags and transported to the laboratory within 1 h. Before isolation, the roots were gently shaken to remove excess soil and vortexed for 10 min in sterile distilled water (1 g per 10 ml). Samples were serially diluted with sterile distilled water from 10^{-1} to 10^{-6} dilutions and 100 µl of each dilution was plated onto nutrient agar at pH 9.0. After incubation for 24 h at 37°C, 27 colonies were picked based on divergence in morphology, size and color from dilution plates and maintained as pure cultures in nutrient agar slants with periodic transfers to fresh medium for further study.

Screening of potent protease producer

Screening assay performed for all the isolates on standard nutrient agar medium maintained at different pH (5.0-11.0) with supplemented 1% of skim milk was used as a substrate in the media for all the ingredients except skim milk autoclaved at 121°C for 20 min at 15 lbs pressure; skim milk was autoclaved for separately for 6 min to prevent the coagulation of milk. After cooling at 45°C, the skimmed milk was mixed with media and poured in the plates and allowed to solidify. After incubation at 37°C for 24 h (to remove moisture and check sterility), the plates were streaked with bacterial cultures using sterile needles. After incubation, seven isolates namely IAS1, IAS2, IAS3, IAS4, IAS5, IAS6 and IAS7 exhibited the prominent zones of clearance on skim milk agar plate at pH 9.0. Therefore, they were collected for further quantitative estimation of protease production when compared with standard culture *B. subtilis* (MTCC-1789).

Inoculum preparation for quantitative estimation of protease production

A volume 25 ml of nutrient broth taken in a 100 ml Erlenmeyer flask was inoculated with a loop full of pure culture from 24 h old plate and kept at 37°C in a rotary shaker. After 24 h of incubation, 1 ml of this nutrient broth culture was used as the inoculums found to be 3×10^6 CFU/ml and was added to the protease production medium containing: Glucose 6%, Soybean meal 2%, $CaCl_2$ 0.04% and $MgCl_2$ 0.02%. Media were autoclaved at 120°C for 20 min and later kept at 45°C in water bath to add 1 ml of inoculum for incubation at different culture condition namely pH (5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11), temperature (30, 35, 37, 40, 45, 50 and 55°C), incubation period (24, 36, 48, 60, 72, 84 and 96) and agitation (80, 100, 120, 140, 160, 180 and 200 rpm). After incubation, the culture was centrifuged at 1000 x*g* for 30 min and the supernatants were used for quantitative estimation of proteolytic activity.

Determination of proteolytic activity and protein content

Protease activity was determined using casein as a substrate. The reaction mixture containing 2 ml of 1.0% casein solution in 0.05 M Glycine-NaOH buffer having pH 11 and 1 ml of an enzyme solution were incubated at 60°C for 15 min. The reaction was then stopped with 3 ml of 10% tri-chloroacetic acid and the reaction mixture was allowed to stand for 10 min. The reaction mixture was then centrifuged at 9000 x*g* for 10 min at 4°C to get the clear solution. Absorbance of the liberated tyrosine in the solution was measured at 660 nm against blank. Amount of tyrosine released by the action of protease was determined from the tyrosine standard factor, according to the following relationship. Sample (µg/ml) = Absorbance of sample x Average standard factor, Where average standard factor was determined from the concentration/absorbance ratios of all points of the standard curve. One proteolytic unit was defined as the amount of the enzyme that released 1 µg of tyrosine per ml per min under the assay conditions (Kembhavi et al., 1993; Yang and Haung 1994). The protein content in the samples was estimated by following the method of Lowry et al. (1951). The amount of protein present in the sample was calculated from the standard curve.

Determination of cell biomass

The cell biomass was determined from a known amount (100 ml) of sample centrifuged at 10000 xg for 15 min at 4°C and the cell pellet was collected and washed with sterilized normal saline three times to remove the suspended particles. The washed cell pellet was transferred to a dried pre-weighed filter paper and then kept in oven at 105°C till the constant weight was achieved. The dry cell biomass was calculated by X = Weight of dry filter paper + cell biomass (g) - Weight of dry filter paper (g).

Identification of the bacterial strain for biochemical and 16s rRNA analysis

The highest enzyme producing isolate was identified as based up on the morphological and biochemical test carried out using basic method.

Morphological study

SEM was used to investigate the morphology of isolated strain. The sample for SEM was prepared by transferring the microbial strain to a clean Eppendorf tube containing approximately 1.5 ml of 3.5% glutaraldehyde solution. Then, culture was incubated for 4 h at room temperature followed by wash with phosphate buffer (100 mM, pH 7.2). The culture was then dehydrated using alcohol gradient from 10 to 100%. The dehydrated sample was then air dried and fixed on the stubs using double adhesive tape. A thin layer of gold was coated over the sample using HUS-5GB Hitachi vacuum evaporator for 90 s. These samples were then observed under scanning electron microscopy (Annamalai University, Tamil Nadu) at various magnifications at acceleration voltage of 10.0 KV.

Biochemical study

The selected isolate was identified according to the methods recommended in Bergey's Manual of Systematic Bacteriology (Grimont and Grimont, 1984; Holt et al., 1994) and Diagnostic Microbiology (Betty et al., 2002). The classical biochemical properties were identified by using diagnostic test kit TREK (The Sensititre GPID plate is an *in vitro* diagnostic product for the automated identification of Gram positive bacteria). Molecular identified was carried by 16S rRNA gene sequencing. The genomic DNA was extracted as per the protocol (Babu et al., 2009 and Manufacturer guide) and using microbial DNA extraction kit (insta-gene TM Matrix, Bio –Rad). The amplification of 16S rRNA gene was carried out by using Thermal cycler (PTC-225) in 100 µl reaction mixture containing 2.5 mM each of four dNTP, 10X PCR buffer, 3 U of Taq DNA polymerase, 10 ng template DNA and 400 ng each of primer 27F 5' AgAgTTTgATCMTGGCTCAg-3' primer 1492R 5'-TACggYTACCTTgTTACgACTT-3', were used to amplify 16s rRNA gene. The programs was set as initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min and a final extension at 72°C for 5 min. The sequencing was performed by using Big Dye Terminator Cycle Sequencing Kit with AmpliTag DNA polymerase (FS enzyme) (Applied Biosystems). The sequence producing product were resolved on an Applied bio-systems model 3730 XL automated DNA sequencing system (USA). The purified 16s rRNA gene sequence was compared with the Gen Bank nucleotide database (NCBI) using BLAST and BLASTX algorithms. The sequence alignments and the phylogenetic tree were constructed by using MEGA software version 5.2 (Altschul et al., 1990). The phylogenetic tree was constructed using a neighbor joining method and assessed with 1000 bootstrap replication.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 (SPSS, Inc., Chicago) statistical package. Data were expressed as men standard deviation (SD). One way analysis of variance (ANOVA) followed by Duncan multiple comparison method was used to correlate the difference between the variables. Data was considered statistically significant if $p \leq 0$ 0.05.

RESULTS AND DISCUSSION

Qualitative screening of protease producer

In the present study, a total of 27 morphologically distinct bacterial colonies were obtained from coastal regions of rhizosphere soil screened for the potent alkaline protease producing strain on skim milk plate assay. Seven strains were identified as protease producer by zone of hydrolysis around the colonies at pH 9.0 (Figure 1).

Optimization of process parameter for protease production

The culture condition determines the rate of bioprocess like pH and temperature. In this study, the highest enzyme production was found to be 235.05±2.91 U/ml at pH 9.0, this variation is shown in Table 3. Similar researcher (Sankareswaran et al., 2014) reported that the pH 9.0 is suitable for protease production. Therefore, this may be attributed to the microbial growth and protease activity inactivated at higher and lower level of hydrogen concentration. Table 4 shows 37°C is the suitable temperature for protease production throughout the study. Based on the result Abebe et al. (2014) reported that the temperature, 37-45°C is suitable for protease production. Fermentation period was contacted on every 12 h of incubation up to 96 h. The highest enzyme activity was recorded in period of 72 h, this variation is shown in Table 5. Similar researcher (Ponnuswamy et al., 2014) proved 72 h is suitable for protease production under solid state fermentation condition using cow dung as a substrate. Therefore, the results of this study showed that protease production when increased with incubation time up to 72 h, after 72 h of incubation, the enzyme activity was considerably decreased due to nutrient depletion. Agitation rang was investigated for protease production upto (80-200 rpm), the result is shown in Table 6. The highest enzyme activity was recorded after 140 rpm. Therefore the agitation rate gradually increase the protease production.

Quantitative analysis and identification/support protease producer

The selected seven primary bacterial isolates were separately checked for quantitative analysis of protease production in selected media (Genckal and Tari, 2006) at 72 h incubation, 37°C and 140 rpm under shack flask fermentation condition. After incubation and assay, it was found that the isolate IAS01 produced highest yield of protease activity (332.08±18.82 U/ml), protein (3.30±0.07) and cell biomass (2.99±0.01) followed by standard culture of *B. subtilis* (MTCC-1789) with their enzyme activity of 326.06±18.85U/ml, protein 2.52±0.09 and cell biomass 2.05±0.01, this variance is shown in Table 7. Similar researcher (Vanitha et al., 2014) reported the food waste isolate for *B. subtilis* "168" exhibited enzyme activity of 170.32 ± 1.5l U/ml. Another researcher (Hanan, 2012) reported that the marine isolate *Bacillus* sp. 2 EHN produced maximum yield of protease with the enzyme activity of 243 U/ml. Another

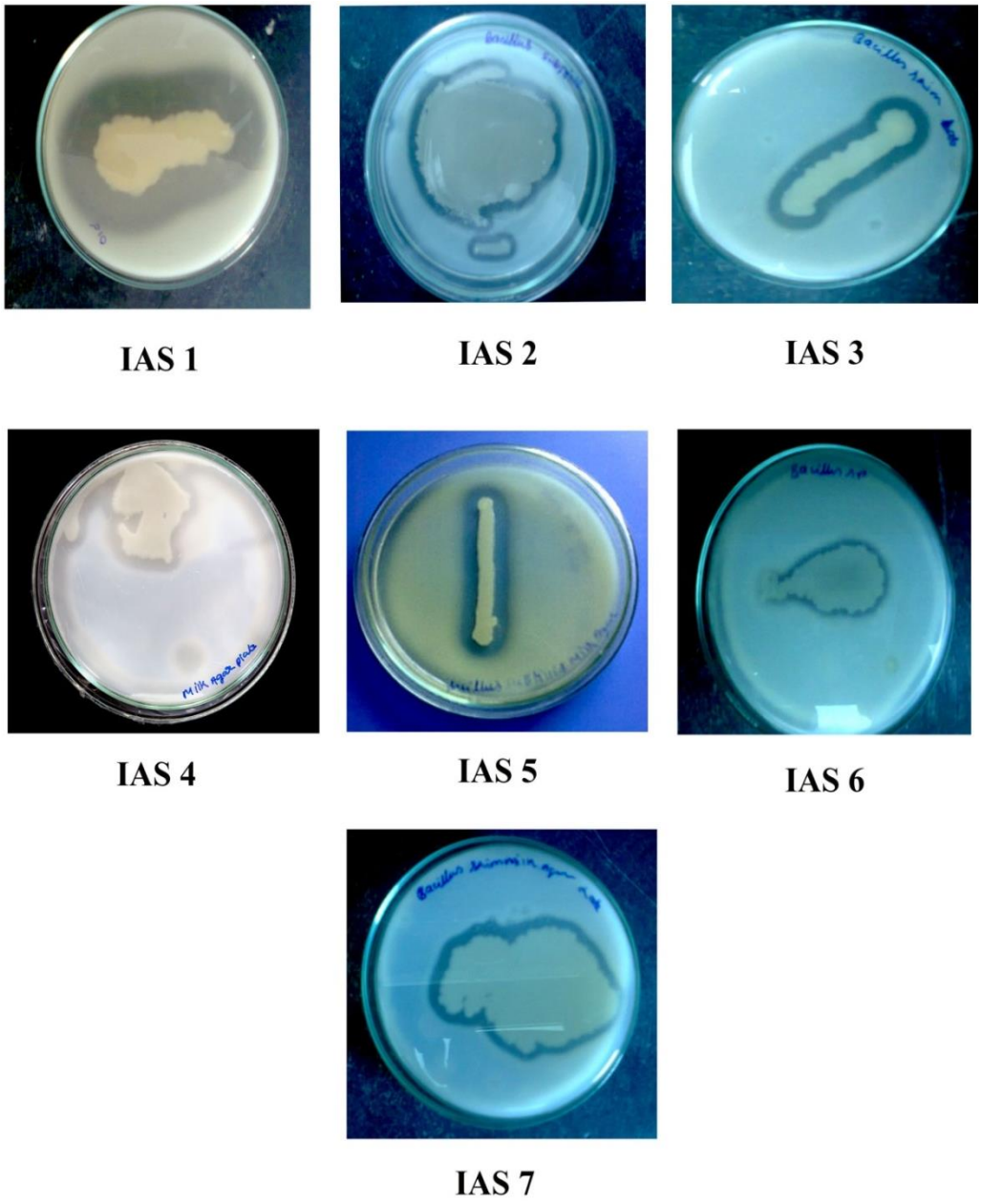

Figure 1. Effect of protease activity on skim milk agar plate.

researcher (Yun-Zhu et al., 2015) reported statistically produced alkaline protease optimized from *Penicillium citrinum* YL-1 under solid state fermentation exhibited activity of 94.30 U/ml. Earlier, the alkaline protease producing bacteria was also isolated, which shows that both *Bacillus anthraces* S-44 and *Bacillus cereus* S-98 exhibited their maximum ability to biosynthesize proteases within 60 h incubation period since the productivity reached up to 126.09 U/ml for *B. anthracis* S-44 corresponding to 240.45 U/ml for *B. cereus*, *Bacillus* sp. JB-99 respectively (Johnvesly et al., 2012). Therefore, similarly an isolate '1AS01' produced highest yield of protease activity (Table 5), chosen for further study. The selected isolate was confirmed as *B. subtilis* based on morphological SEM (Figure 2), biochemical tests performed according to Bergey's manual (Grimont and Grimont, 1984; Holt et al., 1994) indicating that this isolate (Tables1 and 2) belong to the genus *Bacillus* and 16S rRNA characterization study revealed that the species was confirmed as *B. subtilis* with approximately 1440 bp nucleotide sequence of 16s rRNA gene. The gene sequence was deposited in Gen bank (Accession number KF 761633) also phylogenetic tree was constructed (Figure 3).

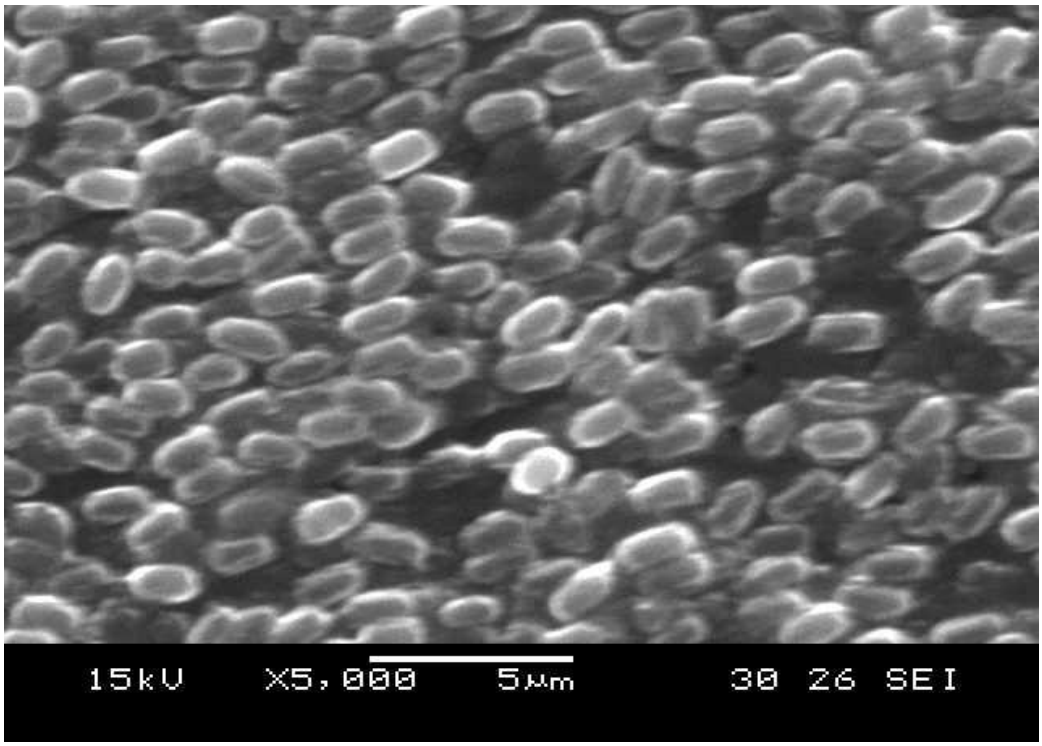

Figure 2. SEM picture revealing rod shape of the Gram positive bacteria.

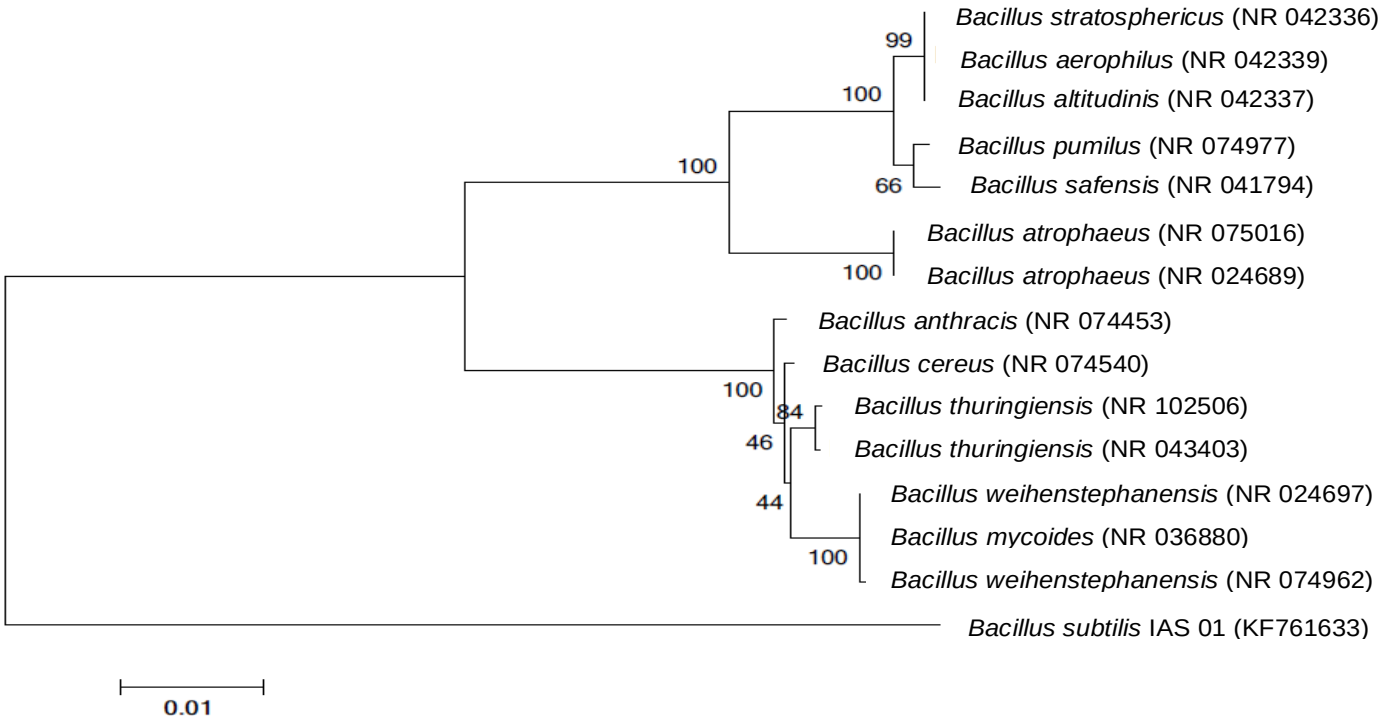

Figure 3. Phylogenetic tree predicted by the neighbour joining method using 16s rRNA gene sequences. The bootstrap considered 1000 replicates. The isolate IAS01 belong to the *B. subtilis*. Taxa are represented by type strain with Gen bank accession number. The scale bar represents the expected number of substitution average to over all the analyzed sites. Number in bracket indicates accession number.

Table 1. Biochemical result for selected strain, IAS01.

Biochemical characteristic	Result
Nitrate reduced to nitrite	+
Voges-Proskauer test	+
Urea test	-
Utilization of citrate test	+
Utilization of propionate	-
Formation of indole test	-
Hydrolysis of casein, starch and gelatin test	+

+, 90% or more strain positive; -, 90% or more strain negative.

Table 2. Classical biochemical test for selected strain IAS01.

Sugar fermentation test		Fluorogenic substrate test		Other specific test	
Parameters	**Result**	**Parameters**	**Result**	**Parameters**	**Result**
Glucose	+			Methionine	+
Glycerol	+			Aesulin	+
Maltose	+	β – D galactopyenoside	-	Ornithin and D alanine	-
Mannitol	+			Arginine	+
Rhamnose	-			Leucine	+
Sucrose	+	Valanine AMC	-	Serine and Urea	-

The selected IAS1 was tested for their biochemical nature by using diagnostic test kit TREK. The Sensititre GPID plate is an in vitro diagnostic product for the automated identification of Gram positive bacteria

Table 3. Effect of pH on growth and alkaline protease production by isolated strain "IAS01" after 48 h of incubation at 30°C temperature and 100 rpm agitation speed in shake flask culture.

pH	Protease activity (U/ml)	Protein (mg/ml)	Cell biomass (g/l)
5	101.88±3.28^{g}	0.88±0.07	1.26±0.02
6	119.33±15.36^{f}	1.27±0.07	1.49±0.03
7	154.83±2.48^{e}	1.57±0.10	1.63±0.03
8	193.74±2.84^{c}	1.67±0.09	1.71±0.02
9	235.05±2.91^{a}	2.45±0.08^{a}	2.04±0.02
10	209.38±2.84^{b}	1.99±0.07	1.93±0.02
11	180.50±3.13^{d}	1.41±0.16	1.47±0.05

Each value is an average of six replicates, ± indicates standard deviation (SD) among the replicates. Values of a given column are statistically compared by employing single factor analysis of variance and Duncan's multiple range test using CoStat software. Values followed by different letters in respective column differ significantly from each other at p≤0.05.

Table 4. Effect of temperature on growth and alkaline protease production by isolated strain "IAS01" at pH 9.0 and agitation speed of 100 rpm after 48 h incubation in shake flask culture.

Temperature (°C)	Protease activity (U/ml)	Protein (mg/ml)	Cell biomass (g/l)
30	210.19±3.10^{d}	2.01±0.09	1.26±0.02
35	246.89±2.21^{c}	2.21±0.11	1.93±0.03
37	272.76±1.64^{a}	2.55±0.10	2.14±0.02

Table 4. Contd

40	257.72±2.45[b]	1.74±0.11	1.97±0.01
45	227.43±2.63[e]	2.12±0.56	1.24±0.02
50	196.55±2.48[f]	1.99±0.07	0.94±0.02
55	163.05±1.26[g]	1.04±0.18	0.56±0.01

Each value is an average of six replicates, ± indicates standard deviation (SD) among the replicates. Values of a given column are statistically compared by employing single factor analysis of variance and Duncan's multiple range test using CoStat software. Values followed by different letters in respective column differ significantly from each other at p≤0.05.

Table 5. Effect of incubation period on growth and production of alkaline protease by isolated strain "IAS01" at pH 9.0, temperature 37°C and agitation speed of 100 rpm in shake flask culture.

Incubation (h)	Protease activity (U/ml)	Protein (mg/ml)	Cell biomass (g/l)
24	167.26±2.94[f]	1.08±0.10	0.57±0.02
36	200.96±2.52[e]	2.06±0.09	0.93±0.02
48	237.26±1.77[c]	2.52±0.09	2.05±0.01
60	261.93±1.45[b1]	2.63±0.12	2.18±0.02
72	289.81±2.33[a]	3.23±0.07	2.30±0.12
84	262.93±4.54[b]	2.02±0.18	2.51±0.02
55	221.82±1.96[d]	1.46±0.09	1.84±0.02

Each value is an average of six replicates, ± indicates standard deviation among the replicates. Values of a given column are statistically compared by employing single factor analysis of variance and Duncan's multiple range test using CoStat software. Values followed by different letters in respective column differ significantly from each other at p≤0.05.

Table 6. Effect of agitation speed on growth and production of alkaline protease by isolated strain "IAS01" at corresponding pH 9.0 and temperature37°C after 72 h of incubation in shake flask culture.

Agitation (rpm)	Protease activity (U/ml)	Protein (mg/ml)	Cell biomass (g/l)
80	230.04±1.77[f]	2.50±0.06	2.48±0.01
100	244.08±1.92[e]	2.62±0.12	2.66±0.02
120	285.80±1.65[c]	3.20±0.05	2.92±0.02
140	332.13±1.31[a]	3.30±0.07	2.99±0.01
160	305.25±2.36[b]	1.78±0.09	3.06±0.01
180	272.36±2.10[d]	2.56±0.12	2.30±0.05
200	200.16±2.10[g]	2.08±0.09	1.57±0.31

Each value is an average of six replicates, ± indicates standard deviation (SD) among the replicates. Values of a given column are statistically compared by employing single factor analysis of variance and Duncan's multiple range test using CoStat software. Values followed by different letters in respective column differ significantly from each other at p≤0.05.

Table 7. Quantitative estimation of protease production by test cultures as compared to standard culture, *B. subtilis* (MTCC-1789) under optimizing condition: pH 9.0, temperature 37°C, incubation period 72 h and 140 agitation in shake flask cultures.

Name of the isolates	Protease activity (U/ml)	Protein (mg/ml)	Cell biomass (g/l)
MTCC-1789	230.04±1.77[e]	2.52±0.09	2.05±0.01
IAS-01	332.13±1.31[a]	3.30±0.07	2.99±0.01
IAS-02	285.80±1.65[c]	3.19±0.05	2.95±0.02

Table 7. Contd

IAS-03	222.59±11.14^{f}	1.99±0.07	1.93±0.02
IAS-04	305.25±2.36^{b}	3.25±0.07	2.30±0.12
IAS-05	272.36±2.10^{d}	2.55±0.10	2.14±0.02
IAS-06	200.16±2.10^{g}	1.67±0.09	1.71±0.02

Each value is an average of six replicates, ± indicates standard deviation (SD) among the replicates. Values of a given column are statistically compared by employing single factor analysis of variance and Duncan's multiple range test using CoStat software. Values followed by different letters in respective column differ significantly from each other at p≤0.05.

Conclusion

The present study achieved the isolation of an effective protease producing *Bacillus* sp. such as *B. subtilis* IAS01 from coastal regions of rhizosphere soil and investigated the ability of the strain based on qualitative and quantitative examination with standard culture of *B. subtilis* (MTCC-1789) under alkaline condition. As a result, the isolate *B. subtilis* IAS01 produced highest yield of alkaline protease (332.13±1.31 U/ml), protein concentration (3.30±0.07 mg/ml) out of twenty seven isolates. Therefore, IAS01 was found to be an efficient isolate and it can be studied further for its biochemical characteristics and applicability in industrial applications.

Conflict of interest

The authors did not declare any conflict of interest.

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Full Length Research Paper

Selenium stress in *Ganoderma lucidum*: A scanning electron microscopy appraisal

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The present investigation explores the effect of selenium supplementation on radial growth and ultrastructural alterations in the hyphae and spores of *Ganoderma lucidum*. A concentration dependent decrease in radial growth was observed on culturing *G. lucidum* on sodium selenate supplemented mushroom minimal agar post 24 h. However, in comparison to control, selenium supplementation slightly increased diameter of radial growth till 10 ppm after 48 h. The Scanning Electron Microscopy (SEM) studies of radial growth showed selenium concentration dependent gradual decrease in hyphal diameter suggesting thinning and extensive branching of mycelia in response to increased Se supplementation. Moreover, SE micrographs also depict selenium concentration dependent decrease in number of spores and spore size. A significant decrease in spore diameter was recorded for mushroom minimal agar supplemented with 20 and 25 ppm (5.64 and 1.26 µm, respectively) as compared to control (10.04 µm). The spore were morphologically altered from spherical to oval or oblong and inflated to constrict deformed on increasing selenium concentration. SEM-energy dispersive X-ray spectroscopy (SEM-EDS) studies of intact mycelia showed no traces of selenium. However, crushed mycelia samples exhibited Se signals probably due to presence of selenium as integral component of cytosolic moieties like selenoproteins. Atomic absorption spectroscopy (AAS) of mycelia showed an increasing trend in the uptake of selenium with increased selenium supplementation. Percentage absorption was found to be in range of 7.2 - 9.9% with maximum absorption at concentrations of 15 and 25 ppm. Hence, sodium selenate supplementation at 10 ppm (with maximum 15 ppm) can be used for Se fortification as *Ganoderma* can grow rapidly without significant alteration in structure and morphology to enhance its biomedicinal properties.

Key words: *Ganoderma*, scanning electron microscopy (SEM), SEM-energy dispersive X-ray spectroscopy (SEM-EDS), selenium, selenoproteins.

INTRODUCTION

Medicinal mushrooms such as *Ganoderma*, *Auricularia*, *Flammulina* and *Lentinus* have great potential for successful bioprospecting through genetic modification or fortification to enhance their anti-cancer properties.

Medicinal mushrooms are the untapped source of novel pharmaceutical products for cancer therapeutics as these mushrooms consist of several bioactive compounds like glycans, glycoproteins, proteoglycans, quinones,

triglycerides and selenium which exhibit potent anti-cancer properties (Ferreira et al., 2010). Selenium is vital to human health and is regarded as the King of anti-cancer agents by WHO (WHO Report, 2003). It possesses excellent antioxidant properties and hence functions as a cellular protector against free radical oxidative damage. Selenium rich diet is thus beneficial and can be obtained from selenium enriched mushrooms in daily diet. The mushroom selenium content can be increased by incorporating selenium fortified substrate to increase its level up to 30 or 110 µg Se/g dw (*Ganoderma* 72 µg Se/g dw) (Falandysz 2008). Moreover, anti-mutagenic activities of selenium involve identification and elimination of cancer cells by activation of the tumor suppressor genes. It forms an integral component of methyl selenol, and inhibits formation of new blood vessels thereby, curbing blood supply to tumor tissue and eventually causing tissue senescence. Selenium also forms a conjugated complex with mushroom polysaccharides that further enhances its anti-tumor, anti-proliferative and scavenging properties (Shi et al., 2010).

The anti cancer therapies must include mushroom selenoprotein and polysaccharide to effectively treat cancer (Zhao et al., 2008). Mushrooms normally contain selenium, however the selenium content could be enhanced by biotransformation of inorganic selenite in substrate to selenoprotein, selepolysacchride and other compounds (Zhao et al., 2004). Organic selenoproteins particulary the water and alkali soluble proteins (molecular mass ≥16 kDa) are the major repositories for storage of organic selenium. Incidentally SeCys forms the active centre of several antioxidant selenoproteins - glutathione peroxidase, thioredoxinreductase, 15 kDa selenoprotein etc. (Rayman, 2005).

Mushroom selenium supplementation is strictly a selenium concentration dependent phenomenon (Stajic et al., 2002). However, selenium concentration in mycelia is not proportional to selenium supplementation in the growth medium. Higher dosage can lead to decrease in hyphal growth due to selenium toxicity. A low concentration of Se (<100 µg/g) in the substrate facilitate the synthesis of total protein and amino acids in *G. lucidum*, but a high concentration of Se (>150 µg/g) played a reverse role (Zhao et al., 2004).

Selenium, hence is an integral component of the mushroom Ionome (that is mineral nutrient and trace element of an organism that involves application of high-throughput techniques to quantitatively and simultaneously measure the elemental composition and changes in response to physiological stimuli, developmental state, and genetic modifications). Electron microscopy (EM) along with Energy Dispersive Spectroscopy (EDS) is a useful tool to decipher altered morphology, topography and ultrastructural changes in a biological sample as well as elemental composition of the sample surface and can provide the relative localization of different elements by X-ray mapping technique. Thus, the aim of our research was to investigate the growth rate, ultrastructural changes in the hyphae and spores of *Ganoderma lucidum* and ability of mycelia to store selenium as a result of selenium supplementation in form of sodium selenate.

MATERIALS AND METHODS

G. lucidum culture was procured from the Mushroom Research Complex, Department of Microbiology, Punjab Agricultural University, Ludhiana on agar slant and was maintained on mushroom minimal agar (MMA) medium at 30°C ± 1°C. MMA was enriched with selenium with different concentrations of 5, 10, 15, 20 and 25 ppm of inorganic sodium selenate (Na_2SeO_3) salt. The Se-MMA plates were inoculated with 5 mm mycelium disc punched from the fresh culture.(5 replications) and incubated at 30°C ± 1°C for 24, 48 and 92 h and radial diameter was measured in centimeter scale on Leica Macroviewer MZ 60.

The mycelia growth was scrapped from the agar surface and the the fungal hyphae were fume fixed in 1% OsO_4 and processed as per standard protocol of Bozzola and Russel (1999) for SEM. The samples were placed on aluminium stub using double sided carbon tape and sputter coated with gold using Hitachi Ion Sputter Coater model E-1010 for 30 s. The samples were then viewed under SEM (Hitachi S-3400N) at 15 kV accelerating voltage in secondary electron (SE) mode. The SEM-EDS was performed to ascertain the extracellular presence of selenoproteins or organic selenium in the cytosol by using Thermo Noran EDS System Six module attached to SEM. The significance of the data collected was checked statistically by calculating critical difference at 5% level using SPSS 20 software.

The concentration of absorbed Se was measured with a graphite generation atomic absorption spectrophotometer (model Avanta GBC GF 3000 system) at various working concentrations of sodium selenate. Fungal sample (0.5 g) was digested with 10 mL of di-acid (conc. HNO_3: perchloric acid in 3:1 ratio). After cooling, the obtained samples were diluted with distilled water to a final volume 50 mL and filtered through Whatman filter paper no. 42.

RESULTS AND DISCUSSION

Effect of selenium on radial growth

G. lucidum strain GL-II was grown on selenium fortified with sodium selenate mushroom minimal agar MMA at different concentrations (5 to 25 ppm). Stereomicroscopic studies revealed a decrease in radial hyphal growth of the mycelium in MMA. After 24 h of incubation, a decrease in radial diameter at the given concentrations of selenium was observed (Table 1 and Figure 2). However, selenium supplementation resulted in similar growth in radial diameter with respect to control at concentration of5 and 10 ppm at 48 h (Figure 1). There was a slight increase

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Table 1. Effect of selenium on radial growth (in cm) of *Ganoderma lucidum*

Incubation time (h)	Selenium Concentration							
	Control	**5 ppm**	**10 ppm**	**15 ppm**	**20 ppm**	**25 ppm**	**Mean**	**R^2**
24	1.375	1.175	1.10	1.25	1.15	1.05	1.18	0.511
48	2.95	2.95	2.93	2.73	2.48	2.35	2.72	0.884
92	6.15	6.45	6.55	6.425	6.15	5.75	6.24	0.303
Mean	3.49	3.52	3.52	3.46	3.25	3.05		
CD @ 5%: A (Selenium concentration) -0.0752, B (Incubation duration) – 0.0532, AB (Interaction) – 0.130								

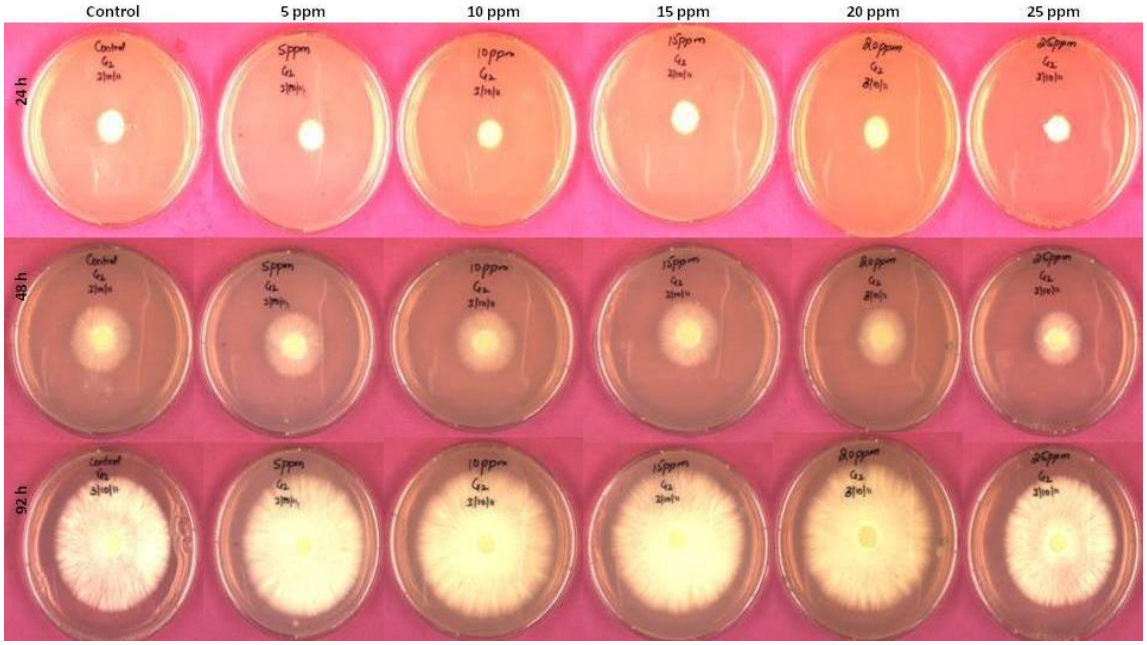

Figure 1. Effect of selenium on radial growth (in cms) of *Ganodeirma lucidum.*

in radial growth at concentrations from 5 to 15 ppm followed by a decreasing trend at higher concentrations at 92 h of incubation (Figure 1). A significant absorption was observed at 48 h at 10 and 15 ppm Se concentrations (R^2 value 0.0885). Thus, 10 and 15 ppm sodium selenate concentrations can be utilized for fortification without any significant change in radial growth. At concentrations higher than 15 ppm, the growth decreased significantly, probably due to enhanced toxic effect of selenium.

Stamets (2000) stated that *G. lucidum* grows radially without making aerial hyphae, and later develops mycelia running parallel to the surface and gained an intensive cover. The culture exhibited initial white color pigmentation than turns to golden yellow. It has been reported that Se concentrations were 4.6 and 9.3 µg/g (d.w) at 5 and 10 µg/g of added Se in the media, respectively (Stajic et al., 2002; Werner and Beelman, 2002). Se accumulation occurs approximately linear in relation to levels of this element added to the substrate.

Mushrooms grown on substrates supplemented with 10 ppm would provide 81.4 µg of Se, representing 116.3% of the daily value (DV) and according to the FDA, a serving size of fresh *P. eryngii* mushrooms produced in Na_2SeO_3 supplemented substrates should be considered an excellent source of Se because it provides more than 20% of the DV (Estrada et al., 2009).

Effect of selenium on spore count

Scanning electron microscopy studies were carried out to decipher the alterations in the spore, hyphal morphology and topography of *Ganoderma lucidum* in response to added selenium. A significant decrease in the spore count with respect to control was recorded with increase in selenium concentration (Table 2, Figure 2). At 25 ppm, SE micrographs showed absence or presence of very sparsely spaced spores while at lower concentration (5 ppm), the spore count was relatively better than

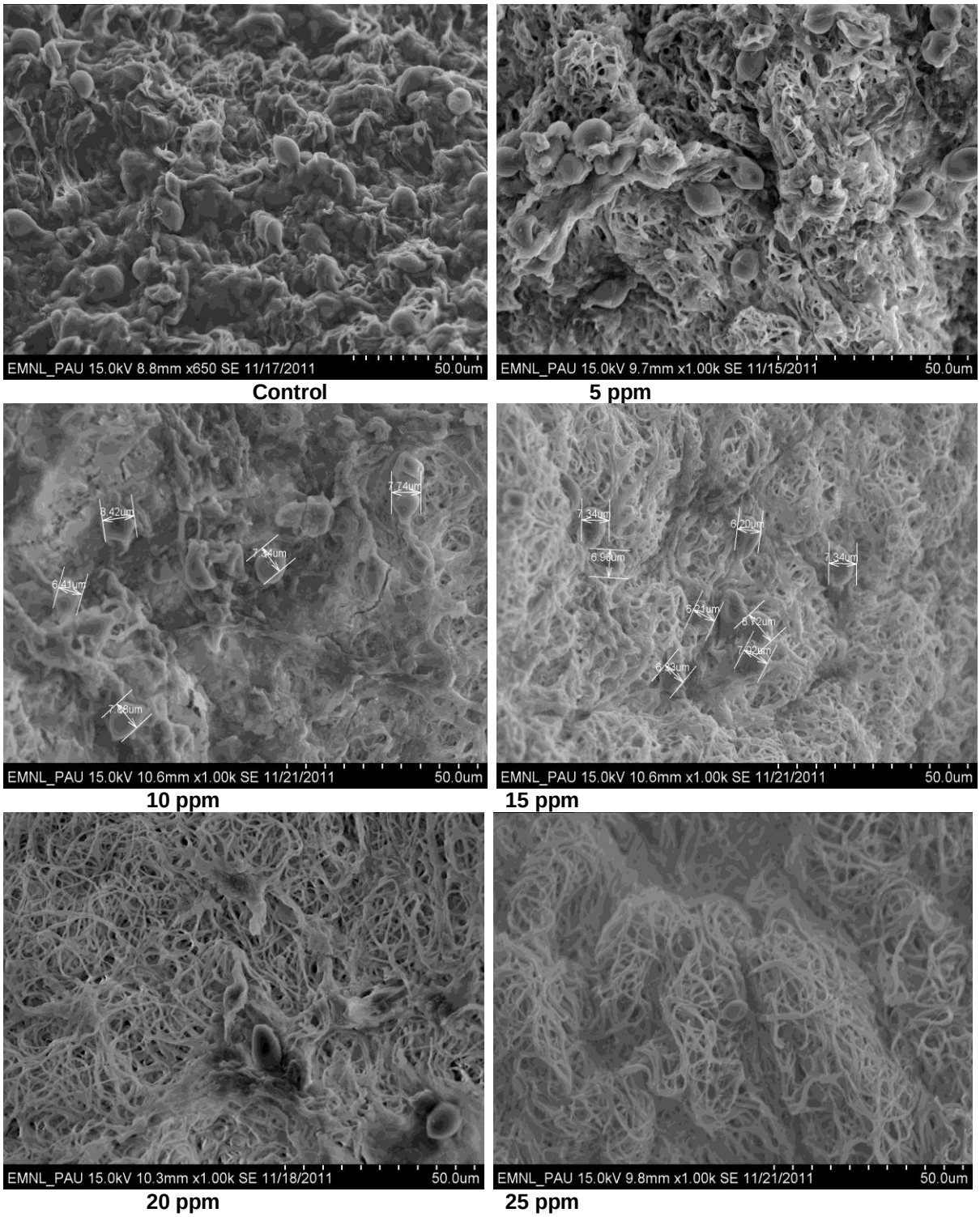

Figure 2. Effect of selenium on spore count of *Ganoderma lucidum.*

consecutive higher concentrations.

Effect of selenium on spore diameter

SE micrographs also revealed the ultrastructural alterations in spore size (diameter) and shape with increase in selenium concentration (Figure 3). The spore structures were altered from spherical to oval or oblong, inflated to constricted deformed and deflated on increasing Se concentration (5 to 25 ppm). Similar trend

Table 2. Effect of selenium on hyphal and spore diameter and number of spores of *Ganoderma lucidum*

Selenium Concentration	Hyphal diameter (nm)	Spore diameter (µm)	Number of spores
Control	861.2 (919 – 756)	10.04	31
5 ppm	632 (728 – 428)	8.87	22
10 ppm	608.2 (705 – 456)	7.7	12
15 ppm	518 (580 – 452)	5.99	9
20 ppm	508.4 (566 – 449)	5.64	8
25 ppm	525 (577 – 434)	5.60	2
CD @ 5%	95.52	1.26	-

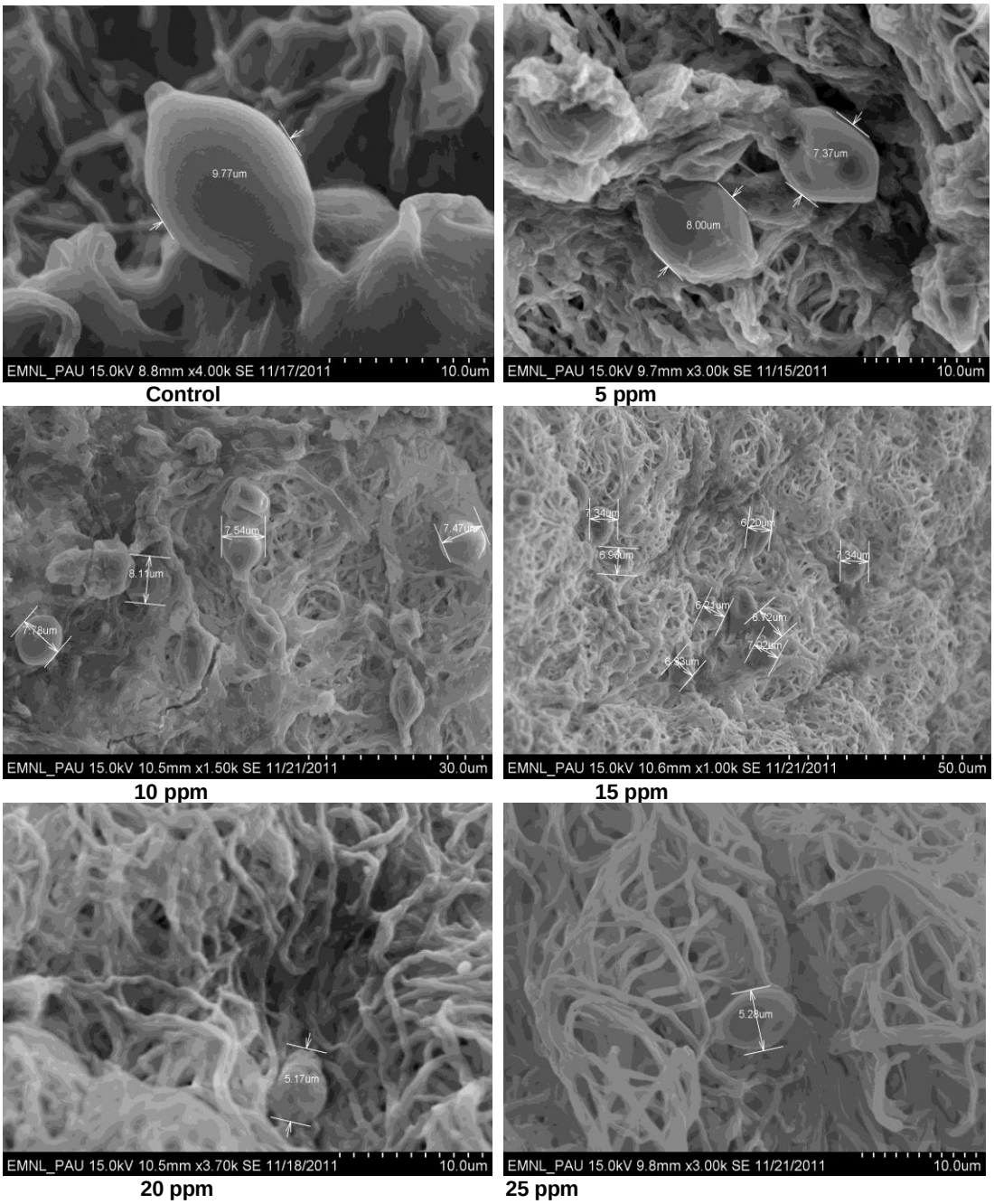

Figure 3. Effect of selenium on spore diameter of *Ganoderma lucidum.*

was recorded in spore diameter studies. A significant decrease in spore diameter was observed in concentration of 20 and 25 ppm (5.60 and 1.26 µm, respectively) as compared to control (10.04 µm). Earlier SEM studies performed by Guler et al. (2011) report presence of septate mycelium and non-smooth spores surface with average size of 92.5-108 µm x 301-331 µm with beaked germinating point in *G. lucidum.*

Tiqiang et al. (1996) studied micro-morphology using optical microscope (OM) and SEM of intact and wall-cracked spores of log-cultivated *G. lucidum*. Optical Micrographs depicted the spore to be ovate-oblong or ovoid with truncated apex or blunt taper and a size of 6.53 - 8.04 × 9.55 -12.56 µm while SEM micrographs provided some sinuous depressions or minute holes on the surface of the spore. The spore size and shape were observed to be similar to our findings (Figure 3).

Effect of selenium on hyphal diameter

SE micrographs exhibited gradual decrease in hyphal diameter with increase in selenium concentration. The hyphae branched out more oftenly with smaller internodes as the selenium concentration increased. At higher concentration, the networking of mycelia was enormous, a phenomenon might be used by growing culture to overcome stress conditions. At 10 ppm (Figure 4, Table 2) concentration no prominent Se stress could be observed and culture grew rapidly without any significant alteration in structure and morphology.

Scanning electron microscope-energy dispersive X-ray spectroscopy study

The SEM-EDS studies were performed to observe the elemental composition of sample surface and to reveal the presence of selenoproteins or organic selenium on the surface of hyphae, however; no selenium was recorded on the hyphal surface thus suggesting selenoproteins or other forms of selenium to be cytosolic moieties. On the contrary, the SEM-EDS results of the samples crushed with liquid nitrogen showed the presence of selenium in samples indicating the uptake of the selenium by the hyphae and sequestration as cytosolic proteins (Table 3).

SEM-EDS studies of the hyphal mass showed difference in the % weight and % atom carbon and oxygen composition. Thus it is noted that a trend of an initial decrease in % carbon till 10 ppm followed by increasing trend with respect to control with maximum % weight carbon was recorded at 15 ppm. On the contrary % weight oxygen exhibited a decreasing trend. Similar observations were observed in % atom C and O composition (Table 4).

Since mushrooms contain relatively high protein levels, and can accumulate large amounts of selenium, it is reasonable to expect that selenium could be incorporated into proteins. The growth of mycelia and fruit body formation of *Pleurotus ostreatus* (Hk-35 and P70) over wide range of concentrations of inorganic form of selenium was examined and showed stimulatory effects (in concentration of 1-50 mg/l) and toxic effects in higher concentration (Savic et al., 2009). It has been reported that the low concentration of Se (<100 µg/g) in the medium leads to enhanced synthesis of total protein and amino acids in *G. lucidum,* but high concentration of Se with more than 150 µg/g was found to have played a reverse role and was fatal (Zhao et al. 2004).

Atomic absorption spectroscopic studies

Atomic absorption spectroscopy of fungal samples exhibited an increasing trend in the uptake of Se by *Ganoderma* hyphal network or hyphae with increased concentration of sodium selenate (Table 5). Percentage of absorption was found to be in range of 7.2% - 9.9% with maximum absorption at concentrations of 15 and 25 ppm.

The Se-enriched Champignon mushroom could contain up to 30 or 110 µg Se/g dw, while the Varnished Polypore (*Ganoderma lucidum*) could contain up to 72 µg Se/g dw). An increasingly growing database on chemical forms of Se-enriched mushrooms indicates that seleno-compounds identified in carpophore include selenoysteine, selenomethionine, Se-methylselenocysteine, selenite, and several unidentified seleno-compounds; though their proportions vary widely (Falandysz, 2008).

The overall study indicated that selenium absorption increased with increasing concentration of sodium selenate. However, selenium uptake induces stress conditions and ultrastructural changes in hyphae and spores of *G. lucidum.* A decrease in hyphal and spore diameter occurred with increase in concentration though a statistically significant change was recorded at 20 ppm and 25 ppm concentrations. Finally, it can be concluded that Se-supplementation till 15 ppm could be considered to be safe and selenium is absorbed as intracellular component in form of organic selenium in selenoproteins.

The above studies conclude that Se supplementation (10 and 15 ppm) is useful for Se fortification in *G. lucidum* to enhance its medicinal properties (by enhanced production of cancer suppressor and antioxidant selenoproteins) without having significant effect on ultra-architectural features on the fungal hyphae and spores.

Conflict of interests

The authors did not declare any conflict of interest.

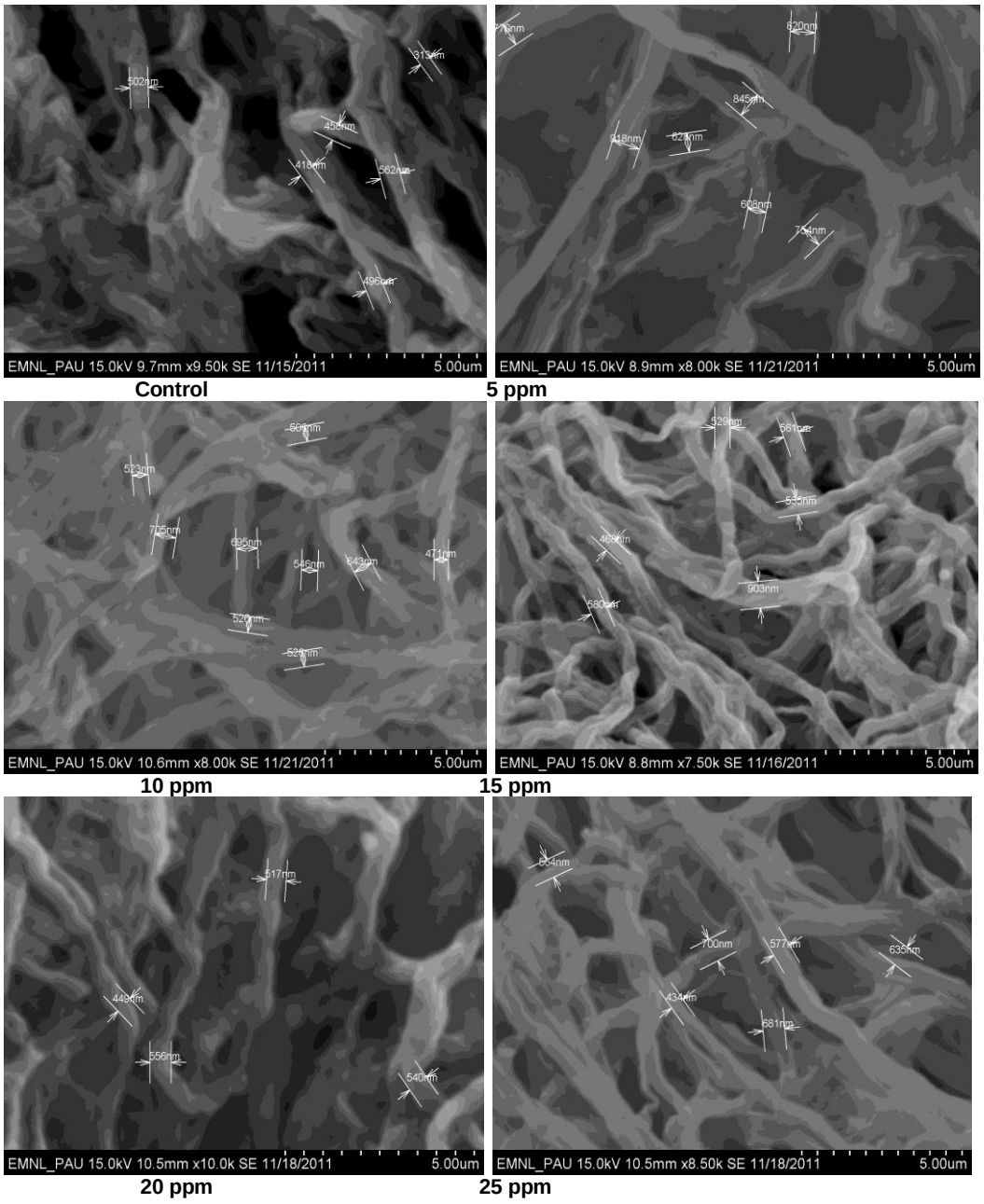

Figure 4. Effect of selenium on hyphal diameter of *Ganoderma lucidum.*

Table 3. SEM-EDS of selenium (%wt) in crushed mycelium of *Ganoderma lucidum*

Parameter	Control	5 ppm	10 ppm	15 ppm	20 ppm	25 ppm	CD @ 5%
% Weight	0.00	0.14	1.14	2.71	0.09	0.30	0.024
% Atom	0.00	0.02	0.21	0.46	0.01	0.05	0.019

Table 4 .Effect of selenium on carbon and oxygen (% weight) of *Ganoderma*

Parameter	Control	5 ppm	10 ppm	15 ppm	20 ppm	25 ppm	CD @ 5%
% carbon	58.91	55.24	57.28	62.12	62.58	62.12	0.178
% oxygen	37.65	30.68	29.64	22.35	23.54	21.14	0.179

Table 5. Total Selenium content (in ppm) in mycelia hyphae of *Ganoderma lucidum*

Selenium concentration	Total selenium content hyphae (ppm)	Percentage absorbtion (%)
Control	0.405	-
5	0.380	7.6
10	0.726	7.2
15	1.474	9.82
20	1.950	9.75
25	2.497	9.9
CD @ 5%	0.065	-

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Choice of DNA extraction protocols from Gram negative and positive bacteria and directly from the soil

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DNA extraction is a fundamentally important step for the implementation of genotypic techniques in microbial identification, and the use of such techniques has become essential for the analysis of soil microbial diversity. Considering culture independent methodologies, it is still necessary to ensure that DNA is extracted in appropriate amounts and that extracted DNA is inhibitor-free. This study aimed at selecting a single protocol suitable for the extraction of total DNA from Gram positive and negative bacteria isolated from different sources, as well as a protocol for the direct extraction of DNA from soil. Four experimental protocols and a commercial kit were tested for the extraction of total DNA from isolated bacteria. Among the protocols, the detergent + salt + thermal incubation method (based on Harju et al., 2004) was considered the most promising because it produced satisfactory yields of DNA, with adequate quality for all isolates studied, especially *Staphylococcus aureus,* without the need to use enzymes and glass beads which can make the extraction process more expensive. Three experimental protocols and the commercial kit were tested for the direct extraction of DNA from soil. Regarding PCR amplification, the amount of total DNA extracted is less limiting than its quality. Thus, commercial kit PowerMax™ Soil DNA Isolation (MoBio) offered more promising results, because although this provided low yields of DNA, it was sufficient for polymerase chain reaction (PCR) amplification.

Key words: Genotypic characterization, bacterial diversity, polymerase chain reaction (PCR), agroforestry system, organic farming.

INTRODUCTION

The biodiversity of microbes within soil is significant for the maintenance of healthy soil because these microbes are involved in many vital functions like crucial cycles of C, N, P, formation of soil, toxin removal and so on.

Microbial characteristics of soils are being evaluated increasingly as sensitive indicators of soil health because of the clear relationships between microbial diversity, soil and plant quality, and ecosystem sustainability (Doran

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and Parkin, 1994). While the understanding of microbial properties such as biomass, activity and diversity are important to scientists in furthering knowledge of the factors contributing to soil health, results of such analyses may also be useful to extension personnel and farmers in devising practical measures of soil quality.

Traditional microbiological methods for diversity studies are based on the cultivation of microorganisms prior to phenotypic and genotypic identification. Isolation and purification of nucleic acids from bacteria requires its effective separation from other cellular constituents. Furthermore, it is essential to maintain the integrity of these molecules, which should remain unchanged throughout the extraction procedure, because the information contained in DNA depends on its sequence. Although the extraction method does not affect the sequence directly, some protocols result in breakage of DNA polymers, causing the loss of such information. Therefore, it is extremely important that the extracted nucleic acids remain as intact as possible.

Unlike Gram negative bacteria that are readily lysed by standard protocols, the Gram positive species are comparatively more resistant to cell lysis because of a greater concentration of peptide and cross-bond peptides in the cell wall. For example, *Staphylococcus* spp. has a thick cell wall of (20-80 nm in diameter) (Prescott et al., 1999). This hinders cell lysis, often requiring the addition of enzymes such as lysostaphin and acromopeptidase to the lysis buffer (Ezaki and Suzuki, 1982; Mason et al., 2001; Schindler and Schuhardt, 1964; Zschöck et al., 2000). This burdens the extraction process. On the other hand, Gram negative bacteria, such as *Escherichia coli,* with diameters of 1-3 nm and a thin layer of peptidoglycan (Prescott et al., 1999), may have the crosslinking cleaved by EDTA or lysozyme and are generally lyzed with boiling or SDS (Sambrook and Russel, 2001).

Characterization studies of microbial diversity in highly diversified environments, such as soil, have revealed the difficulties of application of specific DNA extraction protocols for each bacterial group. This makes it desirable to establish a single protocol for Gram positive and negative bacteria that enables the extraction of nucleic acids in quantity and quality required for subsequent application techniques, such as polymerase chain reaction (PCR) to identify them (Baratto and Megiolaro, 2012; Kramer and Coen, 2001; Sambrook and Russel, 2001; Weissensteiner et al., 2004).

According to the estimates, c. 99% of the microorganisms present in nature are not cultivable by standard techniques. Therefore, the genetic information and biotechnological potential of the majority of the organisms would be untapped by conventional approaches (Chernitsyna et al., 2008; Green and Keller, 2006). In order to circumvent limitations of the culture method, DNA-based approaches, have been adopted to explore the entire microbial community (Nordgard et al., 2005). Many of these techniques rely on PCR, such as nucleic acid hybridization, DNA cloning and sequencing, and denaturing gradient gel electrophoresis. However, the quantitative and qualitative efficiency of these techniques in obtaining DNA need to be guaranteed.

Soil is an extremely complex environment, with a multitude of colloids having electric charges capable of adsorbing DNA molecules. Thus, it is a limiting factor in obtaining DNA from this environment. Another critical issue is the presence of humic substances, which are acidic macromolecules that precipitate in the same pH range as DNA, and which can inhibit the activity of various enzymes, such as Taq DNA polymerase, and restriction enzymes used in various nucleic acid-based techniques (Tebbe and Vahjen, 1993; Torsvik, 1995).

Many protocols for the extraction of environmental DNA have been published, and some of them are commercialized as soil DNA extraction kits. The methods vary with respect to shearing, purity and quantity of the isolated DNA. However, the basic concept of cell lysis by enzymatic and hot detergent (SDS) treatment is still the core of many DNA extraction methods (Rondon et al., 2000). Besides, some protocols also apply mechanical forces generated by bead beating, freeze-thawing and sonication methods to disrupt the rigid cell structure (Kennedy and Marchesi, 2007; Sharma et al., 2007; Voget et al., 2003).

The soil extraction protocols are generally classified as direct and indirect DNA extraction procedures. Direct DNA isolation is based on cell lyses within the sample matrix and subsequent separation of DNA from the matrix and cell debris (Voget et al., 2003). While the indirect approach involves the extraction of cells from the environmental material prior to the lytic release of DNA (Kauffmann et al. 2004; Santosa 2001), direct DNA extraction protocol involves soft and harsh lysis methods.

Soft lysis method is based on the disruption of microorganism solely by enzymatic and chemical means, whereas harsh lysis approach involves the mechanical cell disruption by bead beating, sonication, freeze-thawing and grinding. Because the composition of different habitats varies with respect to their matrix, organic and inorganic compounds and biotic factors, standardization of total DNA extraction technique is desirable. Improved DNA extraction techniques could help to ensure analyses that adequately represent the entire community's genome without inhibitory substances.

Hence, the aim of this study was to evaluate the comparative effectiveness of different protocols available in the literature and commercial kits to select a single protocol for the extraction of DNA from Gram positive and negative bacteria isolated from different sources and a protocol for the direct extraction of DNA from soil from different regions and cropping systems in Bahia (Brazil).

Table 1. Morphotinctorial characteristics and sources of bacterial isolates used in this study.

Isolates	Species	Morphotinctorial characteristics	Source
30A	*Vibrio cholerae*	Gram-negative curved-rod shape	Mussels
40B	*Vibrio cholerae*	Gram-negative curved-rod shape	Oysters
BP11	*Pseudomonas* sp.	Gram-negative bacilli	Soils
BN5	*Bacillus* sp.	Gram-negative bacilli	Soils
BT108	*Bacillus* sp	Gram-negative bacilli	Soils
BP1	*Bacillus* sp.	Gram-negative bacilli	Soils
SA	*Staphylococcus aureus*	Gram-positive cocci	Milk
MecA	*Staphylococcus aureus*	Gram-positive cocci	Milk
BN1	*Staphylococcus epidermids*	Gram-positive cocci	Soils

MATERIALS AND METHODS

Protocols for bacteria DNA extraction

Five protocols for DNA total extraction (detergent + thermal incubation, glass beads, detergent + enzymatic + thermal incubation, detergent + Salt + thermal incubation method and commercial kit QuickExtract™ Bacterial DNA Extraction - Epicentre) from nine bacterial samples collected from different sources were evaluated (Table 1).

Bacteria were inoculated into 5 ml of Brain-Heart Infusion Broth (Merck) and incubated at room temperature on a shaker at 150 rpm for 24 h. The concentration of cells was adjust to OD_{600} = 1.0. Subsequently, 1.5 ml of the culture was transferred to microcentrifuge tubes and centrifuged at 12,396 ×*g* for 5 min. The supernatant was discarded. This step was repeated three times.

Cell lysis step (except for commercial kit) are shown in the Table 2, and deproteinization and precipitation steps were common to them. Deproteinization was performed using the same volume of phenol/chloroform : isoamyl alcohol [1-1 (24:1)] and the same volume of chloroform : isoamyl alcohol (24:1) and to the precipitation, two volumes of iced ethanol 100% were added, followed by incubation at -20°C for 2 h. The microtubes were centrifuged at 14,549 ×*g* for 30 min, and the sediments were washed with 500 µl of 70% ethanol, dried at room temperature, and resuspended in 30 µl of TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)].

For the commercial kit QuickExtract™ Bacterial DNA Extraction Kit (Epicentre), the sediments were resuspended in 50 µl of Quick Extract plus 0.5 µl of Bacterial Extraction of Ready-Lyse Lysozyme Solution. After 2 h at room temperature, the microtubes were incubated at 80°C for 2 min. Subsequently, the samples were centrifuged at 12,396 ×*g* for 2 min, and the supernatant was transferred to a new microtube.

Protocols for soil DNA direct extraction

For the direct extraction of DNA from soil, four protocols (glass beads, enzymatic, glass beads + PEG8000 method and commercial kit PowerMax™ Soil DNA Isolation - MoBio) were tested using four soil samples from two farms in southern Bahia and two areas with different cocoa planting systems. Soil samples collected from each area were composed of 10 subsamples collected at a depth of 0-10 cm in July 2011.

Cell lysis step (except for commercial kit) are shown in the Table 2, and deproteinization and precipitation steps were common to them. One gram of soil was added to 1 ml of extraction buffer followed procedure described in Table 2. Deproteinization was performed using the same volume of phenol/chloroform : isoamyl alcohol [1-1 (24:1)] and the same volume of chloroform : isoamyl alcohol (24:1). For precipitation, the sample was added to a same volume of 100% iced isopropanol and incubated at -20°C for 2 h. Subsequently, the microtubes were centrifuged at 14,549 ×*g* for 30 min, and the sediments were washed with 500 µl of 70% ethanol, dried at room temperature, and resuspended in 30 µl of TE.

For the commercial kit PowerMax™ Soil DNA Isolation (MoBio Laboratories, Inc), two hundred and fifty milligrams of soil was added to the PowerBead tube, and DNA extraction and purification was performed according to the protocol provided by the manufacturer.

Yield and purity of the DNA

All samples were stored at -20°C, and the amount and quality of DNA obtained were measured using a spectrophotometer (NanoDrop ND-1000 spectrophotometer, Thermo Fisher Scientific). To estimate the purity of the extracted nucleic acids, the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}) was used. The A_{260}/A_{280} values between 1.7 and 2.0 indicate DNA samples with good quality. Values below this range indicate contamination with proteins, and values above this range indicate the presence of RNA or polysaccharides.

To DNA extracted from soil, the ratio of absorbance at 260 to 230 nm (A_{260}/A_{230}) was evaluated. The A_{260}/A_{230} values can help evaluate the level of salt and organic compounds, like humic acids carryover in the purified DNA. The A_{260}/A_{230} ratio should be greater than 1.5, ideally close to 1.8 (Moore et al., 2004).

PCR of the 16S rDNA region

PCR was performed in a 20 µl volume containing 1 U of Taq DNA polymerase (Fermentas), 1x reaction buffer, 200 µM of each dNTP, 3.0 mM $MgCl_2$, 0.5 mM of primers 27f (Suzuki and Giovannoni, 1996) and 1512r (Kane et al., 1993) and 10 ng of DNA. Blank and positive controls were included alongside each set of PCR reactions. Amplifications were performed according to the following parameters: 5 min initial denaturation at 94°C, 30 cycles of 94°C for

Table 2. Cell lysis step employed in the DNA extraction protocols.

Protocols	Lysis step	Reference
	Bacteria DNA extraction	
Detergent + thermal incubation method	- 800 µl of saline solution-EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0). SDS was added to a final concentration of 2%, followed by incubation at 60°C for 10 min.	Marmur (1961)
Glass beads method	- 400 µl of STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and centrifuged at 14,549 ×*g* for 5 min. The supernatant was discarded, and the pellets were resuspended in 200 µl of TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)]. To this mixture, 50 mg of glass beads (150–212 µm in diameter) and 100 µl of saturated phenol in Tris-HCl (pH 8.0) were added, and the mixture was vortexed for 60 s.	Cheng and Jiang (2006)
Detergent + enzymatic + salt + thermal incubation method	-570 µl of TE, SDS (final concentration, 0.5%), and proteinase K (final concentration, 100 mg mL^{-1}), and incubated at 37°C for 1 h. To this mixture, 100 ml of 0.8 M NaCl and 80 µl of CTAB/NaCl (10% CTAB in 0.7 M NaCl) were added, and the microtubes were incubated for 10 min at 65°C.	Wilson (1997)
Detergent + salt + thermal incubation method	- 600 µl of extraction buffer [200 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 25 mM NaCl, 1% SDS] and incubated at 65°C for 30 min.	Harju et al. (2004)
	Direct extraction of total DNA from soil	
Glass beads method	- 1 ml of extraction buffer [50 mM Tris-HCl (pH 7.6), 50 mM EDTA, 50 mM NaCl, 5% SDS], 0.4 g of glass beads (150–212 µM in diameter), and 1 µl of 1 M ditiotreitol, and vortexed for 3 min. The samples were incubated at 65°C for 30 min and centrifuged at 14,549 ×*g* for 10 min. The supernatant was transferred to a new microtube.	Costa et al. (2004)
Enzymatic method	- 1 ml of extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 100 mM NaH_2PO_4, 1.5 M NaCl, 1% CTAB, pH 8.0) and proteinase K (final concentration, 0.1 mg mL^{-1}). The samples were incubated by shaking at 250 rpm for 30 min. SDS was added to a final concentration of 3%, and the samples were incubated at 65°C for 2 h. The samples were incubated at 65°C for 30 min and centrifuged at 14,549 ×*g* for 10 min. The supernatant was transferred to a new microtube.	Hang et al. (2006)
Glass beads + PEG8000 method	- 1 ml of extraction buffer [0.1 M Tris-HCl (pH 8.0), 0.1 M EDTA, NaH_2PO_4, 1.5 M NaCl, 1% CTAB)] and 0.4 g of glass beads (150–212 µM in diameter). SDS was added to a final concentration of 5%. The samples were incubated at 65°C for 1 h and centrifuged at 14,549 ×*g* for 10 min. The supernatant was transferred to a new microtube, and the same volume of iced isopropanol was added, followed by incubation at room temperature for 15 min. The samples were centrifuged at 14,549 ×*g* for 10 min, and the pellets were resuspended in 80 µl of TE, 1 mL of NaCl, and PEG 8000 (final concentration, 1.6 M). These were again incubated for 2 h at room temperature and centrifuged at 14,549 ×*g* for 10 min. The pellets were resuspended in 400 µl TE and $NH_4C_2H_3O_2$ (final concentration, 2.5 M) and incubated on ice for 5 min. Subsequently, the samples were centrifuged at 14,549 ×*g* for 20 min, and the supernatant was transferred to a new microtube.	Yeates et al. (1998)

Table 3. Estimated amount (ng μl^{-1}) and purity (A_{260}/A_{280} ratio) of DNA extracted from gram-positive and gram-negative bacteria isolated from different sources.

Isolates[1] / Protocols	DNA yield (ng μl^{-1})									
	30A	**40B**	**BP11**	**BN5**	**BT108**	**BP1**	**SA**	**MecA**	**BN1**	**Average**
Detergent + thermal incubation method	1019 bc	1149^{c}	3517	506^{c}	263^{b}	283^{b}	199^{b}	190^{c}	184^{c}	812
Glass beads method	1924 a	800^{cd}	5270	1437^{ab}	125^{b}	101^{b}	135^{b}	226^{c}	1508^{a}	1281
Detergent + enzymatic + salt + thermal incubation method	1353 abc	3626^{a}	299	107^{c}	69^{b}	321^{b}	25^{b}	24^{c}	29^{c}	650
Detergent + salt + thermal incubation method	1614 ab	298^{d}	5039	813^{bc}	596^{b}	534^{b}	1339^{a}	1754^{a}	550^{b}	1393
KitCE*	839 c	2003^{b}	3170	1908^{a}	1618^{a}	1717^{a}	1041^{a}	575^{b}	465^{b}	1482
Protocols	A_{260}/A_{280} ratio									
Detergent + thermal incubation method	2.29	2.10	2.05	2.00	1.95	2.06	1.95	1.98	2.01	2.04
Glass beads method	2.04	2.01	1.99	2.03	2.02	2.00	2.04	2.03	2.06	2.02
Detergent + enzymatic + salt + thermal incubation method	2.01	2.00	1.86	1.73	2.09	1.13	1.78	1.67	1.69	1.79
Detergent + salt + thermal incubation method	2.00	1.93	1.71	2.10	2.05	2.06	1.92	1.87	2.02	1.97
KitCE	1.60	1.96	1.61	1.51	1.38	1.56	1.64	1.58	1.35	1.72

*KitCE: QuickExtract™ Bacterial DNA Extraction Kit (Epicentre). [1]Bacterial isolates: 30A = *Vibrio cholera*; 40B = *V. cholerae*; BP11 = *Pseudomonas* sp.; BN5 = *Bacillus* sp.; BT108 = *Bacillus* sp.; BP1 = *Bacillus* sp.; SA = *Staphylococcus aureus*; MecA = *S. aureus*; BN1= *S. epidermidis*. The results shown represent the average of triplicates. The averages followed by the same letter in each column do not differ by Tukey's test at 5% probability.

60 s, 58°C for 60 s, 72°C for 60 s, followed by a final elongation at 72°C for 10 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel plus SYBR Safe DNA Gel Stain (Invitrogen), and the gel was visualized under UV light at 254 nm.

Statistical analysis

All the experiments were carried out in triplicates. Data were subjected to analysis of variance, and when significant, Tukey's test ($P < 0.05$) was used. The statistical program SAEG (SAEG 2009) was used for analysis.

RESULTS AND DISCUSSION

Choice of a single protocol for Gram positive and negative bacteria DNA extraction

Gram positive and negative bacteria isolated from soil samples, mussels, oysters and milk were subjected to five DNA extraction protocols, including the QuickExtract™ Bacterial DNA Extraction Kit (KitCE), with subsequent qualitative-quantitative assessment of the extracted DNA. Although total DNA was successfully extracted from all bacteria with all protocols used, differences were observed in the efficiency of extraction processes considering the different bacterial groups (Table 3). Among the Gram negative bacteria, the KitCE yielded the highest quantity of DNA, 1908, 1618 and 1717 ng μl^{-1} DNA for *Bacillus* isolates. For *Vibrio* sp., the best method for one isolate was detergent + enzymatic + thermal incubation, for another it did not have difference between evaluated methods. For *Pseudomonas* sp. isolates, glass beads and detergent + salt + thermal incubation methods yielded the highest quantity of DNA, 5270 and 5039 ng μl^{-1} DNA, respectively. Among the Gram positive bacteria, detergent + salt + thermal incubation method yielded the highest

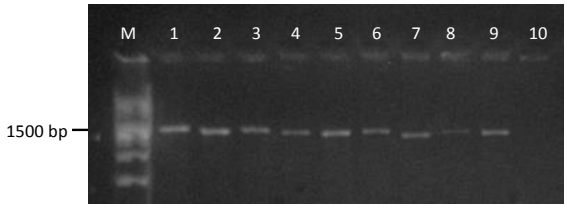

Figure 1. Electrophoresis of amplification products of the rDNA 16S region of the DNA extracted using the detergent + salt + thermal incubation method based on Harju et al. (2004) on a 1.5% agarose gel. M: Molecular weight marker 1 Kb (Amresco) 1: SA = *Staphylococcus aureus*; 2: MecA = *S. aureus*; 3: BT108 = *Bacillus* sp.; 4: BP1 = *Bacillus* sp.; 5: BN1 = *Staphylococcus epidermidis*; 6: 30A = *Vibrio cholerae*; 7: 40B = *Vibrio cholerae*; 8: BP11 = *Pseudomonas* sp.; 9: BN5 = *Bacillus* sp.; 10: Blank.

quantity of DNA for *Staphylococcus aureus* isolates, 1339 and 1754 ng μl^{-1} DNA, respectively. For *Staphylococcus epidermidis,* the method that yielded the highest quantity of DNA was the glass beads with 1508 ng μl^{-1} DNA. On average, the best results was with KitCE, detergent + salt + thermal incubation method and glass beads method that yielded 1482, 1393, and 1281 ng μl^{-1} DNA, respectively.

The ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}) was used as an estimate of nucleic acid purity. On average, detergent + salt + thermal incubation, detergent + enzymatic + salt + thermal incubation method and KitCE yielded better quality DNA (A_{260}/A_{280} between 1.7 and 2.0) with ratios of 1.97, 1.79, and 1.72, respectively (Table 3).

The detergent + salt + thermal incubation method based on Harju et al. (2004) was considered the most promising because it produced satisfactory yields of DNA, with adequate quality for all isolates studied, especially *S. aureus,* without the need to use enzymes and glass beads which can make the extraction process more expensive.

The quality of extracted DNA using the detergent + salt + thermal incubation method, based on Harju et al. (2004) was confirmed by PCR amplification using universal bacterial primers, and all samples were amplified generating a fragment of approximately 1500 bp (Figure 1).

Various protocols have been described for the extraction of DNA from specific groups of microorganisms; however, the efficiency of these protocols varies among different groups (Baratto and Megiolaro, 2012; Chapaval et al., 2008; Ligozzi and Fontana, 2003; Rivera et al., 2003; Wilson, 1997). This variation is mainly due to the inherent characteristics of the different bacterial groups and the structure of their cell walls, which reflects the efficiency of lysis. The presence of capsular polysaccharide makes it difficult to separate DNA, and the association of DNA with proteins influences its purification (Marmur, 1961; Navarre and Schneewind, 1999).

Choice of a protocol for direct extraction of DNA from soil

Direct DNA extraction from soil has three basic objectives: lysis of representative microorganisms within the sample, obtaining intact DNA with high molecular weight, and removal of inhibitors from the extracted DNA for subsequent molecular manipulations, such as for PCR amplification.

Four protocols, including the commercial kit PowerMax™ Soil DNA Isolation (KitCMB) were used for the direct extraction of DNA from different soil samples. The highest yields were observed for DNA samples extracted using enzymatic and glass beads method, on average, 258.04 and 233.39 µg g^{-1} of soil, respectively (Table 4). Samples extracted using glass beads + PEG8000 method and KitCMB exhibited the lowest yield of DNA of approximately 7.81 and 6.29 µg g^{-1} of soil, respectively (Table 4).

Although samples extracted using enzymatic and glass beads method gave higher yields of DNA, they resulted in the worst A_{260}/A_{280} ratio, on average, 1.38 and 1.32, respectively, suggesting contamination with proteins. The glass beads + PEG8000 method resulted in a low A260/A280 ratio too. A better A_{260}/A_{280} ratio, on average 1.87, was observed in the samples extracted using KitCMB (Table 4).

All samples showed low value of A_{260}/A_{230} ratio indicating high humic acid (Table 4). The ratio of absorbance at 260 and 230 nm (A_{260}/A_{280}) is used as a secondary measure of nucleic acid purity (Boehm et al., 2009; Lim et al., 2009; Ning et al., 2009; Wilfinger et al., 2006).

To confirm the quality of the extracted DNA samples and the impact of the presence of contaminants on the PCR samples, the samples were subjected to PCR amplification using universal primers for bacteria. None of the samples extracted using enzymatic and glass beads methods were amplified. Two samples extracted using glass beads + PEG8000 method and all samples extracted using KitCMB were amplified, generating a fragment of approximately 1500 bp (Figure 2).

The enzymatic and glass beads protocols produced the highest yields of DNA for all soils; however, these extracted samples were not sufficiently pure for PCR amplification using universal primers for bacteria. Despite the lower yield of DNA, KitCMB protocol gave the best quality of DNA, enabling its amplification by PCR.

In non-amplified samples, it was observed that despite producing amounts of DNA suitable for PCR amplification (several dilutions were tested), the purity of DNA was compromised, as evidenced by the A_{260}/A_{280} ratios. It is likely not all contaminants that adhered to the DNA have been removed, which may have led to the inhibition of amplification by Taq DNA polymerase (Roh et al. 2006).

Table 4. Estimated amount (µg µg^{-1}) and purity (A_{260}/A_{280} and A_{260}/A_{230} ratio) of total DNA extracted from soil samples collected from different cocoa planting systems

	DNA yield (µg g^{-1})				
Protocols	**[1]Conv.1**	**ASF**	**Conv.2**	**Org.**	Average
Glass beads method	384.38^{a}	193.47^{b}	243.65^{b}	112.05^{b}	233.39
Enzymatic method	99.17^{b}	268.74^{a}	287.74^{a}	376.52^{a}	258.04
Glass beads + PEG8000 method	29.94^{c}	0.54^{c}	0.31^{c}	0.46^{c}	7.81
KitCMB*	0.33^{d}	12.07^{c}	6.35^{c}	6.40^{c}	6.29
	A_{280}/A_{260} ratio				
Glass beads method	1.31	1.37	1.25	1.36	1.32
Enzymatic method	1.34	1.38	1.42	1.37	1.38
Glass beads + PEG8000 method	1.41	1.38	1.27	1.21	1.32
KitCMB	1.69	1.97	1.85	1.98	1.87
	A_{260}/A_{230} ratio				
Glass beads method	0.79	0.73	0.85	0.78	0.79
Enzymatic method	0.79	0.73	0.82	0.70	0.76
Glass beads + PEG8000 method	0.66	0.47	0.71	0.35	0.55
KitCMB	1.09	0.42	1.11	0.26	0.72

*KitCMB: - PowerMax™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc). [1]Soils: Conv. 1. - Conventional system, Farm 1; ASF. - Agroforesty system, Farm 1; Conv.2 - Conventional system, Farm 2; Org. - Organic Farming, Farm 2. The results shown represent the average of triplicates. The averages followed by the same letter in each column do not differ by Tukey's test at 5% probability.

Figure 2. Electrophoresis of amplification products of the 16S rDNA region of the DNA extracted directly from soil on 1.5% agarose gel. Lanes 1-4: Glass beads method (Costa et al., 2004); Lanes 5-8: Enzymatic method (Hang et al., 2006); Lanes 9-12: Glass beads + PEG8000 method (Yeates et al., 1998); Lanes: 13-16: Commercial kit - PowerMax ™ Soil DNA Isolation (MO BIO Laboratories, Inc.); 17: Positive control; 18: Blank. Soils: 1, 5, 9 and 13: Conv. 1 - Conventional system, Farm 1; 2, 6, 10 and 14: Conv. 2 - Conventional system, Farm 2; 3, 7, 11 and 15: ASF. - Agroforesty system, farm 1; 4, 8, 12 and 16: Org - Organic Farming, Farm 2;. M: 100 bp DNA ladder (Fermentas).

Regarding PCR amplification, the amount of total DNA extracted is less limiting than its quality. Thus, KitCMB protocol offered more promising results, enabling PCR amplification. Soil contaminants, particularly humic substances, might preclude PCR amplification, interfere with DNA hybridization, and increase the background in microarray hybridization (Braida et al., 2003; Lemarchand et al., 2005; Niemi et al., 2001; Zhou et al., 1996).

Some of the most popular DNA purification methods involve removal of humic material through agarose gel electrophoresis, polyvinylpolypyrrolidone (PVPP), size exclusion chromatography or silica-based DNA binding (Berthelet et al., 1996; Miller et al., 1999; Miller, 2001). The commercial kits like KitCMB essentially rely on silica gel spin columns for purification of the DNA and it was effective in this case, providing the amplification of DNA of soil samples.

Conflict of Interests

The authors have not declared any conflict of interests.

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Trickle and micro sprinkler fertigation on soil microbial population in cocoa (*Theobroma cacao* L.)

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Cocoa, the 'Food of Gods' is one of the important plantation crops consumed worldwide and around 40 - 50 million people depend on cocoa for their livelihood. An experiment was conducted during 2010 and 2011 to investigate the impact of N, P and K fertilizers through fertigation on soil microbial population of cocoa at Tamil Nadu Agricultural University, India. The study was laid out in randomized block design with 13 treatment combinations. The study shows that, fertigation with 125% recommended fertilizer dose as water soluble fertilizer through fertigation by micro sprinkler irrigation (T_{10}) had the highest soil bacterial population (60.10×10^{-6} CFU g^{-1}), fungal population (16.61×10^{-4} CFU g^{-1}) and actinomycetes population (8.07×10^{-3} CFU g^{-1}). The same treatment recorded higher yield characters viz., beans per pod (47.81), bean length (2.47 cm), bean girth (3.57 cm), single bean wet weight (3.15 g), single bean dry weight (1.31 g), dry bean weight per pod (62.23 g), dry bean yield per tree (3273.63 g).

Key words: Fertigation, drip irrigation, micro sprinkler irrigation, bacteria, fungi, actinomycetes.

INTRODUCTION

Cocoa is cultivated mainly in Africa, Asia, Central America and South America and major cocoa producing countries are Ivory Coast, Ghana, Indonesia, Nigeria, Cameroon, Brazil, Ecuador and Malaysia. The annual production is around 4.8 million tonnes with an estimated value of $ 8.3 billion (World Cocoa Foundation, 2012). Ivory Coast leads in production occupying 38% of total world cocoa production followed by Ghana (21%), Indonesia (13%), Nigeria (5%), Cameroon (5%), Brazil (4%), Ecuador (3%), Malaysia (1%) and others (10%). West Africa alone contributes nearly 70% of the world cocoa production (World Cocoa Foundation, 2011).

India offers considerable scope for cocoa cultivation, production and further development. Though cocoa has been known as the beverage crop even before tea and coffee, it is a relatively new crop to India. Cocoa is inter-cropped in coconut and arecanut and is a good companion to these crops. Cocoa readily responds to applied fertilizers to meet its nutrient requirements (Armando et al., 2001; Owusu et al., 2010).

Fertigation ensures 40% higher fertilizer use efficiency than the surface irrigation, besides providing scope for making soil amendments and even biological methods of plant protection. In the fertigation method, fertilizers can be applied throughout the crop growing season in a phased manner, in various split doses, in any desired

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concentration. This is in contrast to the conventional practice where larger amounts of fertilizers are placed on the soil at the beginning of the season in one or very few split doses (Dangler and Locascio, 1990; Kadam and Karthikeyan, 2006). Many countries have recognized fertigation to usher a second Green Revolution for enhancing productivity (Beard, 2000).

Through fertigation, nutrients are added to the soil in adequate doses and intervals through which a qualitative improvement of produce can also be attained. Production of quality beans in cocoa (single bean weight of more than 2 g) will enable the farmers to earn more income. Being relatively demanding in terms of soil fertility, cocoa requires frequent doses of fertilizers coupled with soil moisture to utilize the nutrients more effectively (Noordiana et al., 2007; Soumya et al., 2012). Drip and micro sprinkler irrigation are innovative approaches to precisely meet the water requirements of many crops (Selvaraj et al., 1997; Salo et al., 2000; Gupta et al., 2012).

In Tamil Nadu, a dose of 100:40:140 g NPK tree^{-1} year^{-1} is generally recommended for cocoa (Anonymous, 2004). The tap roots (1.2 m deep) in cocoa act as physical support and only lateral roots (20 - 30 cm) absorb the nutrients. As cocoa is very sensitive to moisture stress and water logging, irrigation should be at its optimum level for better growth.

The fertility of soil depends not only on its chemical composition but also on the qualitative and quantitative nature of microorganisms inhabiting it. Soil microorganisms in the rhizosphere influence the plant growth in many ways. Most of them play a role in the carbon, nitrogen, phosphorus and sulphur cycles and availability of certain trace elements like manganese, copper and iron in the soil. Some soil microbes act as antagonists for soil borne pathogens, thus aiding normal growth of plants. Besides, the soil microbes influence the permeability, water holding capacity and tilth of the soil (Balasubramanian, 2007; Govindan and Nair, 2011).

The present study was aimed to evaluate the fertigation system involving drip and sprinkler irrigation methods; various levels of fertilizers with a comparison of the farmers practice (surface irrigation + soil application of RDF) on soil microbial population in the rhizosphere of a cocoa plantation.

MATERIALS AND METHODS

Six year old cocoa trees were selected for the study. In a coconut plantation of 30 years old, the cocoa plants were intercropped with a spacing of 3 x 3 m. In case of drip irrigation, two emitters were installed with a discharging rate of 8 lph (litres per hour). Two micro sprinklers transmitting @ 60 lph micro sprinkler^{-1} were installed to cover the entire basin. The micro sprinkler type is half sub circle with a height of 30 cm and it has sprinkling capacity of 60 cm area (Figure 1). The venturi was used for mixing of fertilizer with water. The study was laid out in randomized block design with 13 treatment combinations replicated thrice (Table 1).

An annual application of 100 g N, 40 g P_2O_5 and 140 g K_2O through the mode of surface irrigation (T_1) is recommended for annual basis per tree in two splits (1st dose in 1st week of April and 2nd dose in 1st week of September). Surface irrigation was carried out once in seven day's interval. The fertilizers were applied through drip and micro sprinkler irrigation system (fertigation) at weekly intervals for drip and micro sprinkler treatments (T_2 to T_{13}) and the irrigation was carried out once in a day (20 L tree^{-1} day^{-1}). The rhizosphere soil sample from cocoa was analysed for bacteria, fungi and actinomycetes.

Serial dilution of soil sample

Ten grams of rhizosphere soil sample was transferred to 90 ml of sterile distilled water to get 10^{-1} dilution. After thoroughly mixing it, 1 ml of this dilution was transferred to 9 ml water blank to get 10^{-2} dilution. Likewise, sample was diluted serially with 9 ml water blanks till appropriate dilution was obtained (Srinivas et al., 2011).

Bacteria

The total bacterial population was enumerated by planting 1 ml of 10^{-6} dilution in sterile Petri plates using soil extract medium. The bacterial colonies appearing on the plates after 48 h of incubation at 30°C were counted and expressed per g of dry weight of the soil.

Fungi

For the enumeration of fungal population, 1 ml of 10^{-4} dilution of the soil sample was plated in sterile plate with potato dextrose agar medium. After 72 h of incubation, the fungal colonies were counted and expressed per g of dry weight of soil.

Actinomycetes

The total actinomycetes population was enumerated by plating 1 ml of 10^{-3} dilution with starch casein nitrate agar medium. The powdery colonies of actinomycetes appearing after 5 days were counted and expressed per gram of dry weight of soil.

RESULTS AND DISCUSSION

Data recorded on the soil bacterial populations during first and second season in 2010 and 2011 showed significant effect of the treatments applied. The highest bacterial population was registered by fertigation with 100% RDF as WSF using micro sprinkler (T_9) of 63.06 x 10^{-6} cfu g^{-1} soil and T_{11} (62.28 x 10^{-6} cfu g^{-1} soil) during first season in 2010 and 2011. The treatment T_{10} recorded highest bacterial population (66.76 and 62.40 x 10^{-6} cfu g^{-1} soil) during second season in 2010 and 2011. The lowest bacterial population was recorded in control (45.30 and 41.58 x 10^{-6} cfu g^{-1} soil, 34.38 and 39.08 x 10^{-6} cfu g^{-1} soil) during first and second season in 2010 and 2011 respectively (Table 2). Data on pooled mean (2010 and 2011) showed that, the highest soil bacterial population of 60.10 × 10^{-6} CFU g^{-1} was registered by T_{10} (125 % RDF as WSF through fertigation by drip irrigation) which was on par with T_9 (59.90 × 10^{-6} CFU g^{-1}). The

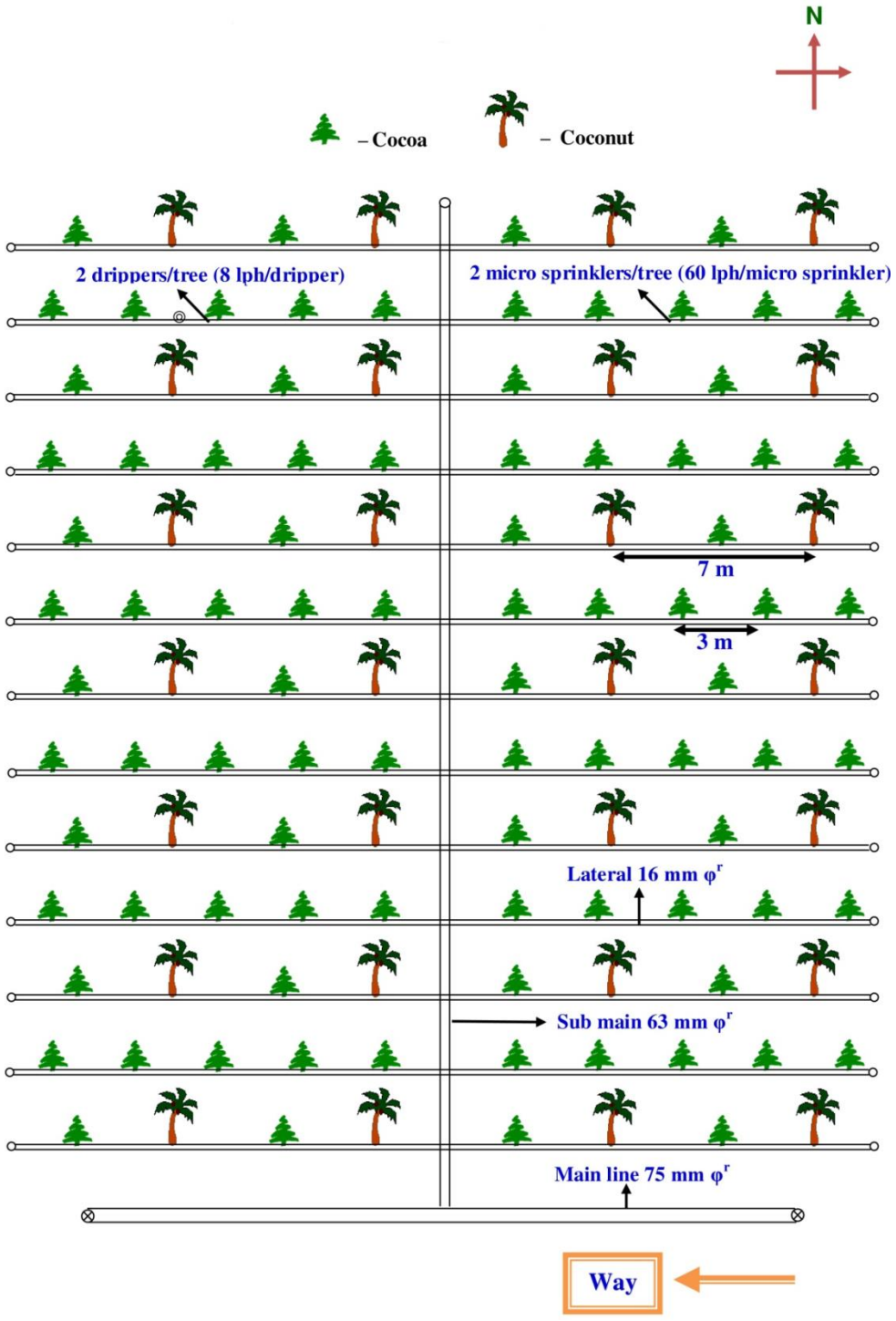

Figure 1. Lay out of drip and micro springer in cocoa.

lowest bacterial population was recorded in control (40.09 × 10^{-6} CFU g^{-1}) (Figure 2).

Significant difference was noticed among the treatments in relation to soil fungal population. The highest soil fungi population of 18.64 and 16.67 x 10^{-4} cfu g^{-1} soil was registered by T_{10} (125 % RDF as WSF through fertigation by micro sprinkler irrigation) during first season in 2010 and 2011 respectively. During second season in 2010 and 2011, the treatment T_9 registered highest soil fungi population of 18.72 and 17.08 x 10^{-4} cfu g^{-1} soil. The lowest fungal population was recorded in control (11.61 and 12.23 x 10^{-4} cfu g^{-1} soil, 10.52 and 11.76 x 10^{-4} cfu g^{-1}

Table 1. Treatment details of the experiment.

Treatment number	Dosage	Method of application / irrigation
T_1	100% RDF	Surface application + flood irrigation (control)
T_2	75% RDF as WSF	Drip
T_3	100% RDF as WSF	Drip
T_4	125% RDF as WSF	Drip
T_5	75% RDF as straight fertilizers	Drip
T_6	100% RDF as straight fertilizers	Drip
T_7	125% RDF as straight fertilizers	Drip
T_8	75% RDF as WSF	Micro sprinkler
T_9	100% RDF as WSF	Micro sprinkler
T_{10}	125% RDF as WSF	Micro sprinkler
T_{11}	75% RDF as straight fertilizers	Micro sprinkler
T_{12}	100% RDF as straight fertilizers	Micro sprinkler
T_{13}	125% RDF as straight fertilizers	Micro sprinkler

RDF, Recommended dose of fertilizer; WSF, water soluble fertilizer.

Table 2. Effect of drip and micro sprinkler fertigation on soil bacterial population ($\times 10^{-6}$ CFU g^{-1}) at various seasons.

Treatments	2010			2011			(Pooled analysis for the year 2010 and 2011)
	1st season	2nd season	Mean	1st season	2nd season	Mean	
T_1	45.30	41.58	43.44	34.38	39.08	36.73	40.09
T_2	49.48	45.31	47.40	45.91	40.31	43.11	45.26
T_3	47.92	49.69	48.81	45.64	44.94	45.29	47.05
T_4	54.37	51.27	52.82	43.07	48.62	45.85	49.34
T_5	48.11	40.38	44.25	40.68	39.19	39.94	42.10
T_6	56.68	49.14	52.91	47.17	42.74	44.96	48.94
T_7	45.76	55.02	50.39	49.35	41.68	45.52	47.96
T_8	57.43	52.19	54.81	55.29	52.27	53.78	54.30
T_9	63.06	56.00	59.53	61.00	59.53	60.27	59.90
T_{10}	52.45	66.76	59.61	58.76	62.40	60.58	60.10
T_{11}	56.28	52.13	54.21	62.28	56.96	59.62	56.92
T_{12}	50.16	56.94	53.55	59.46	50.72	55.09	54.32
T_{13}	55.12	52.55	53.84	60.11	49.85	54.98	54.41
SEd	0.976	1.029		1.140	1.041		0.998
CD (0.05)	2.014	2.123		2.353	2.148		2.059
CD (0.01)	2.745	2.893		3.206	2.927		2.806

soil) during first and second season in 2010 and 2011 respectively. Pooled mean data showed that the highest fungal population (Table 3) was registered by T_{13} (16.61×10^{-4} CFU g^{-1}), followed by T_9 (16.38×10^{-4} CFU g^{-1}). The lowest population was recorded in T_1 (11.53×10^{-4} CFU g^{-1}) (Figure 3).

Soil actinomycetes were significantly influenced by the different treatments during both the years. During first and second season in 2010, the actinomycetes colonies were found to be at a higher level (8.42 and 8.13 x 10^{-3} cfu g^{-1} soil) when the plants were fertigated with micro sprinklers with 100 % RDF as WSF (T_9). The treatment T_{10} recorded the highest actinomycetes population of 9.10 and 8.65 x 10^{-3} cfu g^{-1} soil during first and second season in 2011. The lowest population (3.07 and 3.98 x 10^{-3} cfu g^{-1} soil, 4.04 and 3.16 x 10^{-3} cfu g^{-1} soil) was recorded in control during first and second season in 2010 and 2011 respectively (Table 4). Pooled mean values showed that T_{10} recorded the highest soil actinomycetes population (8.07×10^{-3} CFU g^{-1}). The trees which received 100 % RDF as soil application recorded lowest soil actinomycetes population (3.57×10^{-3} CFU g^{-1})

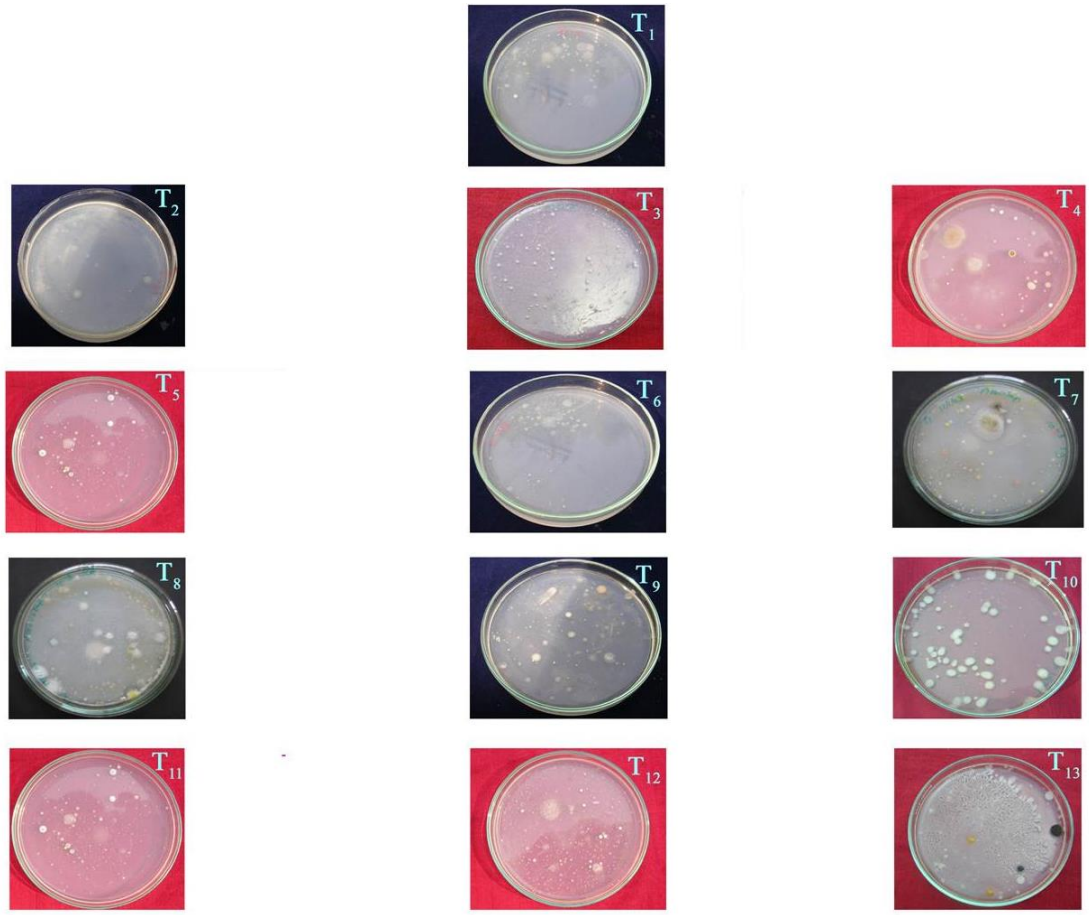

Figure 2. Effect of drip and micro sprinkler fertigation on bacterial population.

Table 3. Effect of drip and micro sprinkler fertigation on soil fungal population (× 10^{-4} CFU g^{-1}) at various seasons.

Treatments	2010			2011			(Pooled analysis for the year 2010 and 2011)
	1st season	2nd season	Mean	1st season	2nd season	Mean	
T_1	11.61	12.23	11.92	10.52	11.76	11.14	11.53
T_2	15.05	14.81	14.93	15.66	14.28	14.97	14.95
T_3	12.32	15.39	13.86	12.79	13.37	13.08	13.47
T_4	16.84	15.74	16.29	14.31	12.51	13.41	14.85
T_5	12.19	13.52	12.86	11.76	13.00	12.38	12.62
T_6	14.00	16.36	15.18	10.93	13.68	12.31	13.75
T_7	15.63	14.03	14.83	14.07	11.78	12.93	13.88
T_8	16.86	17.68	17.27	13.39	14.33	13.86	15.57
T_9	15.27	18.72	17.00	14.44	17.08	15.76	16.38
T_{10}	17.28	16.96	17.12	15.30	16.89	16.10	16.61
T_{11}	16.53	14.22	15.38	15.29	14.32	14.81	15.10
T_{12}	16.06	16.41	16.24	14.52	15.14	14.83	15.54
T_{13}	18.64	12.39	15.52	16.67	14.46	15.57	15.55
SEd	0.311	0.319		0.277	0.271		0.269
CD (0.05)	0.642	0.659		0.571	0.559		0.554
CD (0.01)	0.875	0.898		0.779	0.762		0.756

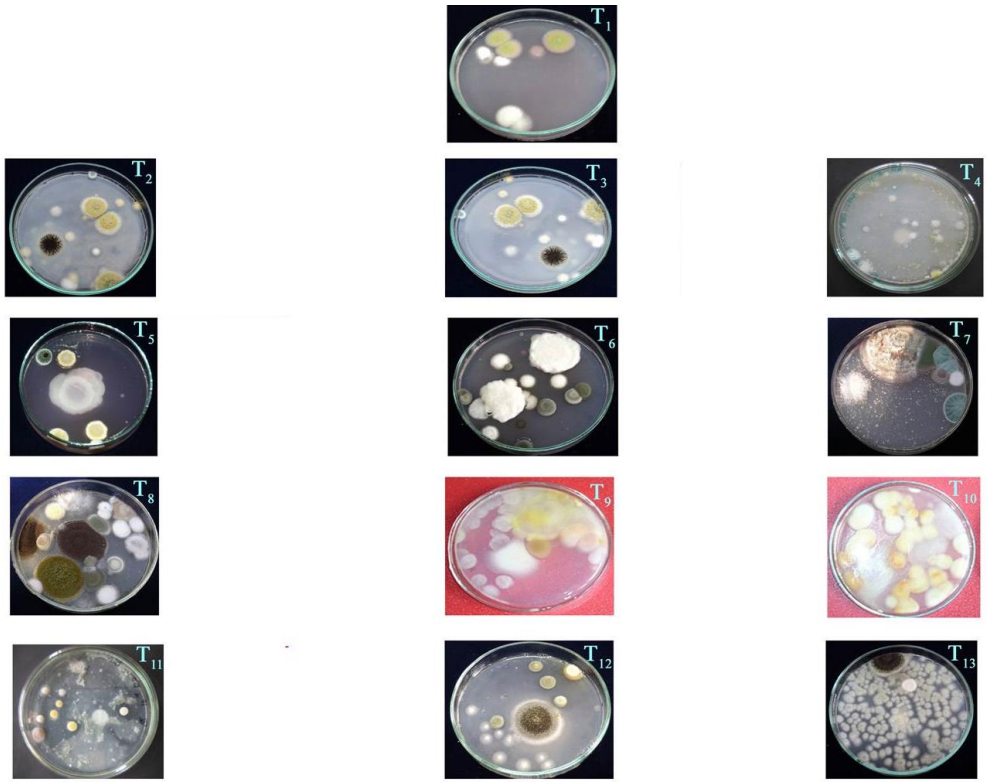

Figure 3. Effect of drip and micro sprinkler fertigation on fungal population.

Table 4. Effect of drip and micro sprinkler fertigation on soil actinomycetes population (× 10^{-3} CFU g^{-1}) at various seasons.

Treatments	2010			2011			(Pooled analysis for the year 2010 and 2011)
	1st season	2nd season	Mean	1st season	2nd season	Mean	
T_1	3.07	3.98	3.53	4.04	3.16	3.60	3.57
T_2	3.51	4.82	4.17	6.28	7.02	6.65	5.41
T_3	5.50	3.99	4.75	6.49	5.38	5.94	5.35
T_4	4.50	6.12	5.31	8.16	6.14	7.15	6.23
T_5	6.34	4.50	5.42	5.51	4.82	5.17	5.30
T_6	6.89	4.65	5.77	6.69	5.93	6.31	6.04
T_7	4.97	6.44	5.71	8.34	7.50	7.92	6.82
T_8	7.18	4.77	5.98	7.85	5.47	6.66	6.32
T_9	8.42	8.13	8.28	7.09	8.22	7.66	7.97
T_{10}	7.58	6.92	7.25	9.10	8.65	8.88	8.07
T_{11}	6.84	6.38	6.61	8.56	6.07	7.32	6.97
T_{12}	6.06	7.15	6.61	6.16	5.94	6.05	6.33
T_{13}	7.10	7.67	7.39	7.49	6.00	6.75	7.07
SEd	0.166	0.155		0.167	0.160		0.146
CD (0.05)	0.343	0.321		0.344	0.330		0.301
CD (0.01)	0.468	0.437		0.469	0.450		0.409

(Figure 4).

In the present study, micro sprinkler irrigation had more significant influence on soil microbial population than drip irrigation. In micro sprinkler irrigation, the leaf litter was decomposed quickly by water sprinkled on leaf litter along the tree basin. The decomposed plant residue in

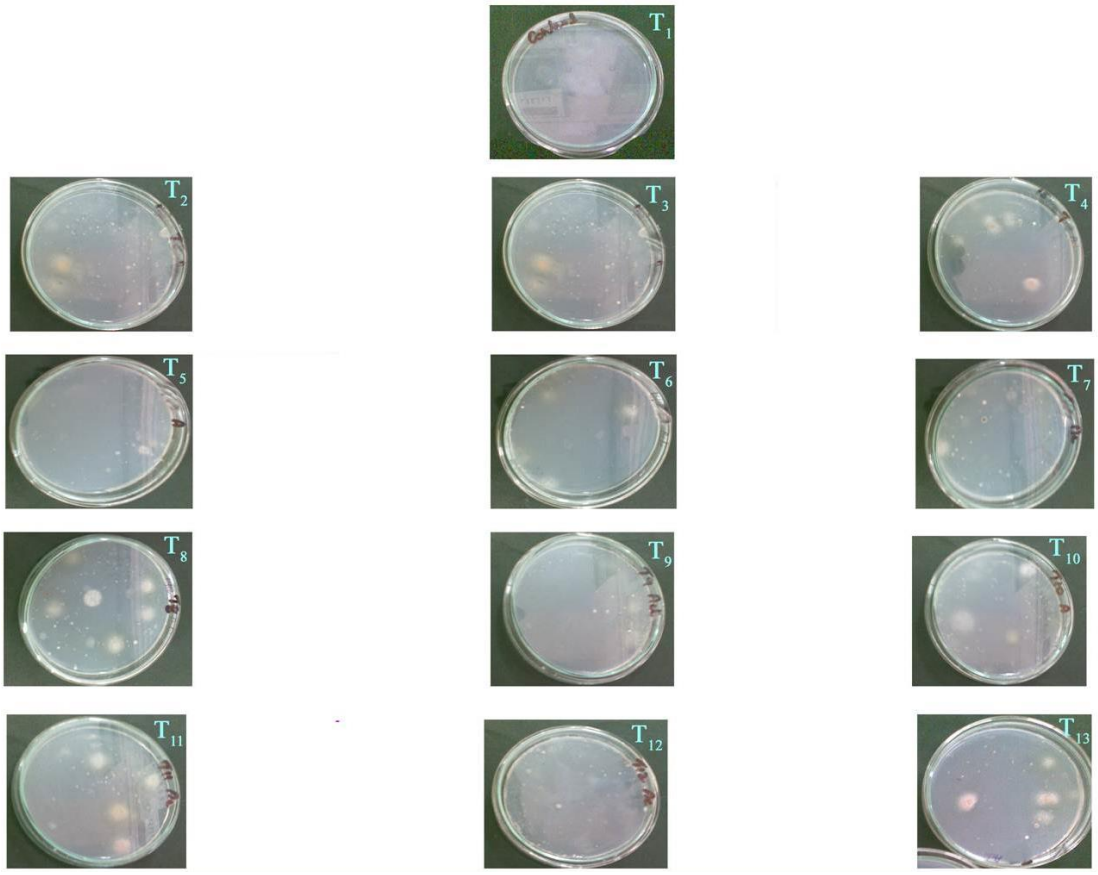

Figure 4. Effect of drip and micro sprinkler fertigation on actinomycetes population.

the tree basin would have been helpful for microbial growth (Hebbar et al., 2010; Shobana et al., 2012). In contrast, in the drip irrigated plots, water applied slowly directly to the soil rather not sprayed in the tree basin. These findings are supported by Shivanand (2003) in tomato and Nguyen (2003) who reported that high above ground biomass yield are obviously accompanied by an active root system, which releases an array of organic compounds into the rhizosphere. Plant roots release about 17% of the photosynthate captured, most of which is available to soil organisms. These compounds support the growth of the microbial community and result in dense population in micro sprinkler fertigation plot over the other systems of fertilization.

Conclusions

Fertigation studies on cocoa through micro sprinkler irrigation with a dose of 100 or 125% RDF as water soluble fertilizer (WSF) has shown to increase the soil bacterial population (60.10×10^{-6} CFU g^{-1}), fungal population (16.61×10^{-4} CFU g^{-1}) and actinomycetes population (8.07×10^{-3} CFU g^{-1}) respectively. It can be concluded that, application of 100 or 125% RDF as water soluble fertilizer (WSF) through micro sprinklers increases microbial growth, nutrient transformations inside the roots, degrade biomass and destroy xenobiotic contaminants (such as residual herbicides).

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Characterization of probiotic bacteria isolated from regional chicken feces

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The present study was aimed to isolate probiotic bacteria from poultry feces and to study their physiological and biochemical as well as probiotic properties. Analysis of morphological, physiological and biochemical properties confirmed that all the bacteria were Gram positive, endospore negative, catalase negative and non-motile those are the characteristics of typical probiotics. Sugar fermentation profiling of 16 important sugars ensured the presumptive identification of *Lactobacillus acidophilus*, *Lactobacillus brevis* and two *Bifidobacterium* species. All bacteria were resistant to artificial gastric juice environment at pH 2.2 and 6.6 but their resistance capacity decreased after 24 h of incubation at 37°C. These bacteria were found to multiply after 24 h of incubation at 0.3% of artificial bile salt, and to grow moderately even at 9% of NaCl. This study suggests that the isolated bacteria possess feasible physiological and biological properties to be good candidates for formulating probiotic mix for livestock and chicken.

Key words: Probiotics, chicken feces, livestock, *Lactobacillus*, *Bifidobacterium*.

INTRODUCTION

The digestive flora in avian species is frequently a complex mixture of microbial populations variously colonizing in gastrointestinal (GI) tract areas. Hundreds of diverse microorganisms are reported to recognize to subsist in the flora of the chicken's GI tract in which a few are responsible for providing nutritional benefits (Gong et al., 2002). Thus researchers put considerable attention to find out these host-friendly microorganisms and to use them directly in convenient ways. The latest inclination is the use of blend of these live bacteria with nutrients usually sugars in livestock to control undesirable intestinal pathogens, especially in view of the concern over the use of antibiotics in livestock feed. Probiotics are homo/hetero-geneous culture of live microbes that help a host to nourish nutritionally by improving the percentage of indigenous beneficial microbes in host's gut through

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competitive exclusion and antagonism (Fuller, 1989), by improving feed ingestion and digestion (Nahanshon et al., 1993) and by varying bacterial metabolism (Cole et al., 1987; Jin et al., 1997). Considering the significant contribution in healthy nutrition, probiotic lactic acid bacteria (LAB) especially species within the genera *Lactobacillus* and *Bifidobacterium* have been using frequently in functional food manufacturing (Holzapfel et al., 1998).

Analysis of previous studies concerning isolation of probiotic bacteria, especially LAB, showed that they can be found in dairy and meat products, sewage, plants, and in human and animal feces (Kandler and Weiss, 1986; Bayane et al., 2006). LAB isolated from chicken and poultry samples include *L. aviaries* (Fujisawa et al., 1984), *L. fermentum* sub sp. *cellobiosus* and *L. animalis* (Gusils et al., 1999) from gastrointestinal tracts of chicken, *L. gallinarum* and *L. Johnsonii* (Fujisawa et al., 1991), and *Lactobacillus casei* (Bayane et al., 2006) from chicken feces. These selected bacteria from chickens can be used as potential ingredients for chicken probiotic feed formulation intended to control salmonellosis and to improve poultry sanitation (Qin, et al., 1995; Gusils et al., 1999; Pascual, et al., 1999). The already carried out research in number of countries encouraged us to focus our aim of constituting a collection on LAB with the hope to formulate later a nutritionally effective chicken probiotic livestock feed.

MATERIALS AND METHODS

Sample collection

Chicken feces samples were collected from two local poultry farms located at Jessore and Satkhira districts of Bangladesh. Two samples were chosen from two different regional farms for maximum bacterial species variability. Feces samples were alienated from other trashes and stored at 4°C in sterile poly-bags discretely to protect from deterioration and contagion.

Isolation of LAB from sample

LAB were isolated from the samples using adapted GYP (Glucose Yeast Peptone) media at pH 6.8 according to the technique followed by Bayane et al. (2006). Five grams of sample were mixed with 100 ml of GYP broth medium to prepare suspension and was incubated anaerobically at 30°C for 24 h. Then 100 µl of the suspension was diluted up to ten logarithmic (10^{-10}) fold and spread onto GYP agar medium. The culture was incubated aerobically at 30°C for 24 h. LAB were finally purified by repetitive streaking on agar plate and by microscopic assessment.

Identification of bacterial isolates

Morphological, physiological and biochemical properties of isolated bacteria were analyzed by some common tests. Colony morphology (color, shape and size) were normally examined with open eyes, sometimes microscopic assessment was considered to separate colonies. Gram staining was carried out according to the protocol of Harley and Prescott (2002). For sugar fermentation test, bacterial culture was prepared in 10 ml MRS (De Man Rogosa and Sharpe) medium at 37°C, and further inoculation and incubation were carried out according to Erkus (2007). Motility-Indole-Lysine (MIL) partially broth medium was equipped and supplementary exemption was done according to Reller and Mirrett (1975) for motility test. Endospore test and catalase test were also executed for accuracy of categorization by Schaeffer and Fulton (1933) and Holt et al. (1994) methods correspondingly.

Analysis of probiotic properties

NaCl tolerance test was carried out using test tubes containing MRS broth furnished with special concentrations (1-10%) of NaCl, according to Hoque et al. (2010). Gastric juice tolerance capability was determined by a slight moderated procedure described by Graciela and Maria (2001) at pH 2.2 and pH 6.6. Phenol tolerance was performed in MRS broth with different concentration (0.1-0.4%) of crude phenol and 1% (v/v) of fresh overnight culture as described by Hoque et al. (2010). MRS broth medium with bile salt (0.05, 0.1, 0.3 and 0.6%) was utilized to determine the tolerance and growth rate of isolated bacteria. Agar plates were equipped by 0.5% (w/v) sodium salt of taurocholic acid to establish bile salt hydrolase activity test. To examine milk coagulation property, 1% (v/v) culture of isolated bacteria was inoculated into pure milk and incubated for 24 h.

RESULTS

Morphological, physiological and biochemical characterization

From the morphological, physiological and biochemical investigation, the isolated bacteria were identified as *Lactobacillus acidophilus, Lactobacillus brevis* and *Bifidocterium spp. L. acidophilus* and *Bifidocterium* spp. were isolated and identified from sample 1 whereas sample 2 was endowed with both *Lactobacillus brevis* and *Bifidocterium spp.* Among 16 sugars, all were fermented by *L. acidophilus* excluding sorbitol, mannitol, rhamnose while *L. brevis* did not ferment salicin, rhamnose and sorbitol. The sugar fermentation outline of *Bifidobacterium* spp. was also found positive apart from rhamnose and sorbitol.

All the four bacteria were gram positive and found non-motile during growth motivation down the inoculation line. Colony morphologies showed, very small circle shaped non-transparent colonies for *L. acidophilus* and small bar shaped non-transparent colonies for *L. brevis* (Saccaro et al., 2011). Triangular minute watery circle with white center colonies examined for *Bifidocterium* spp. were similar with the findings of expert group of Japanese association of fermented milks and fermented milk drinks. In light microscopic examination, deficient of endospores specify that all the isolates were non-endospore forming. Due to production of no gas during addition of H_2O_2, all bacterial species were claimed as catalase negative. The transformation of purple to yellow color of media was the indication of particular sugar fermentation performed by the isolated bacteria. It was examined that each bacterium had distinct carbohydrate fermentation model which has been presented in Table 1. Carbohydrate fermentations

Table 1. Carbohydrate fermentation profiles of isolated bacteria.

Sugar used	Lactose	Mannitol	Sucrose	Fructose	Salicin	Ribose	Cellubiose	Glucose	Maltose	Xylose	Rhamnose	L-Arabinose	D-Sorbitol	D-Mannose	Raffinose	Galactose
L. acidophilus	+	+/-	+	+/-	+	+	+	+	+	+	-	+	-	+	-	+
L. brevis	+	-	+	+	+	+	+	+	+	+	-	+	-	+	+	+
Bifidocterium spp.(1)	+	+	+	+/-	+	+	+	+	+	+	-	+	-	+	+	+
Bifidocterium spp. (2)	+	+	+	+/-	+	+	+	+	+	+	-	+	-	+	+	+

'+'indicates good fermented; '+/-' indicates moderately fermented; '-'indicates not fermented.

Table 2. NaCl tolerance test of isolated bacteria.

NaCl (%)	*L. acidophilus*	*L. brevis*	*Bifidocterium* spp. (1)	*Bifidocterium* spp. (2)
1	++	++	++	++
2	++	++	++	++
3	++	++	++	++
4	++	++	++	++
5	++	++	++	++
6	++	++	++	++
7	++	+	++	+
8	+	+	+	+
9	-	-	+	+
10	-	-	-	-

'+' indicates low level growth; '++' indicates normal growth; '-' indicates no growth.

found little incongruity which could be association of frequent environmental factors.

Characterizations for probiotic properties

Current work showed that isolated bacteria were good enough for growing at 1-7% of NaCl concentrations but at 8 and 9% of concentrations each bacterium showed moderate growth enlightened at Table 2. No expansion was found at 10% of NaCl concentration. Isolated bacteria had the competence to settle fit in mock gastric acid atmosphere at low pH (pH 2.2) and approving pH (pH 6.6) but their stamina were decreased after 24 h of incubation at 37°C. The uphill shapes in Figure 1a indicate tolerability of the bacteria at pH 2.2. All isolated species showed excellent proliferative ability at 0.1 and 0.2% of phenol and moderate ability at 0.3 and 0.4%. The lines in Figure 1b and c specify tolerability of the bacteria in 0.2 and 0.4% of crude phenol respectively. Data was expressed as average value of isolated bacteria at various concentrations after 12 and 24 h of incubation at 37ºC. The isolated bacteria were too competent to proliferate in the above mentioned concentrations of bile acid after 24 h of incubation at 37ºC. The optical density averages were diagramed in Figure 1d for symbolizing tolerance ability of the bacteria to synthetic bile salt at highest concentration (0.6%). The isolated bacteria were able to deconjugate 'taurine-conjugated' bile acid and to generate deoxycholic acid. The activity of isolated bacteria turns their colonies into intense rough white or impulsive halos signifying the bile salt hydrolase positive. Coagulation of milk was observed due to formation of lactic acid while isolated bacterial culture was supplemented with fresh skim milk. All the isolated bacteria were competent to clot milk and turned into curd which is one of the most important properties of probiotic bacteria.

DISCUSSION

To be an attractive probiotic enough to attract livestock industries, LAB should possess better biological activities as well as physicochemical attributes resistance to adverse conditions in digestive tracts, desiccation and conservation parameters (Bayane et al., 2006). The LAB used as starters play an essential role to inhibit the growth of food spoilage bacteria by producing lactic acid

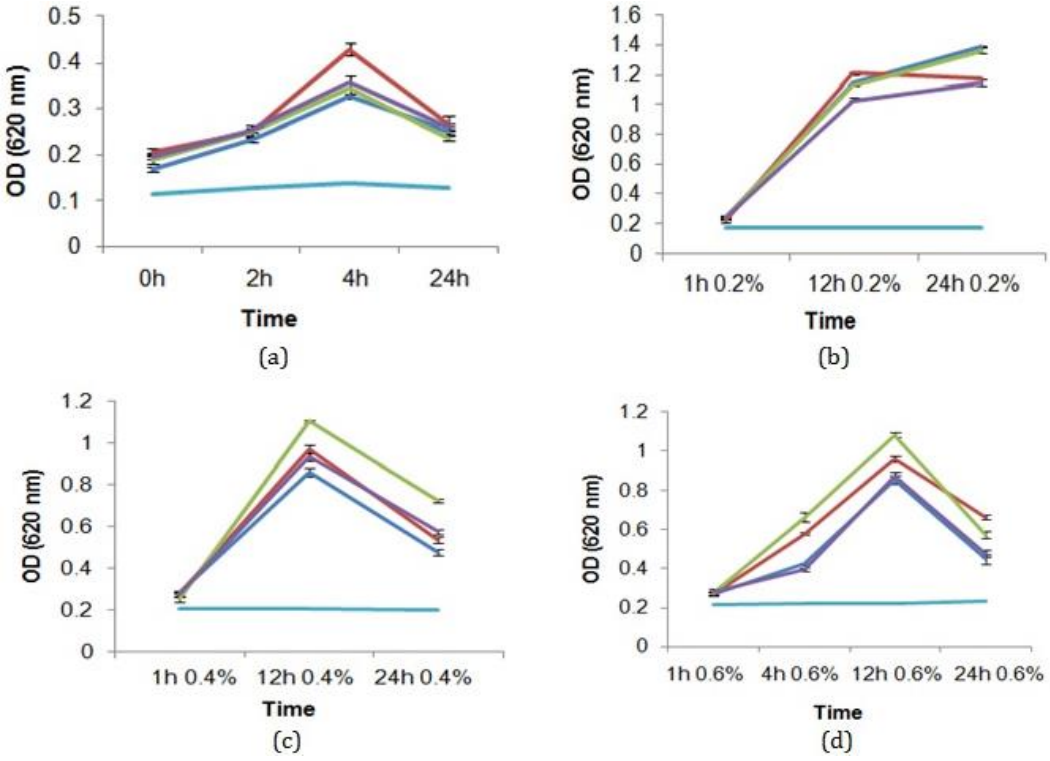

Figure 1. Survival and multiplication abilities of isolated bacterial species in **(a)** artificial gastric juice at pH 2.2; **(b)** crude phenol (0.2%); **(c)** crude phenol (0.4%), and **(d)** artificial bile salt (0.6%). Here, for *L. acidophilus*; for *L. brevis*; for *Bifidocterium spp.* (1); for *Bifidocterium spp.* (2); and for control.

and occasionally antimicrobial compounds like bacteriocins (Buckenhüskes, 1993; Gomez and Malcata, 1999). Isolation and identification of different probiotic strains from poultry feces indicate that poultry are good source of probiotic bacteria. They did exhibit good probiotic characteristics which might be considered as excellent probiotic candidate for feed producing industry that could be beneficial for poultry and animal health. There is an urgent need for development of indigenous probiotic strains for expressing optimal functionality and that reasoned the present experiment to consider isolating probiotic bacteria from regional poultry feces.

L. acidophilus, *L. brevis* and two *Bifidobacterium* spp. were isolated from two chicken fecal samples. Different types of probiotic bacteria originate in different types of chicken samples from different locations due to various reasons. The expansion of probiotic bacteria varies mainly for various ecological ambiences. Among them optimal temperature, convenience of carbohydrates, and favorable pH condition significantly vary the proliferation outline of specific bacteria. For instance *L. acidophilus* grows actively at low pH values (below pH 5.0) and an optimal growth temperature of around 37°C but *Lactobacillus delbrueckii* subspecies *bulgaricus* is very susceptible to O_2 contact with 45°C optimal growth temperature. Besides, *Lactobacillus brevis* has favorable temperature fixed between 40 and 45°C but remain active at 60°C for 30 min.

According to Food and Agricultural Organizations (FAO) and World Health Organization (WHO), one of the main properties of probiotic bacteria is salt tolerance, mainly NaCl (FAO/WHO, 2002). In this study the isolated bacteria had terrific tolerance against 1-7% NaCl. They showed stumpy altitude of growth at 8 and 9% NaCl while no expansion was found and at 10%. Hoque et al. (2010) reported to isolate *Lactobacillus* spp. from yoghurt samples that experienced different concentrations of NaCl from 1 to 10% with positive growth. The NaCl tolerance test results of the present study were found analogous with that. Another experiment of Elizete and Carlos (2005) showed that isolated *Lactobacilli* from gastrointestinal tract of swine were endurable to 4-8% NaCl. The research of Schillinger and Lucke (1987) showed expansion of *Lactobacilli* isolated from animal protein and meat products in the presence of 7.5% NaCl, and the outcome is nearly similar to our study.

FAO/WHO (2002) reported that probiotic bacteria have to proliferate at various pH because gut have to experience a fair range of acidic conditions depends on food type. In synthetic gastric fluid the isolated bacteria in this study showed good acceptance of overnight growth. After 24 h at pH 2.2, all the isolates confirmed lowest

survival capacity compared to prior hours. In addition, at pH 6.6, all the isolated bacteria showed more or less similar survival and multiplication abilities that was considered as favorable environment. This result was parallel to the findings of Maniruzzaman et al. (2010).

After 12 and 24 h of incubation, enhanced resistance and multiplication competence were observed against 0.1 and 0.2% of crude phenol. With increasing the concentration of crude phenol at 0.4%, tolerance of the bacteria was found to decrease significantly. Xanthopoulos, et al., (2000) found the same experimental result. According to Havenaar and Huis (1992), bile salt tolerability was the most common phenomenon for probiotic bacteria. Prasad, et al., (1998) showed that resistance of bacterial isolates was excellent against 0.05, 0.1, 0.15 and 0.3% of artificial bile acid after 24 h of incubation. The present research work found the same consequences. The bile salt hydrolase activity test result of the present study was analogous to Dashkevicz and Feighner (1989) who expanded an agar plate to recognize bile salt hydrolase activity in *Lactobacilli*.

Conclusion

With the findings of the present research work, it would be possible to provide preliminary information for production of probiotic feed products for poultry. It is also anticipated that the deliverables of the research work would promote establishment of community based environmentally sustainable probiotic industries by wider participation of vulnerable, poor and destitute women of the society through financial support from the Government and/ or donors.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Biological corrosion inhibition of steel alloy by pani nano fiber

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Sulfate-reducing bacteria are recognized as a major group of microorganisms linked to anaerobic corrosion. Polyaniline is a good inhibitor for microbially influenced corrosion. The polyaniline nanofibres were prepared by interfacial polymerization method and the prepared polyaniline nanofibres were coated on steel coupons. These steel coupons were used for corrosion studies using sulphate reducing bacteria and compared with and without polyaniline nanofibre by weight loss measurements. 5th day polyaniline coated steel coupon inoculated with sulfate-reducing bacteria gave a low corrosion rate and high inhibition efficiency. The polyaniline nanofibre inhibits the corrosion level of the steel coupons induced by sulphate reducing bacteria.

Key words: Microbially influenced corrosion (MIC), sulfate reducing bacteria (SRB), polyaniline (PANI) nanofibre.

INTRODUCTION

Corrosion can be classified regarding the nature of the process. Physicochemical interactions between a metallic material and its environment can lead to corrosion. Electrochemical corrosion is a chemical reaction involving the transfer of electrons from zero violent metal to an external electron acceptor, causing release of the metal ions into the surrounding medium and deterioration of the metal (Beech, 2004; Javed et al., 2015). Usually corrosion is of an electrochemical nature, but there is also a chemical corrosion not involving charge transfer, like the corrosion of steel in liquid sodium. Presently, oil recovery and transportation are accompanied by serious problems connected with corrosion fracture of the equipment and pipelines of the oil and gas industry. The first signs of deterioration of pipelines were detected in the 1970s. However, the causes and mechanisms of stress corrosion of steel structures have not been determined exhaustively until the present despite the intense study of the problem (Dowling and Guezennec, 1997; Gangloff and Kelly, 1994; Videla, 1996a; Wilmott et al., 1996; Wilmott, 1997). In the first turn, this is connected with the complex nature of the stress corrosion process

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where numerous dissimilar factors interact. The main volumes of oil are extracted by the method of keeping the formation pressure with the use of highly mineralized formation waters. Oil-field brines contain a high amount of chloride ions, oxygen, carbon dioxide and hydrogen sulfide and stimulate the appearance and development of local electrochemical processes that produce the most dangerous corrosive and fracturing effects on the metal. In practical, oil recovery formation pressure is often kept with the help of surface waters of close-lying sources, which contain numerous microorganisms. Adapting themselves to the conditions of the formation, the micro-organisms produce sulfate-reducing bacteria (SRB). Precipitating on the surface of the metal SRB emits hydrogen sulfide and organic acids, for example, acetic acid, which is very aggressive with respect to carbon steel (Nizhegorodov et al., 2008). Biocorrosion is a result of interactions, which are often synergistic, between the metal surface, abiotic corrosion products and bacterial cells and their metabolites (Beech and Sunner, 2004). Bacterial adhesion and bio-film formation are commonly encountered both in natural environments and in industrial processes (Marshall et al., 1994). The heterogeneous bio-film and the associated bacteria form complex biological systems that can cause several chemical changes at the metal/bio-film interface, such as producing gradients in pH, dissolved oxygen, chloride and sulfate (Lewandowski, 1994; Boronstein, 1994). Under aerobic conditions, the microbial colonization usually leads to the formation of differential aeration and concentration cells due to the metabolism of the bacterial colony. The generation of these concentration cells is widely recognized to be detrimental to the integrity of the passive film and to facilitate the initiation of pitting or crevice corrosion (Little et al., 1992). The above process is well known as micro-biologically influenced corrosion (MIC) or a biocorrosion phenomenon. The use of coatings is the most common means of corrosion control for materials susceptible to environmental interactions. A coating may act as an ionic filter with sufficiently high electrical resistance to mitigate current transfer between anodic and cathodic sites when water permeates the coating. Coatings may also act as a barrier to oxygen diffusion to impede the cathodic reaction. Overall, the chief function of such coatings is to provide an effective environmental barrier to the substrate, thereby preventing corrosion (Shifler, 2005). However, much is unknown on the specifics of how organic coatings fail. There is a lack of understanding of the complex, multi-variable degradation processes leading to coatings failure. Water and oxygen can permeate, at least to some extent, through any amorphous polymer film, even when the film has no imperfections such as cracks or pores (Charaeklis, 1990). Polyaniline (PANI) ranks among electrically conducting polymers. Its high conductivity and chemical variability makes it suitable for a number of applications (Samui and Phadnis, 2005; Sathiyanarayanan et al., 2005). In the course of polymerization, PANI has the ability to create thin conducting films with very good adhesion on various base materials. So this polyaniline nanofibres was used as an corrosion inhibitor for the corrosion inhibition studies.

MATERIALS AND METHODS

Collection of samples

The corroded material was scraped from the metal rods placed in sterile container and was transferred to the laboratory for further use.

Isolation and cultivation of sulphate reducing bacteria

The sulphate reducing bacteria present in the collected corroded material was isolated and cultivated. One gram of the scrapped material was aseptically inoculated on 100 ml of sterile lyngby medium (peptone: 20.0 g/L, agar: 12.0 g/L, sodium chloride: 5.0 g/L, beef extract: 3.0 g/L, yeast extract: 3 g/L, L-cysteine: 0.6 g/L, ferric citrate: 0.3 g/L and sodium thiosulphate: 0.3 g/L). After inoculation, it was incubated at 37°C for 24 h, in a shaker incubator. A loopful of lyngby medium broth culture was inoculated in 100 ml of sterile postgate medium (Videla, 1996b) (pottasium dihyrogen phosphate: 0.5 g/L, ammonium chloride: 1.0 g/L, sodium sulphate: 4.5 g/L, calcium chloride: 0.6 g/L, magnesium sulphate: 0.6 g/L, sodium lactate: 6.0 g/L, yeast extract: 1.0 g/L, ferrous sulphate: 0.04 g/L and sodium citrate: 0.003 g/L). After inoculation, it was incubated at 37°C for 24 h, in a shaker incubator. A loopful of bacterial culture grown in Postgate medium was streaked on sterile postgate agar plates. It was incubated at 37°C for 24 h. After incubation, the plates were observed for morphologically different bacterial cultures.

Identification of the isolates

The bacterial isolates grown on postgate agar medium were identified based on the microscopic, morphological and biochemical characters (Holt et al., 1994).

Preparation of polyaniline (PANI) nanofibres

PANI nanofibre was synthesized by interfacial polymerization method as described by Huang and Kaner (2004). 5.6 g (60 mmol) of aniline was dissolved in 200 ml of chloroform and 3.4 g (15 mmol) of ammonium per sulphate was dissolved in 200 ml of 1.5 mol/L HCl solution. These two solutions were carefully transferred in a 1000 ml beaker, generating an interface between two layers. The reaction was carried out in an interface of the two phases at room temperature for 24 h. The protonated PANI nanofibres were obtained and then converted into emeraldine base form by treatment with 10% weight of aqueous ammonium solution for 24 h.

Preparation of steel coupon

Approximately 5.2 x 1.3 x0.5 cm steel coupons was obtained from local steel agencies and was used for the biocorrosion studies. To remove the impurities, the steel coupons were immersed in concentrated HCl for overnight and were washed with distilled water. The washed steel coupons were washed in acetone for 30 min and were dried in air. The cleaned steel coupons were sequentially ground with 180 and 500 grit emery papers to get

finally polished smooth surface steel coupons.

Preparation of PANI- polyvinyl acetate (PVA) coating

Polyvinyl acetate (PVA) was used as a base matrix for dispersing PANI (2.5% weight) over the steel coupons. 2.5% (weight) of the conducting polymer in the coating was sufficient for achieving good corrosion resistant (Cao et al., 1989). So, in the present study, the same procedure was used for the preparation. 2.5% of PANI was dispersed in 99% of chloroform. The suspension was vigorously agitated using shaker for 24 h. In another beaker, PVA (10 weight %) was dissolved in chloroform. The two solutions were mixed and stirred (4.5%) until a homogenous dispersion was formed. Film thickness can be controlled by varying the duration of the substrate dipping time (Avlyanov et al., 1995); the cleaned steel coupons were coated with the prepared PANI-PVA matrix by dipping for 1, 3 and 5 days. After coating, the coated steel coupons were placed in hot air oven at 65°C for 12 hfor curing process. After curing, the steel coupons were used for the biocorrosion studies.

Biocorrosion inhibition by PANI-PVA matrix

The biocorrosion inhibition level of PANI-PVA matrix was analyzed using sulphate reducing bacterial isolate. To compare the level of biocorrosion inhibition, the same numbers of coupons were exposed to the sterile nutrient rich medium which had exactly the same composition but the exception is without inoculating any microorganisms. All the experiments were carried out in a batch mode under stagnant condition in an incubator at 25°C. A loopful of 24 h SRB broth culture was aseptically inoculated on to 50 ml of the sterile postgate broth. After the OD value reached a level of 1.0, the steel coupons (with and without coating) were hung on the nylon string and were aseptically introduced into the 50 ml of grown broth cultures. To maintain the bacterial density, the steady-state growth phase throughout the experiment, a semi-continuous mode were employed, that is, 75% of the medium were drained and replaced with an equal amount of fresh medium for every 7 days. On days of 7, 14, 21, 28, 35 and 49, the coupons were retrieved from the inoculated medium for weight loss measurement and bacterial colonization studies with stereo trinocular microscope.

Analysis of corrosion

Measurement of weight loss (Umoren et al., 2007)

The steel coupons were immersed in 20% NaOH solution containing 200 mg zinc dust, scrubbed with bristle brush under running water in order to remove the corroded particles, dried and reweighed. The weight loss was taken as the difference between the weight at the given time and the initial weight of the test coupon was determined, using the digital balance. The measurement was carried out for the blank, with anti-corroding agent coating and without coating. The inhibition efficiency was evaluated using the equation:

$$I\ \% = (1-W_1/W_2) \times 100$$

Where W_1 and W_2 are the weight loses (mg) for mild steel in different time exposure both coated PANI nanofibres and uncoated coupons have been determined from the weight loss measurement using an expression. The corrosion rate was evaluated using the equation:

$$\text{Corrosion rate (mg cm}^{-2}\ \text{h}^{-1}) = \Delta w / AT$$

Where, Δw is the weight loss (mg) (obtained as a difference between initial weight and weight at a given time), A is the area of specimen (cm^2) and T is the exposure time (h).

Analysis of bacterial colonization in steel coupons using fluorescence microscope (Walker and Keevil, 1994)

At the predetermined period of bacterial incubation, the specimens were retrieved and washed twice with a sterile phosphate buffered saline (PBS) solution, to remove the dead and loosely attached bacteria. The fixation of bacterial cells with 2.5% glutaraldehyde PBS solution for 4 h, the specimens were washed twice with sterile phosphate buffered saline (PBS) solution and deionized water, followed by staining with 0.01% Acridine orange solution for 10 min. The excess dye was removed by washing with 70% alcohol solution. The specimens with immobilized bacterial cells were imaged under 1000x magnification using a stereo trinocular microscope.

RESULTS AND DISCUSSION

The beginning of the anaerobic period is associated with an increase of the corrosion rate attributed to micro-organisms; SRB (Enning et al., 2015). The corroded material was scrapped from the metal rod and used in the present study for the isolation of sulphate reducing bacteria. The SRB was isolated by inoculating the corroded material in lyngby medium. The grown cultures was further sub cultured on postgate medium and finally streak plated on postgate agar medium. The morpho-logically different colonies observed in postgate agar medium were used for the identification procedure. Cultural characters of the isolates grown on lyngby medium and postgate agar medium were observed. Based on the morphological, microscopical and biochemical characters of the isolates, they were identified as sulphate reducing *Desulphovibrio* sp. (Table 1 and Figure 1). Desulfovibrio profundus was one such SRB associated in carbon steel corrosion (Lanneluc et al., 2015).

Biocorrosion inhibition by PANI-PVA matrix

MIC of steel is a serious problem in the marine environ-ment and many industries, such as power generation, petrochemical, pulp and paper, with serious safety and economic concerns (Walsh et al., 1993). The use of coatings is the most common means of corrosion control for materials susceptible to environmental interactions. Electrically conducting polymers have been shown to be effective for corrosion prevention (Liu and Levon, 1999). The biocorrosion inhibition level of PANI-PVA matrix was analyzed using sulphate reducing *Desulphovibrio* sp. Yuan et al. (2012) also observed that PVAn-PANI bilayer coating is having good corrosion inhibition against SRB. The corrosion rate of uncoated sterile and SRB containing steel coupon were analyzed in various days (Table 2 and Figure 2) and the corrosion rate were

Table 1. Morphological and biochemical characters of the isolate.

Culture	Growth in lyngby broth			Growth in Postgate medium		
	Morphological characters of the isolate					
SRB	H_2S production (black color formation)			Small colonies		
	Biochemical characters of the isolate					
Gram staining	**Shape**	**Lactate utilization**	**Malate utilization**	**Pyruvate utilization**	**Actate utilization**	**Growth at NaCl**
Gram negative	Rod	positive	positive	positive	positive	No growth

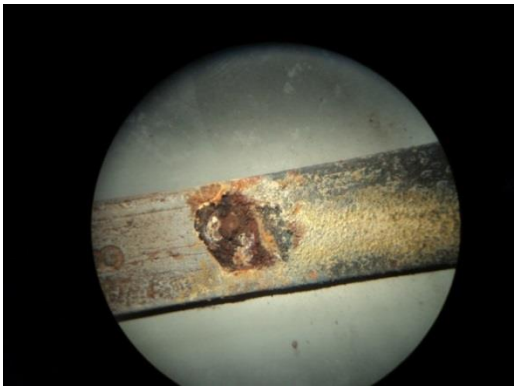

Figure 1. Stereo trinocular microscopical view of sulphate reducing *Desulphovibrio* sp.

Table 2. Comparison of corrosion rate in sterile medium and with SRB (without coating).

	Corrosion rate at different days (mg cm^{-2} h^{-1})						
	1st day	**7th day**	**14th day**	**21st day**	**28th day**	**35th day**	**42nd day**
Corrosion rate in sterile medium	-	-	0.0017	0.0035	0.0052	0.0056	0.0063
Corrosion rate in SRB containing medium.	0.010	0.0272	0.0348	0.0349	0.0391	0.0419	0.0422

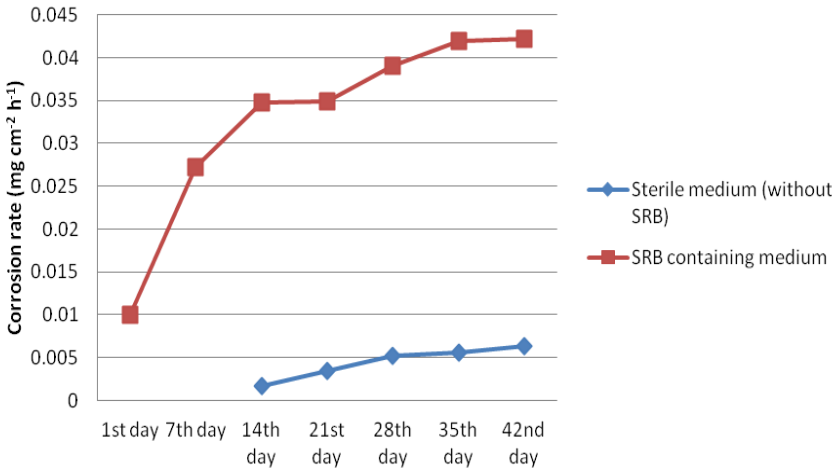

Figure 2. Comparison of corrosion rate in sterile medium and with SRB (without coating).

Table 3. Corrosion rate in SRB containing medium at different days with PANI nanofibre coating and without coating.

SRB containing medium	Corrosion rate at different days (mg cm^{-2} h^{-1})						
	1st day	7th day	14th day	21st day	28th day	35th day	42nd day
Uncoated coupons	0.010	0.0272	0.0348	0.0349	0.0391	0.0419	0.0422
1st day coated coupons	-	0.007	0.007	0.011	0.013	0.015	0.014
3rd day coated coupons	-	0.003	0.004	0.009	0.009	0.010	0.010
5th day coated coupons	-	-	0.004	0.009	0.009	0.009	0.01

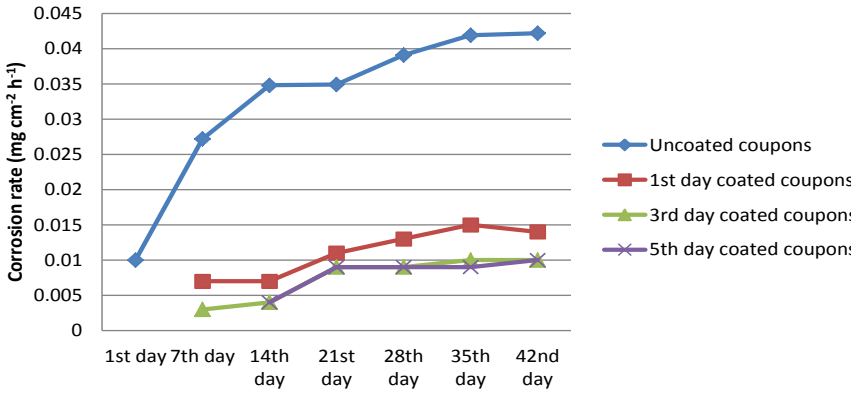

Figure 3. Corrosion rate in SRB containing medium at different days with PANI nanofibre coating and without coating.

Table 4. Inhibition efficiency for PANI nanofibres steel coupons for 1, 3 and 5 days.

	Inhibition efficiency at different days (%)						
	1st day	7th day	14th day	21st day	28th day	35th day	42nd day
Sterile medium	-	-	-	68.2	33.8	21.8	21.4
1st day	-	77.3	76	76.3	66.3	62.6	65.9
3rd day	-	88.2	88.1	79.6	76.0	75.6	74.3
5th day	-	-	88.5	80.2	76.8	75.2	70.9

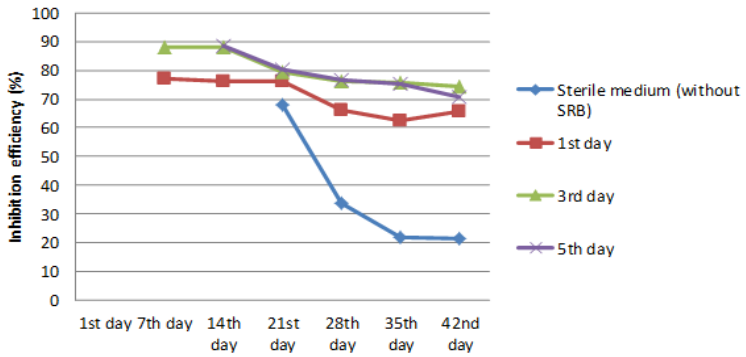

Figure 4. Inhibition efficiency for PANI nanofibres steel coupons for 1, 3 and 5 days.

increased, with time of the incubation period. With the corrosion rate in the presence or absence of SRB (Table 3 and Figure 3), there is increase in corrosion rate in the presence of SRB. When the same study with the coated PANI steel, there was increasing corrosion inhibition efficiency (Table 4 and Figure 4).

When comparing the level of coating (1, 3 and 5 days of PANI exposure), the maximum corrosion inhibition was seen in 5 day PANI exposure steel coupon (88.5%). Thus, the present study show that the 5 day PANI expose steel coupon have maximum corrosion inhibition rate (88.5%) against SR bacteria. The inhibition efficiency of the PANI nanofibre was decreased when they are exposed for long time duration. The decreasing level of corrosion rate and inhibition efficiency led to increase in the corrosion of steel by SR bacteria.

Conclusion

Corrosion is one of the most serious problems to the environment and to mankind. In this context, the present study aims to prepare an anti-corrosion agent such as PANI– nanofibre. The inhibition efficiency of the PANI-nanofibres coated steel coupons was studied with different day exposure of PANI- nanofibre for 1, 3 and 5 days. The PANI- nanofibre inhibits the corrosion level of the steel coupons induced by SRB. In this observation, the PANI- nanofibre coated steel coupons shows best results when compared with the sterile medium and SRB containing medium. This work will pave way for the uncorroded environment. Corrosion of the metals by sulphate reducing bacteria leads to serious problem to mankind that can be controlled by treating the steel coupons with PANI- nanofibres.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Effect of adding different levels of probiotics to broilers' diets on gastrointestinal tract development and production performance

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Probiotics are used as alternative in diets. Probiotics, as defined by many authors, are food additives consisting of living microorganisms that have beneficial effects on the physiology and health of organisms. Microorganisms are most commonly used by lactic acid bacteria which are part of the bio-preparations, for poultry animals in improving their health and production parameters. The objective of this work is to determine the effect of different doses of probiotics on broiler Ross 308 in terms of improving its production and digestive tract development. The study evaluated the addition of different doses of probiotics offered orally in relation to weight gain, feed intake and feed conversion in broilers. Comparison was also made in development of gastrointestinal tract, based on villi level of intestinal walls. In feed intake, differences were not significant (P >0.05). Daily weight gain of treatments with higher level of probiotic was higher (P < 0.05). However, in feed conversion, despite being excellent, treatments were not different (P > 0.05). Measurements of intestinal villi in duodenum were not different (P > 0.05). In jejunum and ileum, villi length and extent of muscle layer in treatment three were different compared to other treatments (P <0.05). It was concluded that 1.5 ml of probiotics supplement improves body weight gain and measurement of the villi and muscle layer of jejunum and ileum.

Key words: Broilers, organ weight, performance, probiotic, villi measurements.

INTRODUCTION

Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit to the host through improvements of the intestinal microbial balance (FAO/WHO, 2002; Foulquié Morenoa et al., 2006). Also they are defined as live microbial feed supplement which beneficially affects host animal by improving its intestinal microbial balance (Fuller, 1989).

Salminen et al. (1998) propose that probiotics are "microbial cells preparations, or components of microbial cells that have a beneficial effect on health and welfare".

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Milian (2005) mentions that probiotics are natural products used as growth promoters in animals, allowing higher yields, higher immune resistance and reduced amount of pathogens in the gastrointestinal tract (GIT). These bacteria represented by *Lactobacillus acidophilus, Lactobacillus bulgaris, Bifidobacterium bifidum, Bifidobacterium infantis*, and other beneficial micro-organisms are the first line of defense of the body against potentially harmful microorganisms that are inhaled or swallowed. Probiotics possess immunomodulatory properties, hypolipidemic capabilities, protective properties of the gastric mucosa and can inhibit intestinal pathogens,

The scientific and technological research of these properties will optimize production processes of functional foods containing lactic such crops as well as lead to the understanding of the mechanisms by which these bacteria exert their beneficial effect on the host (Pía et al., 2005).

Probiotics are supplied once they develop in the GIT, through several mechanisms which contribute to the balance of intestinal microorganisms and provide an improvement in the digestive processes in host. These positive effects in the GIT are also reflected in the yield of animals (Patterson and Burkholder, 2003). There is evidence that the use of probiotic *Lactobacillus* strains mainly, either pure or mixed, increases nutrients retention in diet. Apparent nutrient retention is favored with using probiotics, primarily by retention of N, P and Ca (Nahashon et al., 1994; Schneitz et al., 1998; Angel et al., 2005). Probiotics used in birds, such as *Lactobacillus*, are bacteria that grow more rapidly in the intestine (Moreno et al., 2002). Likewise, the use of *Bacillus* sp. endospores can help to reduce the acidity of the gut in birds, favoring the growth of *Lactobacillus* in the GIT, stimulating the immune system and controlling microbial growth of pathogenic bacteria (Moreno et al., 2002). Awad et al. (2006) concluded in their study that probiotic supplemented with broilers' diets of 10 mg/kg of DON reduce and may enhance the histological alterations in intestinal wall of duodenum and jejunum, caused by mycotoxins on diet.

Alkhalf et al. (2010) concluded that early supplementation of probiotic in broilers' diet enhances their immune response in their work evaluated size and weight of the lymphoid organs such as spleen, bursa of Fabricius and thymus.

Direct-fed microbials (DFM) did not significantly modify BW gain and most failed to affect serum antibody levels in response to immunization with a recombinant *Eimeria* protein. However, altered intestinal morphometric measurements were readily apparent in DFM-fed chickens as revealed by increased villus height and crypt depth compared with non-DFM-fed controls. In addition, serum levels of α-1-acid glycoprotein as an inflammatory marker were reduced in DFM fed birds. These results provide a rational scientific basis for future studies to investigate DFM as immunomodulating agents to enhance host protective immunity against enteric pathogens in broiler chickens (Lee et al., 2011).

Moreover, Brisbin et al. (2011) measured the immune response in chickens. The objective of their study was to examine the effects of these bacteria individually or in combination on the induction of antibody- and cell-mediated immune responses *in vivo*. These results indicate that systemic antibody- and cell-mediated immune responses can be modulated by oral treatment with lactobacilli but that these bacteria may vary in their ability to modulate the immune response.

Also, the same authors (Alkhalf et al., 2010b) in another study, conclude that in the hemoglobin content, the change was not significant. Likewise, concentrations of total lipids and albumin protein were not affected by probiotics supplement. Furthermore, in probiotic supplemented chicks, cholesterol content significantly decreased compared to the control group. Probiotic supplement also increased body weight and average daily weight gain in the phase of 3-6 weeks of age. They conclude generally that probiotics improve productive parameters and reduce serum cholesterol of broilers.

Mountzouris et al. (2010) conducted a research using probiotics. The aim of this work was to investigate the effect of inclusion levels of a 5-bacterial species probiotic in broilers' nutrition. In this work, it was concluded that probiotic inclusion level had a significant effect on broilers' growth responses, nutrient ADC, AMEn and cecal microflora composition.

MATERIALS AND METHODS

Experimental design and treatments

The experiment was conducted in the area of experimental poultry production, Faculty of Veterinary Medicine at the Autonomous University of Nuevo Leon, in Escobedo, Nuevo Leon, Mexico from April 15 to May 28, 2013. Facilities were adequate with environmental conditions, where temperature was 18 to 27°C. One day old of Ross 308 line chicks were assigned to 34 experimental pens (1.5 x 3m^2). There were 30 birds per pen for the end density of ten birds/m^2. Lighting was provided by incandescent heat lamps to provide initial temperature. At the beginning of the second week of age, feeders (20 kg capacity) and automatic waterers were provided. Broilers were randomly distributed in a completely randomized statistical design considering control treatment (negative control) and two levels of probiotics (Performance ®) applied orally to chickens. The commercial probiotic as specified for other species was offered in water. The birds were vaccinated for Marek's disease and Newcastle disease at hatching. Three birds per replicate were used for sampling of organs of birds at the slaughter. The treatments were as shown: T1 = Negative control (no added probiotic); T2 = adding probiotic orally, 1 ml per bird; T3 = adding probiotic orally, 1.5 ml per bird. The commercial probiotic contains a mix of microorganisms of 1.3 billion/g CFU of *Saccharomyces cerevisiae*, *Enterococcus faecium*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Bifidobacterium thermophilum*, *Streptococcus faecium*, *Bacillus subtilis*. For application of the probiotic, there was a dilution of 25 g of product in 50 ml of bidistilled water, 1.0 ml of which was administered orally to T2 and 1.5 ml at T3. This product was administered orally to poultry randomly selected, at first day of age, and applied every 14 days until day 28. Birds received feed and

Table 1. Feed intake, body weight gain and feed efficiency of broilers feed diets with two probiotics levels.

Parameter	Treatment			SEM	*P value*
	0.0 ml	1.0 ml (6.5^{11} UFC)	1.5 ml (9.75^{11} UFC)		
Feed intake, g/d	59.342	64.942	67.902	3.81	0.2611
weight gain, g/d	45.159^{B}	48.644^{AB}	49.876^{A}	1.42	0.0514(*)
Feed efficiency	1.54	1.56	1.56	0.03	0.9382

Means with different letters (A, B, AB) were statistically different (P <0.05).

water *ad libitum* until termination of growth period. Feeds offered in the diet were corn gluten meal, soybean meal and yellow corn, and were offered with 3200 kcal/kg metabolizable energy, 23% crude protein, 1% Ca, 0.45% P, 0.5% lysine, 0.1% methionine, 1500 IU of vitamin A and 10 IU of vitamin E. Other nutrient levels were based on NRC (1994).

Experimental parameters measured

Body weights were individually recorded and feed intake for each cage was measured weekly starting at day seven. Weight gain and feed consumption were determined weekly, and gain: feed ratio was calculated cumulatively.

Small intestine, liver, spleen, pancreas and bursa sampling

At the end of the experiment, 2 birds per pen were randomly selected (12 birds per treatment) and killed by cervical dislocation. The length of the small intestine was measured (Uni et al., 2003) in a vertical rule surface that allows gravity, and segments (1 cm) were removed from the duodenum, jejunum, and ileum: 1- form the apex of duodenum; 2- the midway between the point of entry of the bile ducts and Meckel´s diverticulum (jejunum); 3-10 cm proximal to the cecal junction. Clean and empty intestine was put in saline solution; liver spleen and pancreas were excised, weighed and frozen until further processing.

Morphometric indices

Intestinal samples from 12 birds per treatment of duodenum, jejunum, and ileum of approximately 1.5 cm were taken from the loop of the duodenum, midpoint between the bile duct entry and Meckel's diverticulum (jejunum), and midway between Meckel´s diverticulum and the ileo-ceacal junction (ileum). Segments were flushed with saline solutions (0.9% NaCl) to remove contents and were fixed in neutral buffered formalin solution for histology; samples were dehydrated, cleared, and paraffin embedded. Twelve sections with the twelve parts of each tissue and same treatment (only one tissue per bird) were cut at 5 µm and placed per glass slide and processed by hematoxylin eosin for examination by light microscope. Morphometric analysis was performed on 15 villus chosen by a random digits table in each segment (12) per slide using a computer-aided light microscope image with openlab software (Openlab Ver 2.2.5 Improvision Inc. Lexington, MA). Parameters measured include villus height from the tip of the villus to the crypt, crypt depth from the base of the villi to the submucosa, villus width at one third of the villi and the muscularis from the submucosa to the external layer of the intestine, and the crypt: villus ratio (Geyra et al., 2001).

The next step was to look at the cuts on a Carl Zeiss microscope integrated with a computer Fujitsu Siemens, and Axioskop40 Zeiss camera (AxioCam HRC Zeiss); measurements were made through the program Axio Vision Release 4.5., carefully carrying a record of all data.

Statistical analysis was performed using Statistix software (version 9.0.4). Means were compared using Tukey's test.

RESULTS AND DISCUSSION

Productive performance

Results of feed intake grams per day, weight gain in grams per day and feed conversion from day old until the day of sacrifice at 42 days are shown in Table 1. Feed intake shown in T3 (commercial probiotic, 1.5 ml) was highest with 67.9 g/d as compared to T 2 (commercial probiotic, 1 ml) and T1 control group, which had an average daily intake of 64.9 and 59.3 g, respectively. There were no significant differences (P>0.05). Daily weight gain shown in Table 1 (T3) was higher with 49.8g compared to 48.6g of T2 and T1 with 45.1g; it showed that T3 and T2 were statistically equal (P>0.05) but significantly better than T1 (P<0 .05).

In terms of feed conversion, T1 shown had the best value with 1.54 g as compared to T2 and T3 with obtained values of 1.56 g; however these data are not shown to be significant (P>0.05).

Feed intake had no differences between treatments, best value corresponded to treatment with higher level of probiotics, while gain was elevated to the same level of probiotic supplement. This finding is consistent with reports by Hoyos et al. (2008), that weight gain in chickens treated with probiotics was higher in the study period. This shows that probiotic bacteria help in the improvement of intestinal bacterial flora, improve nutritional characteristics of food and thus improve digestibility, which affects weight gain of birds. Although the report contrasts with that of Cortes et al. (2000) and Araujo (2005) who observed a significant difference in chickens treated with probiotics.

Another study agrees with these findings (Mountzouris et al., 2010), where it is concluded that probiotic inclusion level had a significant effect on broilers' growth responses. In contrast, Alkhalf et al. (2010b) showed that probiotic supplement in broilers' diet increased body weight and average daily weight gain of 3-6 week old bird. This is consistent with the present study, in terms of the increase in production parameters of broiler influenced by the addition of probiotics. This is inconsistent with the results; however, with the results shown here, we can say that addition of probiotics to broiler diets has great value in feed efficiency.

Table 2. Measurement of duodenal portion during the study of broilers fed diets with different levels of probiotics.

Parameter	Treatment			SEM	*P value*
	0.0 ml	1.0 ml (6.5^{11} UFC)	1.5 ml (9.75^{11} UFC)		
Villus height (µm)	11.266^{A}	12.037^{A}	11.993^{A}	0.34	0.1936
Crypt depth (µm)	2.2631^{A}	2.2355^{A}	2.0743^{A}	0.08	0.1962
Submucosa	0.3740^{A}	0.3845^{A}	0.4096^{A}	0.02	0.4044
Muscular	1.5051^{A}	1.5405^{A}	1.6185^{A}	0.07	0.3903
Distal width	1.0529^{A}	1.1114^{A}	1.1315^{A}	0.06	0.5916
Proximal width	1.0814^{A}	1.0037^{A}	1.0615^{A}	0.03	0.3728

P values were not different between treatments (P <0.05).

Table 3. Measurement of jejunum portion during the study of broilers fed diets with different levels of probiotics.

Parameter	Treatment			SEM	*P value*
	0.0 ml	1.0 ml (6.5^{11} UFC)	5 ml (9.75^{11} UFC)		
Villus height (µm)	7.7466^{B}	7.8508^{B}	8.7244^{A}	0.20	0.0018**
Crypt depth (µm)	1.5902^{A}	1.6306^{A}	1.3695^{B}	0.05	0.0030**
Submucosa	0.4282^{A}	0.4542^{A}	0.4566^{A}	0.015	0.5246
Muscular	1.2311^{B}	1.5154^{A}	1.6471^{A}	0.07	0.0008**
Distal width	1.0079^{A}	0.9073^{A}	0.9773^{A}	0.03	0.1494
Proximal width	0.8761^{A}	0.8357^{A}	0.9205^{A}	0.02	0.1028

P values marked with (**) were statistically different (P <0.01).

Table 4. Measurement of ileum portion during the study of broilers fed diets with different levels of probiotics.

Parameter	Treatment			SEM	*P value*
	0.0 ml	1.0 ml (6.5^{11} UFC)	5 ml (9.75^{11} UFC)		
Villus height (µm)	5.0278^{C}	6.3371^{B}	7.0583^{A}	0.18	0.0001**
Crypt depth (µm)	1.1184^{B}	1.5433^{A}	1.4794^{A}	0.04	0.0001**
Submucosa	0.4188^{A}	0.4298^{A}	0.4387^{A}	0.01	0.6818
Muscular	1.2638^{B}	1.4541^{B}	1.7660^{A}	0.060	0.0001**
Distal width	0.9624^{A}	1.0380^{A}	0.9742^{A}	0.040	0.3480
Proximal width	0.8569^{A}	0.9189^{A}	0.8987^{A}	0.30	0.3783

P values marked with (**) were statistically different (P <0.01).

Development of the GIT

Table 2 shows the means of measures that were determined in different sections of small intestine, morphology of 3 treatments in 42 days. In this case, the duodenum sections are along the villi, crypt, submucosa, muscle, distal width of the villus and proximal width of the villi, in which the analysis of variance showed that there was no difference between treatments (P>0.05). In Table 3, portions of the small intestine and jejunum were evaluated. This table shows that the length of the villi of T3 was higher than that of treatments 1 and 2 (P<0.05).

The measurements of the crypt showed that treatments 1 and 2 were similar (P>0.05), but significantly greater than treatment 3 (P<0.05). In the measurements of muscle layer, it was observed that treatments 2 and 3 were identical (P>0.05), but higher than treatment 1 (P<0.05). Measurements in submucosa of jejunum, distal width and proximal width were equal in all the treatments (P>0.05).

Table 4 shows the evaluation of morphology of ileum. It was also observed that the length of villi of treatment 3 was significantly higher than that of treatments two (P <0.05) and 1 (P<0.05). In crypt, treatment 3 is equal to 2 treatment (P>0.05), but greater than the control treatment (P<0.05). In muscular layer of intestine, treatments 1 and 2 were similar (P>0.05), but treatment 3 was better (P<0.05). Measurements of submucosa of ileum, distal width and proximal were equal in all treatments (P>0.05).

Table 5. Weight of organs and records of measurement during the study of broilers with diets of several probiotics levels.

Parameter	Treatment			SEM	*P value*
	0.0 ml	1.0 ml (6.5^{11} UFC)	5 ml (9.75^{11} UFC)		
Length of the small intestine	161.60	165.40	157.60	8.82	0.823
Liver	37.300	37.300	37.889	4.97	0.995
Spleen	1.400	1.400	1.800	0.24	0.409
Páncreas	2.700	3.400	2.700	0.060	0.401
Bursa of Fabricius	5.800	6.200	5.800	0.040	0.717

P values were not different between treatments (P <0.05).

Table 5 shows the length of small intestine and organ weights collected during the study. Length of small intestine did not differ (P>0.05) in all treatments. On the other hand, weight of liver, spleen, and pancreas did not differ (P>0.05) in all treatments. Size of bursa of Fabricius of broilers receiving different levels of probiotics orally did not differ (P>0.05) in all treatments. Relative weights of liver, spleen and pancreas were not (P>0.05) affected by dietary treatments, and were similar with findings of Hashish et al. (1995) who tested supplementation of antibiotic, zinc bacitracin, alone or combined with an enzyme complex, kemzyme to barley-based broiler diets. Sarica et al. (2005), reported that weights of liver, spleen and pancreas were not (P>0.05) affected by dietary treatments, when they used antibiotic as growth promoter in wheat based broiler diets.

The probiotics administered to broilers result in substantial improvement of the intestinal villi, but is not reflected in the duodenum. If there are differences in the length of the villi and thickness of the muscle layer of jejunum and ileum, these are stimulated by the highest level of probiotic. This enhances nutritional characteristics, which mainly promote secretion of digestive enzymes and improve development and performance of digestive system. This coincides with this statement, expressed by various authors that it decreases malabsorption syndrome in bird (Perez et al., 2003).

The length of the intestine showed no difference in treatment, as well as in the liver organ weights spleen, pancreas, and in the size of the bursa of Fabricius. One example is that of Awad et al. (2009) which is consistent with reports where these parameters were included. However, significant growth of villi of jejunum and ileum makes the bird have a larger surface area to absorb nutrients. This leads to its greater physiological development and provides greater health during production period.

Conclusion

Probiotics as supplement, as shown in the present study, improves weight gain of broilers as well as the villi and thickness of muscular layer of jejunum and ileum in small intestine. Furthermore, dietary supplementations result in an increase in villus height and crypt depth of intestinal mucosa of broilers. Therefore, these products might be used as substitution of antibiotic growth promoters in broiler, leading to higher feed efficiency.

Conflict of interests

The authors declare there is no conflict of interest.

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