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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Review

An overview of fungal and bacterial biopesticides to control plant pathogens/diseases

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Chemical agents are extensively used in all countries of the world in1960s, for controlling the agricultural pest and pathogens, it became apparent that agricultural chemicals were responsible for causing environmental pollution, they were present in the food chain, they were capable of inducing pest resistance, development of disease resistance, toxic hazards to man, plants, domestic animals and wild life, as a result they are regarded as ecologically unacceptable. Therefore, from the last two decades, scientists are looking for environmentally and toxicologically safe and more effective methods to control plant pathogens /pests and they increased social pressure to replace them gradually with biopesticides which are safe to humans and non-target to other beneficial organisms and cheaper than the chemicals. The isolates of the biocontrol agents are formulated by using different organic and inorganic carriers either through solid or liquid fermentation. They are applied as a seed treatment, bio-priming, seedling dip, soil application, foliar spray, fruit spray, sucker treatment and sett treatment.

Key words: Biopesticides, eco-friendly, management, pathogen/disease.

INTRODUCTION

Due to plant diseases, every year nearly 10-20% of the total world food production decreases and this lead to loss of billions of dollars. Agriculture has been facing the destructive activities of numerous pests and pathogens from early times which lead not only to reduction of the yield of the crop, but also lose in terms of money and reduce the aesthetic value. However, the extensive use of the synthetic chemicals during the last three decades has raised a number of ecological problems. In the recent years, scientists have diverted their attention towards the potential of beneficial microbes. The disease causes a significant reduction of seed germination, seed quality thereby limiting its potential yield. Biocontrol agents act through the mechanisms of antibiosis, secretion of volatile toxic metabolites, mycolytic enzymes, parasitism and through competition for space and nutrients. Among different biological approaches, use of the microbial antagonists like fungi, and bacteria is gaining popularity (Eckert and Ogawa, 1988; Droby et al., 1991; Wisniewski and Wilson, 1992; Droby, 2006; Korsten, 2006). Fungi belonging to the genus *Trichoderma* and bacteria such as *Pseudomonas*, *Bacillus subtilis* are the most promising bio control agent which acts against a wide range of plant pathogens. *Trichoderma* spp. are capable of controlling a number of diseases of plants; they control large number of foliar and soil borne diseases (Papavizas, 1985).

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Majority of the isolates were identified as *Trichoderma viride* and *Trichoderma harzianum*. Many species of *Trichoderma* namely *T. harzianum*, *T. viride*, *Trichoderma virens* etc, isolates from rhizosphere, were good antagonistic potential against many soil borne fungi, such as *Fusarium oxysporum*, *Sclerotium rolfsii*, *Rizoctonia solani*. *Trichoderma* spp. produce antibiotics and antifungal toxic metabolites viz trichodermin, viridin, etc and also inhibit pathogens by secreting enzymes like glucanase, cellulase, chitinase, protease, etc, which disintergrate the cell wall of pathogen. *Pseudomonas* spp. which survive in the rhizosphere of the crop are also known as plant growth promoting rhizobacteria (PGPR). *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas cepacia* are the important biocontrol agents and they produce antibiotics like Pyrol, Nitrin, Oomycin-A etc and hormones like indol acetic acid, Giberllic acid, and Siderophores that inhibit the growth of pathogens. A combination of *P. fluorescens* and *T. harzianum* provides effective control of disease of vanilla crop. *Trichoderma*-based agriculture products can be found as registered in many countries, and are sold and applied to protect and improve yield of vegetables, ornamentals and fruit trees (Lortio, 2005). “Biological control of soil borne plant pathogens by *Trichoderma* spp. and other bioagents is a vital area of plant pathological research all over the world these days”. Biological plant protection is an important component in the eco-friendly management of plant diseases all over the globe.

Advantages of biopesticides

1. Avoids environmental pollution (soil, air and water).
2. Avoids adverse effects on beneficial organisms, that is, maintain healthy biological control balance.
3. Less expensive than pesticides and avoids problems of resistance.
4. Biopesticides are self maintaining in simple application and fungicide needs repeated application
5. Biopesticides are very effective for soil borne pathogen where fungicidal approach is not feasible.
6. Biopesticides are eco-friendly, durable and long lasting.
7. Very high control potential by integrating fungicide resistant antagonist.
8. Biopesticides help in induced system resistance among the crop species. Eg: *Trichoderma* sp. resistant to fungicide like, Benomyl and Metalaxyl etc.

Identification

Trichoderma (Plate 1) is one of the common fungal biocontrol agent, and is used worldwide for suitable management of various foliar and soil borne plant pathogens. Biocontrol agents like *Trichoderma* spp. are acclaimed as effective, eco-friendly and cheap, nullifying the ill effects of chemicals. Therefore, these biocontrol agents are identified to act against on array of important soil borne plant pathogens causing serious diseases of crops (Khandelwal et al., 2012; Motlagh and Samimi, 2013; Babu and Pallavi, 2013).

Trichoderma viride (Plate 2)

1. Conidia: 1 celled, green or brownish, subglobose to ellipsoid, rough spored.
2. Conidiophore: Narrow, flexous, primary branching at regular interval, paired or in whorls of three, short or extensively branched.
3. Phialides: Mostly in verticles of 2-3.
4. Chlaymdospores: Present in mostly isolates.
5. Colony character: Grow rapidly 5-9 cm diameter after 4 days at 20°C.
6. Reverse pigment: Colourless to dully yellowish.

Trichoderma harzianum (Plate 3)

1. Conidia: Subglobose to obvoid or short ellopsiod, 1.7-3.2x1.3-2.5 µm.
2. Conidiophore: Hyaline, smooth walled, straight flexuous up to 8 um wide near base 2.5-4.5 µm wide for most of the length, high branched. Primary branching at right angles, whorls of two or three secondary branching of whorl of 2.4, ultimate branching single celled.
3. Phialides-Ampuliform to subglobose or lengiform 3.5-7.5 x2.5-3.8 µm arise mostly in the crowded and diverse whorls of 2-6.
4. Colony character-grow rapidly, floccase aerial mycelium.
5. Reverse pigment: Colourless to dull yellowish.

Identification of bacterial antagonists

Identification was carried out as per Buchanan and Gibbons (eds) 1974 (Bergey’s manual of systematic bacteriology). Fluorescent Pseudomonads (Plate 4) produce fluorescent pigment on King’s B medium but not on $FeCl_3$ - amended King’s B medium. All of them show positive reaction for Kovac’s oxidase test, arginine dihydrolase and gelatin liquefaction except for *P. putida* which is negative for gelatin liquefaction. Identification can also be carried out at the Microbial Type Culture Collection Centre (MTCC) of Institute of Microbial Technology, Chandigarh.

Bacillus spp. are bacillus-shaped under the electron microscope, Gram-positive, and motile. Many strains of *B. subtilis* have been shown to be potential biocontrol agents against fungal pathogens. Evidence to date suggests that the principal mechanism of this antifungal action involves the production of antibiotics, especially

Plate 1. Different cultures of *Trichoderma* isolates.

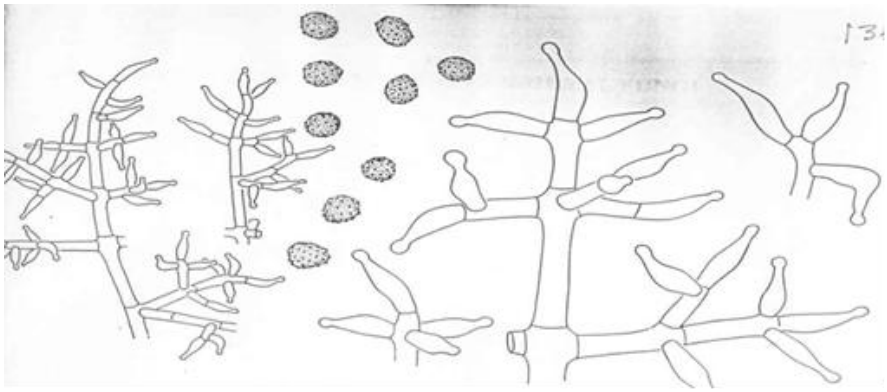

Plate 2. Conidia and conidiophore *Trichoderma viride*.

within soil microsites (Fravel, 1988). However, it is likely that several mechanisms act in concert to achieve control, including the production of volatiles, which have a significant effect on soil microbiology (Kim et al., 2003).

MECHANISMS OF CONTROL

Antibiosis

It occurs during interactions with other microorganisms involving low molecular or it is mediated by the specific or non-specific metabolite of microbial origin, by lytic enzyme, volatile compound or other toxic substance (Marfori et al., 2002; Howell, 2003; Limon et al., 2004; El-Ghaouth et al., 2004; Singh and Sharma, 2007). It produces:

a) Antibiotic: These are the microbial toxin at low concentration, poison or kill other microorganisms. For example, *Gliocladium virens* producing Gilotoxin is responsible for the death of the *Rhizoctonia solani* on potato tubers. Colonization of pea seed by *T. viride* result *in* significant amount of antibiotic producing viridin in the seed controlling *Pythium* spp. Phenazine antibiotic (Phz) produced by *Pseudomonas fluorescens* strain 2-79 has been implicated in control of take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici.* (Handelsman and Parke, 1989).

b) Bacteriocin: These are the antibiotic like substance that help to control the crown gall by the related *Agrobacterium radiobacter* strain K84.

c) Volatile compound and metabolite production: Micro-organisms which produce metabolite that can interfere with the pathogen on growth and activities. For example, a volatile or water-soluble substance produced by the host fungus serves as a chemoattractant for parasites (Deacon and Berry, 1992). *Trichoderma* spp. is a strain which is known to produce a number of antibiotics such as trichodernin, trichodermol, harzianun and harzianolide (Kucuk and Kivanc, 2004). The combination of hydrolytic enzymes and antibiotics results in a higher level of antagonism than that obtained by either mechanism alone (Howell, 1998).

Hyperparasitisms/mycoparasitism

Direct parasitisms or lysis of the death pathogen by other microorganisms is known as a hyperparasitism. Fungi that are parasitic on other fungi are usually referred to as mycoparasites (Baker and Cook, 1974). For example, Weindling in 1932 observed *Trichoderma lignorum* (*T. viride*) parasitizing the hyphae of *Rhizoctonia solani* and also suggested that inoculating soil with *Trichoderma* spores helps to control damping off of citrus seedling (Lo, 1997.) *Sporidesmium sclerotium*, is a biotrophic parasite and is often found only on sclerotia of plant pathogenic

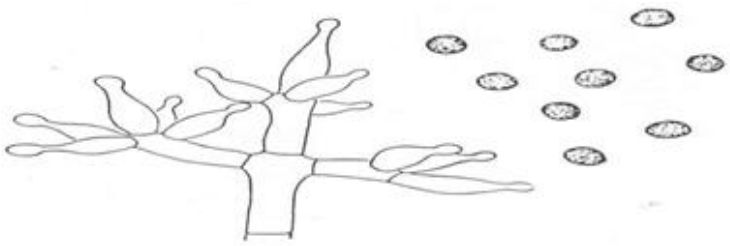

Plate 3. Conidia and conidiophore *Trichoderma harzianum*.

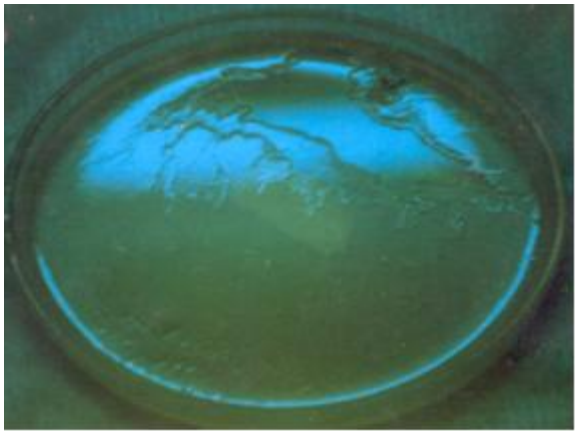

Plate 4. Culture of *Pseudomonas flourescens*.

fungi such as *Sclerotinia minor* and *Sclerotium cepivorum* (the causal agents of lettuce drop) (Adams and Ayers, 1983). The mycoparasitic ability of *Trichoderma* species against some economically important plant pathogens allows for the development of biocontrol strategies (Harman et al., 2004; Motlagh and Samimi, 2013).

Competition

Trichoderma species are generally considered to be aggressive competitors, grow very fast and rapidly colonize substrates to exclude pathogens such as *Fusarium* spp. (Papavizas, 1985). Iron competition in alkaline soils may be a limiting factor for microbial growth in such soils (Costa and Loper, 1994). A few studies have demonstrated that siderophore biosynthesis in *P. fluorescens* plays a role in pathogen suppression (Costa and Loper, 1994; Leong and Expert, 1989). Leeman et al. (1996) have reported that iron-chelating salicylic acid is produced by selected *P. fluorscens* strains at low iron availability and may be involved in the induction of systemic resistance to *Fusarium* wilt of radish. A significant amount of research on the use of the microbial antagonists has been reviewed by several workers (Wisniewski et al., 1989; Droby et al., 1992; Piano et al., 1997; Castoria et al., 1997; Kim et al., 1997; Arras et al., 1998; Kurtzman and Droby, 2001; El-Ghaouth et al., 2004; Grebenisan et al. 2008; Saravanakumar et al., 2008).

PSEUDOMONAS FLOURESCENS ACTS AS A PLANT GROWTH-PROMOTING *RHIZOBACTERIA*

Plant growth-promoting rhizobacterium (PGPR) is present near the roots and colonizes the plant roots, and it reduces the incidence of plant disease(Kloepper and Schroth, 1978) .The PGPR works as an aggressive colonization, plant growth stimulation and biocontrol (Kloepper, 1980; Défago and Haas, 1990; Weller et al., 2002; Vessey, 2003; Lucy et al., 2004). In the mechanisms of PGPR, the process includes nitrogen fixation, phosphate solubilization and the production of phytohormones (such as auxin and cytokinin) and volatile growth stimulants (such as ethylene and 2,3-butanediol) which help to control many pathogens. *Pseudomonas* sp. is ubiquitous bacterium in agricultural soils. *Pseudomonas* spp. controls the diseases by producing siderophore (pyochelin) which has been identified as an antifungal antibiotic in a screening program (Phoebe et al., 2001). The characteristics of *Pseudomonads spp*are: (i) It produces a wide spectrum of bioactive metabolites (antibiotics, siderophores, volatiles and growth- promo-ting substances) (ii) It competes aggressively with other micro-

organisms and it adapts to the environmental stresses also. (iii) Pseudomonads are also responsible for suppression of soil borne pathogens (Weller et al., 2002). Raaijmakers et al. (2002) and Morrissey et al. (2004), reported that some strains of *Pseudomonas* spp. produce more than one antibiotic compounds but they are not related to siderophores. *Invitro* studies revealed that, these antibiotics can inhibit fungal pathogens, but they can also be active against many bacteria. PGPR can also provide protection against viral diseases. Today, the use of PGPR has become a common practice in many regions of the world.

Antifungal activity of PGPR

P. fluorescens produces 2,4-diacetyl phloroglucinol which inhibits the growth of phytopathogenic fungi (Nowak-Thompson et al., 1994). Some PGPR degrade the fusaric acid produced by *Fusarium* sp. causal agent of wilt, which helps to prevent the pathogenesis (Toyoda and Utsumi, 1991). Some PGPR can also produce enzymes that can lyse the fungal cells. For example, *Pseudomonas stutzeri* produces extracellular chitinase and laminarinase which lyses the mycelia of *Fusarium solani*. *P. fluorescens* protects the plants against a wide range of important agronomic fungal diseases such as black root-rot of tobacco (Voisard et al., 1989), root-rot of pea (Papavizas and Ayers, 1974) root-rot of wheat (Garagulia et al., 1974) and damping-off of sugar beet (Fenton et al., 1992).

Pseudomonas shows biocontrol potential against phytopathogenic fungi *in vivo* and *in vitro* conditions from chickpea rhizosphere (Saraf et al., 2008). *P. putida* has potential for the biocontrol of root-rot disease complex of chickpea by showing antifungal activity against *Macrophomina phaseolina* (Laville et al., 1998).

Mass multiplication of fungal and bacterial antagonist

Methods which arE used for mass multiplication:

a) Solid state fermentation
b) Liquid state fermentation

Substrate which are used for solid fermentation

Sorghum grain, wheat straw, wheat bran, wheat bran-saw dust medium, sand and sorghum medium, tapioca rind, coffee husk, sand-corn meal medium, rice bran and vegetable waste.

Procedure: Solid fermentation

The waste substrate cut in the form of pieces is shade dried under the shade. The dried substrate is measured and a gram of flour and yeast extracts is added for nutritional purpose. The moisture level of that mixture is maintained up to 40%. 150 g of substrate are taken (Rice bran, FYM) into polypropylene bag, heat sealed and sterilized at 15 lb of pressure for 2-4 h for 3 successive days. Each bag is inoculated with the mycelia disk/liquid broth of *Trichoderma* and incubated at 28°C for 7-10 days. Solid substrate with *Trichoderma* can be mixed with the sand (1:2) applied to the soil directly

Constraints in solid fermentation

1. Preparation is bulky.
2. There is a greater risk of contamination.
3. Require larger space for growing, incubation and storage.

Liquid fermentation

Media used for liquid fermentation

Molasses-brewer's yeast medium, Richards medium, Czapex Dox broth, V-8 Juice, PDB, Molasses-soy medium and Jaggery medium. *Trichoderma* is generally mass multiplied on molasses yeast broth medium.

Composition: Molasses - 30 g; yeast powder- 5 g; distilled water- 1 L.

Procedure

The ingredients were mixed with the distilled water and poured in a conical flask/horlicks bottle. The flasks were plugged, covered and kept for sterilization in autoclave for 15 min at 15 lbs. After Cooling at room temperature, they were inoculated with the mycelia disk of trichoderma (from 5-6 days old culture). After inoculation, the flasks were incubated byusing two methods:

a) Stationary culture: The flasks containing sterilized molasses and yeast broth are inoculated at room temperature for 10 days. The fungal culture broth is used for the preparation of the formulation.
b) Shaker culture: The flasks containing sterilized molasses and yeast broth are inoculated with *Trichoderma* culture and kept on a rotatory shaker at 150-180°C rpm for 3-5 days. In shaker culture more conidia or chlamydospores are produced. *Trichoderma* could be mass multiplied on large scale in a short period using fermenter.

Methods for preparation of talc based formulation

Trichoderma is multiplied in molasses/yeast medium / nutrient broth. The biomass produced is homogenized in a mix. The homogenized mix of biomass is added to talc

Table 1. Shelf life of bacteria based on the formulations.

Formulation	Shelf life	Bacteria	Reference
Talc	12 month	*P. fluorescens*(P7NF, TL3)	Caesar and Burr (1991)
Talc	8 month (1.3 x 10^7cfu/g)	*P. fluorescens* (Pf1)	Vidhyasekaran and Muthamilan (1995)
alc	45 days (1.0 x 10^6 cfu/g)	*B. subtilis*	Amer and Utkhede (2000)
Talc	30 and 180 6 moths (>1 x 10^8cfu/g)	*P. putida*	Bora et al. (2004)
Lignite	4 months (2.8 x 10^6cfu/g)	*P. fluorescens* (Pf1)	Vidhyasekaran and Muthamilan (1995)
Peat supplemented with chitin	6 months (>1 x 10^9cfu/g)	*B. subtilis*	Manjula and Podile (2001)

powder in 1:2 ratio and mixed thoroughly. The contents are shade dried for 2-3 days. After drying, the mix is grinded to make fine powder. A sticker like carboxy methyl cellulose 0.5% is added to the talc powder and mix. The formulation is ready. The formulation powder is added to the white plastic bags, sealed and labeled.

Advantage of liquid fermentation

1. It requires less space and labour
2. Contamination is not a major problem
3. Large quantities of biomass can be realized in short time
4. Culture can be manipulated
5. Variety of formulations required for various applications can be developed

Commercial products of *Trichoderma* spp.

Bio-Fungus, Binab T, TopShield RootShield T-22G, T-22 Planter Box, (Bio-Trek), Supresivit, Trichodex, Trichopel, Trichoject, Trichodowels, Trichoseal, Trichoderma 2000**.**

Mass multiplication of *Pseudomonas flourescens*

Add 0.6-0.8 g *Pseudomonas* agar media in 250 mL flask. Then it was autoclaved and inoculated with the culture. Then, it was kept on a rotary shaker for 48 h, mixed with 1:2 sterilized talc powder and calcium carbonate was added. It was mixed and packed aseptically.

Carriers used in the formulation

There are two types of carriers which are available in organic or non-organic form. They should be economical and easily available. The organic carriers are peat, talc, lignite, kaolinite, pyrophyllite, zeolite, montmorillonite, alginate, pressmud, sawdust and vermiculite, etc. Carriers increase the survival efficiency of bacteria (Heijnen et al., 1993). Survival of *P. fluorescens* (2-79RN10, W4F393) in montmorillonite, zeolite and vermiculite increases as compared to the phyllite and talc.

Talc based formulations

Krishnamurthy and Gnanamanickam (1998) developed talc based formulation of *P. fluorescens* for the management of rice blast caused by *Pyricularia grisea*, in which methyl cellulose and talc is mixed at 1: 4 ratio and it is used in the concentration of 10^{10} cfu/mL. Talc based formulations were also effective against rice sheath blight (Nakkeeran et al., 2004). Survival period of *P. putida* strain is up to 6 months in talc based formulations (Bora et al., 2004) (Table 1).

Method of application

Time of application of the *Trichoderma* is also important. *Trichoderma* can't tolerate heavy pressure. Therefore, it may be used strictly as a preventive measure, it can't cure infection. *Trichoderma* is least effective against the systematic disease than against more superficial one. It can not control the existing disease. A combination of chemical treatment with *Trichoderma* will be highly effective. A single strain of *Trichoderma* may not be sufficient to be effective under all conditions and against all diseases. A mix of different biocontrol agents is effective against all the diseases.

Seed treatment

Seed treatment is the most effective method. In seed treatment, seed priming is the process in which hydration of seed is controlled to a level that permits pregermi-native metabolic activity to take place without emergence of the radical. Treatment of pigeonpea seeds with talc based formulation of *P. fluorescens* (Pf1) effectively helps to control *fusarium* wilt of pigeonpea (Vidhyasekaran et al., 1997).

Table 2. Commercial formulations of biocontrol agents available in India.

Product	Bio agent	Use
Antagon-TV	*T. viride*	As seed and soil treatment for control of *R. solani* and *M. phaseolina* in pulses and vegetables
Biocon	*T. viride*	Available in broth and dust formulations and used for the control of root and stem diseases of tea.
Bioderma	*T. viride* + *T. harzanium*	As seed treatment, soil treatment, seedling dip and foliar spray against the fungal pathogens of vegetables, pulses and cereals, also in sugar crops.
Biogaurd	*T. viride*	As seed and soil treatment for the control of seed and soil borne diseases of vegetables and pulses.
Biosheild	*Pseudomonas fluorescens*	As seed, soil and seedling dip against fungal pathogens of cereals, pulses and vegetables.
Biotok	*Bacillus subtilis*	Available in broth formulation and used for the control of black rot disease of tea caused by *Corticium invisum* and *C. theae.*
Defence-SF	*T. viride*	As seed and soil treatment for the management of seed and soil-borne diseases of crops

It was reported that numerous strains of fungal and bacterial isolates have biocontrol activity against several plant pathogenic fungi (Tables 3 and 4).

Table 3. Fungal bioagent of various plant pathogens in India.

Pathogen	Crop	Bio-agent
Macrophomina phaseolina	Cowpea	*T. viride, T. harzanium*
Fusarium udum	Pigeon pea	*Trichoderma* spp*. , T. viride*
Pythium	Ginger	*T. viride, T. longibrachitum, T. virens*
Phytophthora	Cardomom	*T. harzanium*
Ustilago segetum var.tritici	Wheat	*T. koningii*
Cercospora moricola	Mulberry	*Trichoderma* spp.
Botrytis cinerea	Rose	*T. viride, T. harzanium*

Seedling dip

Application of *P. fluorescens* strain mixtures by dipping the seedling of rice in bucket of water containing talc based formulation containing mixture of (20 g/l) for 2h and later transplanting it in the field helps to control sheath blight of rice (Nandakumar et al., 2001).

Seed priming

PGPR increases germination and improve seedling establishment. Bio-priming of seeds with bacterial antagonists increase the population load of antagonist to a tune of 10 fold on the seeds thus protected rhizosphere from the ingress of plant pathogens (Callan et al., 1990).

Soil application

Actively growing population is applied in the soil. *Trichoderma* can be applied as granule as well as drench. In transplanted crops granules are applied in nursery. Tomatoes were grown in a potting mix containing the granular formulation of *T. Harzanium*. Vidhyasekaran and Muthamilan (1995) reported that the soil application of peat based formulation with *P. fluorescens* (Pf1) at the rate of 2.5 kg of formulation mixed with 25 kg of well decomposed farm yard manure, helps to control the chickpea wilt caused by *Fusarium oxysporum* f.sp. *ciceris*.

Foliar spray

Delivering of *Pseudomonas* to beet leaves inhibits the amino acids on the leaf surface and inhibits spore germination of *Botrytis cinerea*, *Cladosporium herbarum* and *Phoma betae* (Blakeman and Brodie, 1977). Application of *B. subtilis* to bean leaves reduces the incidence of bean rust (*Uromyces phaseoli*). Seed treatment and foliar application of *P. fluorescens* reduces the severity of rust and leaf spot under field conditions. Some commercially available biocontrol agents are listed in Table 2.

Table 4. Evidence for successful experimental use of biological control agents of various diseases.

S/N	Pathogen	Bioagent	Reference
1	*Fusarium moniliforme*	*T. harzianum*	Harleen and Chander, 2011
2	*Rhizoctonia solani*	*Trichoderma* spp.	Kalita et al., 2012
3	*Macrophomina phaseolina*	*Trichoderma viride*, *T. harzianum*	Adekunle et al., 2001 ; Kaur et al., 2010
4	*Colletotrichum truncatum*	*T. viride*	Pandit & Kaushal, 2011
5	*Colletotrichum capsici*	*T. viride*	Sangeetha et al., 2011; Jagtap et al., 2013
6	*Pythium aphanidermatum*	*T. viride, T. harzianum*	Muthukumar 2009; Triki and Prlou, 1997
7	*Phytophthora capsici,* *Fusarium oxysporum* f. sp. *lycopersici*	*T. harzianum*	Sriram et al., 2010
8	*Alternaria porri*	*T. viride*	Yadav et al., 2013
9	*Fusarium oxysporum* f.sp. udum	*T. viride*	Kapoor et al., 2010
10	*Phytophthora capsici*	*T. viride*	Mathew et al., 2011
11	*Botrytis cinerea*	*Bacillus subtilis*	Zhao et al., 2007
12	*Pythium aphanidermatum*	*Bacillus subtilis* BSCBE4	Nakkeeran et al., 2006; Jayaraj et al., 2005; Ongena et al., 2005
13	*Alternaria alternata*	*B. subtilis*	Yang et al., 2006
14	*Colletotrichum gloeosporioides*	*Bacillus licheniformis*	Govender et al., 2005
15	*Monilinia fructicola*	*Cryptococcus laurentii*	Tian et al., 2004; Qin et al., 2006
16	*Debaryomyces hansenii*	*Penicillium digitatum*	Singh, 2002
17	*Debaryomyces hansenii*	*Rhizopus stolonifer*	Mandal et al., 2007; Singh, 2004, 2005
18	*Colletotrichum musae*	*Trichoderma harzianum*	Devi and Arumugam, 2005
19	*Botryodiplodia theobromae*	*Trichoderma harzianum*	Sivakumar et al., 2001
20	*Erwinia carotovora* sub sp. *carotovora*	*Pseudomonas putida*	Colyer and Mount, 1984
21	*Colletotrichum gloeosporioides*	*Brevundimonas diminuta*	Kefialew and Ayalew, 2008
22	*Rhizoctonia solani*	*T. harzianum, T. hamatum*	Elad et al., 1983; Abada and Abdel-Aziz, 2002
23	*M. fructicola, M. laxa*	*Bacillus subtilis*	Fan et al., 2000; Casals et al., 2010
24	*Ustilago avenae*, *Uncinula necator*	*T. virens*	Schirmbock et al., 1994
25	*Sclerotium rolfsii*	*Trichoderma* spp.	Dubey et al., 1996; Dev and Dawande, 2010
26	*Gaeumannomyces graminis* var. *tritici*	*Pseudomonas Fluorscens* strain 2-79	Weller and Thomashow, 1993
27	*Bipolaris oryzae*	*Pseudomonas sp.* *P. aeruginosa* *Bacillus* sp. *B. subtilis*	Vasudevan et al 2002
28	*Pythium* spp.	*Trichoderma* spp.	Dubey et al., 1996
29	*Sclerotinia sclerotiorum*	*Bacillus* spp.	Zhang et al., 2009; Yang et al., 2009
30	*Thielaviopsis basicola*	*Pseudomonas Fluorscens* strain CHA0	Lam and Gaff ney, 1993

Table 4. Contd.

31	*Macrophomina phaseolina*	*Trichoderma* spp.	Dubey et al., 1996
32	*Peronophythora litchi*	*Bacillus subtilis*	Jiang et al., 2001
33	*Pythium ultimum*	*T. virens*	Howell and Stipanovic, 1995
34	*Aspergillus niger*, *A. flavus*	*Trichoderma* spp	Patale and Mukadam, 2011
35	*Sclerotinia sclerotiorum*	*Pseudomonas aeruginosa*	Deshwal, 2012
36	*Ralstonia solanacearum*	*B. subtilis*	Lemessa and Zeller, 2007; Aliye et al., 2008; Ji et al., 2008; Maketon et al., 2008; Ji et al., 2008; Chen et al., 2012
37	*Fusarium ciceri*	*Trichoderma* spp	Anand and Reddy, 2009
38	*Pseudomonas syringae*	*B. subtilis* strain (ATCC 6051)	Bais et al., 2004
39	Chalara elegans	*Pseudomonas fluorescens*	De´fago and Haas, 1990
40	Endothia parasitica	*Cryphonectria parasitica*(Hypovirulent strains)	Milgroom and Cortesi, 2004
41	*Pythium spp.*	*Pseudomonas fluorescens* F113	Shanahan et al., 1992
42	*Aspergillus flavus*	*Bacillus subtilis* AU195	Moyne et al., 2001
43	*Uromyces phaseoli*	*Bacillus subtilis*	Bettiol and Varzea, 1992
44	*Phytophthora citrophthora* *Phytophthora parasitica*	*Bacillus subtilis*	Kupper, 2009; Amorim and Melo, 2002
45	*Penicillium digitatum*	*Bacillus subtilis*	Leelasuphakul et al., 2008
46	*Hemileia vastatrix*	*Bacillus subtilis*	Haddad et al., 2009
47	*Fusarium oxysporum*	*Bacillus amyloliquefaciens* FZB42	Koumoutsi et al., 2004
48	*Sclerotium oryzae*	*P. fluorescens* *P. aeruginosa* *B. subtilis* *B. pumilus*	Vasudevan et al., 2002
49	*Botryodiploidia theobromae*	*Bacillus* spp	Swain et al., 2008
50	*Aphanomyces cochlioides*	*Lysobacter* sp. strain SB-K88	Islam et al., 2005
51	*Pythium aphanidermatum*	*B. subtilis* (BBG100 & GB03)	Leclere et al., 2005; Corrêa et al., 2010
52	*Pyricularia oryzae*	*Burkholderia cepacia*	Homma et al., 1989
53	*Phytophthora medicaginis* and *P. aphanidermatum*	*Bacillus cereus* UW85	Smith et al. 1993
54	*Colletotrichum lindemuthianum*	*T. harzianum* (T-39)	Harman et al., 2004
55	*Pyricularia oryzae*	*T. harzianum*	Dey et al., 2013
56	*Colletotrichum graminicola*	*T. harzianum* (T-22)	Harman et al., 2004
57	*Pseudomonas syringae* pv *lachrymans*	*T. asperellum* T-203	Grondona et al., 1997
58	*Colletotrichum acutatum,* *Colletotrichum lagenarium*	*T. harzianum*	Svetlana et al., 2010

Table 4. Contd.

	Sphaerotheca fuliginea	*Ampelomyces quisqualis*	Philipp, 1988
60	*Agrobacterium tumefaciens*	*Agrobacterium radiobacter* strain K 84	Kerr et al., 1990
61	*Xanthomonas campestris*pv. *campestris*	*Bacillus* spp.	Monteiro et al., 2005; Wulff et al., 2002
62	*Erwinia amylovora*	*Pantoea agglomerans* strain P10c	Vanneste et al., 2002
63	*C. perniciosa*	*T. harzianum*	De Marco and Felix, 2002
64	*Uncinula necator*	*Verticillium lecanii*	Heintz and Blaich, 1990
65	*Botrytis* cinerea	*Trichoderma* spp	Tucci et al., 2011
66	*Xanthomonas oryzae* pv. *oryzae*	*T. harzianum* (NF-9)	Harman et al., 2004
67	*Cladosporium spp.*	*Bacillus subtilis*	Sivakumar et al., 2007
68	*Sarocladium oryzae*	*P. fluorescens* *B. subtilis* *P. aeruginosa* *Pseudomonas sp.*	Vasudevan et al., 2002
69	*Oidium mangiferae*	*Bacillus* spp.	Nofal and Haggag, 2006
70	*Curvularia lunata*	*T. harzianum*	Svetlana et al., 2010
71	*Gibberella fujikuroi*	*T. harzianum*	de la Cruz and Llobell, 1999
72	*Sclerotinia sclerotiorum*	*Epicoccum nigrum*	Elmer et al., 2002
73	*P. ultimum*	*Bacillus* spp	Idris et al., 2008
74	*Rhizoctonia meloni*	*T. harzianum*	Benítez et al., 2004
75	*Drechslera sorokiniana*	*T. harzianum*	Kucuk and Kivank, 2003
76	Sclerotinia spp	Coniothyrium minitans	Jones et al., 2004
77	*Oidium mangiferae*	*Ampelomyces quisqualis*	Kiss, 2003
78	*Cylindrocladium spathiphylli*	*Bacillus subtilis*	Wit et al., 2009.
79	*Cercospora nicotiana*	*Bacillus* spp	Maketon et al., 2008
80	*Xanthomonas campestris*pv. *vesicatoria*	*Bacillus subtilis*	Roberts, 2008
81	*Botryodiplodia theobromae*	*Bacillus* spp	Swain et al., 2008
82	*X. campestris pv. glycines*	*Bacillus* spp	Salerno and Sagardoy, 2003
83	*Pyricularia grisea*	*Pseudomonas fluorescens*	Vasudevan et al., 2002
84	Rice tungro virus Vector - *Nephotettix* spp.	*P. fluorescens* (for vector)	Vasudevan et al., 2002
85	*Xanthomonas oryzae* pv. *oryzae*	*Streptomyces* spp.	Hastuti et al., 2012
86	*Alternaria alternata*	*Trichoderma harzianum*	Gveroska and Ziberoski, 2012
87	*Curvularia lunata*	*T. viride*	Sumangala et al., 2008
88	*Peronosclerospora sorghi*	*T. Viride* with *T. harzianum, B. subtilis*	Sadoma, 2011
89	*Sclerotium sepivorum*	*T. harzianum*	Abd-El-Moity and Shatla, 1981
90	*Cephalosporium acemonum*	*T. harzianum*	El-Assiuty et al., 1986
91	*Colletotrichum falcatum*	*T. harzianum* strain T 37	Singhet al., 2008
92	*Puccinia sorghi*	*T. harzianum*	Dey et al., 2013

CONSTRAINTS IN THE USE OF A *TRICHODERMA*

Trichoderma spp. is an effective biocontrol agent that effectively controls the soilborne fungal pathogens, but, it produces adverse effects on the fungus arbuscular mycorrhizal (AM). AM fungi is presented in the roots of most herbaceous plants. Another problem has been low field performance of *Trichoderma* as biocontrol agent. *Trichoderma* spp. achieves only transitory localized dominance of the rhizosphere and these are active in only some soil and some season. *Trichoderma* spores are quiescent in active soil. *Trichoderma* spp. are likely to

be effective for seed and seedling diseases, but not against disease of mature crop.

CONCLUSION

Biological control of fungal diseases of plants is eco-friendly and is a potential component of integrated disease management. Biological control of foliar diseases has received less attention, owing to the poor establishment of the introduced biocontrol agents and resulting variations in disease control. Application of synthetic fungicides has been the traditional strategy for the management of plant diseases. The increasing concern for health hazards and environmental pollution due to chemical use has needed the development of alternative strategies for the control of plant diseases. Management of plant diseases by employing microbial agents has been demonstrated to be most suitable strategy to replace the chemicals which are either being banned or recommended for limited use. This review reported the success of some biocontrol agents under laboratory and commercial conditions, and some bioproducts that have been developed for commercial use.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Abada KA, Abdel-Aziz MA (2002). Biological and chemical control of stem canker and black-scurf on potato in Egypt.Bulletin of Faculty of Agriculture, Cairo University 53.115-128.

Abd-El-Moity TH, Shatla MN (1981). Biological control of white rot of onion *(Sclerotium sepivorum)* by *Trichoderma harzianum.* Phytopathology Z., 100:29-35.

Adams PB, Ayers WA (1983). .Histological and physiological aspects of infection of sclerotia of *Sclerotinia* species by two mycoparasites. Phytopathology 73 :1072 .

Adekunle AT, Cardwell KE, Florini DA, Ikotun T (2001). Seed treatment with *Trichoderma* species for control of damping-off of cowpea caused by *Macrophomina phaseolina*. Biological Control Science and Technoiogy 11:449-457.

Aliye N, Fininsa C, Hiskias Y (2008). Evaluation ofrhizosphere bacterial antagonists for their potential to bio-protect potato (*Solanum tuberosum*) against bacterial wilt (*Ralstonia solanacearum*). Biol. Control 47: 282–288.

Amer GA, Utkhede RS (2000). Development of formulations of biological agents for management of root rot of lettuce and cucumber. Can. J. Microbiol. 46: 809-816.

Amorim EPR and Melo IS (2002). Acao antagonica de rizobacterias contra *Phytophthora parasitica P. citrophthora. Revista Brasileira de Fruticultura*, 24(2):565-568.

Anand S, Reddy J (2009). Biocontrol potential of *Trichoderma* sp. against plant pathogens. Inter. J. Agric. Sci. 1(2):30-39.

Babu KN, Pallavi PN (2013). Isolation, identification and mass multiplication of Trichodermaan important bio-control agent.Int. J. Pharm. Life Sci. 4(1):2320-2323.

Bais HP, Fall R and Vivanco JM (2004) Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol.134: 307–319.

Baker KF, Cook RJ (1974). Biological Control of Plant Pathogens.Am. Phytopathol.Soc., St. Paul.MN. p.433.

Benítez T, Rincón A, Carmen Limón M et al. (2004). Biocontrol mechanisms of *Trichoderma* strains. Intl. Microbiol. 7: 249-260.

Bettiol W, Varzea VMP (1992). Controle biológico da ferrugem (*Hemileia vastatrix*) docafeeiro com *Bacillus subtilis* em condições controladas Fitopatolologia Brasileira*,* 17:91-95.

Blakeman JP, Brodie IDS (1977). Competition for nutrients between epiphytic microorganisms and germination of spores of plant pathogens on beet root leaves. Physiol. Plant Pathol.10: 29 - 42.

Bora T, Ozaktan H, Gore E, Aslan E (2004). Biological control of *Fusarium oxysporum* f. sp. *melonis* by wettable powder formulations of the two strains of *Pseudomonas putida*. J.Phytopathology152: 471-475.

Buchanan RE, Gibbons NE (1974). Bergey's manual of determinative bacteriology. 8th Edition. Williams & Wilkins Co. Baltimore, MD p.1246.

Caesar AJ, Burr TJ (1991). Effect of conditioning, betaine, and sucrose on survival of rhizobacteria in powder formulations. Appl. Environ. Microbiol. 57:168-172

Callan NW, Mathre DE, Miller JB (1990). Bio-priming seed treatment for biological control of *Pythium ultimum* pre-emergence damping-off in *sh2* sweet corn. Plant Dis. 74:368-372.

Casals C, Teixido N, Vinas I, Silvera E, Lamarca N, Usall J (2010). Combination of hot water, *Bacillus subtilis* CPA-8 and sodium bicarbonate treatments to control postharvest brown rot on peaches and nectarines. Eur. J. Plant Pathol.*,* 128:51-63.

Chen Y, Yan F, Chai Y,Liu H, Kolter R, Losick R, Jian-hua Guo (2012). Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends onconserved genes mediating biofilm formation. Environ. Microbiol. 1-17

Colyer PD, Mount MD (1984). Bacterization of potatoes with *Pseudomonas putida* and its influence on postharvest soft rot diseases. Plant Disease 68:703–706.

Corrêa EB, Bettiol W, Sutton JC (2010). Controle biológico da podridão radicular (*Pythium aphanidermatum*) e promoção de crescimento por *Pseudomonas chlororaphis* 63-28 e *Bacillus subtilis* GB03 em alface hidropônica. Summa Phytopathologica*,* 36:275-281.

Costa JM and Loper JE (1994).Characterization of siderophore production by the biological control agent *Enterobacter cloacae*. MPMI 7:440-448.

de la Cruz J, Llobell A (1999). Purification and properties of a basic endo-β-1,6-glucanase (BGN16.1) from the antagonistic fungus *Trichoderma harzianum*. Eur. J. Biochem. 265: 145-151

De Marco JL, Felix CR (2002). Characterization of a protease produced by a *Trichoderma harzianum* isolate which controls cocoa plant witches broom disease, BMC Biochemistry3: 3.

De´fago G, Haas D (1990). Pseudomonads as antagonists of soilborne plant pathogens: modes of action and genetic analysis. In: Bollag JM, Stotsky G (eds.) Soil Biochemistry. Marcel Dekker Inc, New York. pp. 249–291

Deacon JW, Berry LA (1992). Models of action of mycoparasites in relation to biocontrol of soilborne plantpathogens. Pages 157-167.in: Biological Control of Plant Diseases: Progress and Challenges for The Future. Tjamos EC, Papavizas GC, Cook RJ, eds., Plenum Press, New York

Défago G, Haas D (1990). Soil Biochemistry V(edsBollag, J. M. & Stotzky, G.) (Marcel Dekker, New York, 6:249–291

Deshwal VK (2012). *Pseudomonas aeruginosa* as biological control agent against Plant pathogenic fungus *Sclerotina sclerotiorum.* Inter. J. Plant*,* Anim. Environ. Sci. 2(1):14-17.

Dev N, Dawande AY (2010). Biocontrol of soil borne plant pathogen *Rhiozoctonia solani* using *Trichoderma* spp. and *Pseudomonas fluorescens*. Asiatic J. Biotech. Res. 01:39-44

Devi AN, Arumugam T (2005). Studies on the shelf life and quality of Rasthali banana as affected by postharvest treatments. Orissa J. Hortic. 33(2):3–6.

Dey Utpal, Harlapur SI, Dhutraj DN (2013). Bioassay of fungicides, botanicals, bioagents and indigenous technology knowledge (itk)

against *Pyricularia oryzae* Cav. causal agent of blast of rice. J. Plant Dis. Sci.. 8(1):102-106.

Dey Utpal, Harlapur SI, Dhutraj DN, Suryawanshi AP, Jagtap GP, Apet

KT, Badgujar SL, Gholve VM (2013). Effect of fungicides, botanicals, bioagents and Indigenous Technology Knowledge (ITK's) on germination of urediniospores of *Puccinia sorghi in vitro*. Afr. J. Agric. Res. 8(39):4960-4971.

Droby S (2006). Improving quality and safety of fresh fruit and vegetables after harvest by the use of biocontrol agents and natural materials. Acta Horticulturae 709, 45–51.

Droby S, Chalutz E, Wilson CL (1991). Antagonistic microorganisms as biocontrol agents of postharvest diseases of fruit and vegetables. Postharvest News Information. Droby S, Chalutz E, Wilson. 2:169–173

Eckert JW, Ogawa JM (1988). The chemical control of postharvest diseases:deciduous fruits, berries, vegetables and root/tuber crops. Annual Review of Phytopathology 26:433–469.

Elad Y, Chet I, Boyle P, Henis Y (1983).Parasitism of *Trichoderma* spp. on *Rhizoctoniasolani* and *Sclerotium rolfsii* scanning electron microscopy and fluorescence microscopy. Phytopathol, 73: 85-88.

El-Assiuty EM, Abd-El-Moity TH, El-Shafey HA (1986).Mycoparasitic effect of *Trichoderma harzianum* Rifai against *Cephalosporium acemonum* Corda.

Elmer PAG, Hoyte SM, Marsden RM, Parry F, Reglinski T (2002). *Epicoccum nigrum*: abiological control agent for the control of *Sclerotinia sclerotiorum* in New ZealandKiwifruit. The 5th International Symposium on Kiwifruit p. 60.

Fan Q, Tian SP, Li YX, Xu Y, Wang Y (2000). Biological control of postharvest brown rot in peach and nectarine fruits by *Bacillus subtilis* (B-912). Acta Botanica Sinica 42(11):1137-1143.

Fenton AM, Stephens PM, Crowley J, O'Callaghan M, O'Gara F (1992). Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. Appl. Environ. Microbiol. 58(Suppl 12): 3873–3878.

Fravel DR (1988). Role of antibiosis in the biocontrol of diseases.Ann. Rev. Phytopathol. 26, 75-91.

Garagulia AD, Kiprianova EA, Boiko OI (1974). Antibiotic effect of bacteria from the genus *Pseudomonas* on phytopathogenic fungi. MikrobioL Zh. (Kiev), 36 (Suppl 2): 197–202.

Govender V, Korsten L, Sivakumar D (2005). Semi-commercial evaluation of Bacillus licheniformis to control mango postharvest diseases in South Africa. Posthar. Biol. Technol. 38(1): 57–65.

Grondona I, Hermosa MR, Tejada M, Gomis MD, Mateos PF, Bridge PD, Monte E, Garcia-Acha I (1997). Physiological and biochemical characterization of *Trichoderma harzianum*, a biological control agents against soil borne fungal plant pathogens Appl. Environ. Microbiol. 63:3189-3198.

Gveroska B and Ziberoski J (2012). *Trichoderma harzianum* as a biocontrol agentagainst *Alternaria alternata* on tobacco. Appl. Technol. Innov. 7(2):67-76.

Haddad F, Maffia LA, Mizubuti ESG, Teixeira H (2009). Biological control of coffee rust by antagonistic bacteria under field conditions in Brazil. Biological Control, 49(2):114-119.

Handelsman Jo, Parke JL (1989). Mechanisms in biocontrol of soilborne plant pathogens. Pages.in:Plant-Microbe Interactions, Molecular and Genetic Perspectives. Kosuge T, Nester EW, eds., McGraw-Hill, New York . 3:27-61.

Harleen K, Chander M (2011). *In vitro* and *in vivo* evaluation of antagonistic potentiality of *Trichoderma* spp. against *Fusarium moniliforme* Sheld.causing stalk rot of maize. Plant Dis. Res. 26(2):173-173.

Harman G E, Petzoldt R, Comis A, Chen J (2004). Interactions between *Trichoderma harzianum* strain T22 and maize inbred line Mo17 and effects of these interactions ondiseases caused by *Pythium ultimum* and *Colletotrichum graminicola*. Phytopathology,94:147-153.

Hastuti RD, Lestari Y, Suwanto A, Saraswati R (2012). Endophytic *Streptomyces* spp. as Biocontrol Agents of Rice Bacterial Leaf Blight Pathogen (*Xanthomonas oryzae* pv. *oryzae*). HAYATI J. Biosci. 19 (4):155-162

Heijnen CE, Burgers SLGE, van Veer JA (1993). Metabolic activity and population dynamics of rhizobia introduced into unamended and betonite amended loamy sand. Appl. Environ. Microbiol.59: 743-747.

Heintz C and Blaich R (1990).*Verticillium lecanii*als Hyperparasit des Rehmchltaus (*Uncinula necator).*Vitis 29.229-232.

Homma Y, Kato Z, Hirayama F, Konno K, Shirahama H and Suzui T (1989). Production of antibiotics by *Pseudomonas cepacia* as an agent for biological control of soilborne plant pathogens. Soil Biol. Biochem. 21:723-728.

Howell CR and Stipanovic RD (1995). Mechanism in the biocontrol of *Rhizoctonia solani* induced cotton seedling disease by *Gliocladium virens*: Antibiosis Phytopathology, 85: 469-472.

Idris HA, Labuschagne N, Korsten L (2008). Suppression of *Pythium ultimum* root rot of sorghum by rhizobacterial isolates from Ethiopia and South Africa. Biological Control, 45(1):72-84.

Islam TM, Hashidoko Y, Deora A, Ito T, Tahara S (2005). Suppression of damping-offdisease in host plants by the rhizoplane bacterium *Lysobacter* sp. strain SB-K88 is linked toplant colonization and antibiosis against soilborne peronosporomycetes. Appl. Environ. Microbiol. 71:3786-3796.

Jagtap GP, Mali AK, Utpal Dey (2013). Bioefficacy of fungicides, bio-control agents and botanicals against leaf spot of turmeric incited by *Colletortricum capsici.* African Journal of Microbiology Research. 7(18):1865-1873

Jayaraj J, Radhakrishnan NV, Kannan R, Sakthivel K, Suganya D, Venkatesan S,Velazhahan R (2005). Development of new formulations of *Bacillus subtilis* for management of tomato damping-off caused by *Pythium aphanidermatum.* Biocontrol Sci. Technol. 15(1):55-65.

Ji XL, Lu GB, Gai YP, Zheng CC, Mu ZM (2008). Biological control against bacterial wilt and colonization of mulberry by an endophytic *Bacillus subtilis* strain. FEMS Microbiol. Ecol. 65: 565–573.

Ji XL, Lu GB, Gai YP, Zheng CC, Mu ZM (2008). Biological control against bacterial wilt and colonization of mulberry by an endophytic *Bacillus subtilis* strain. FEMS Microbiol. Ecol. 65(3):565-573.

Jiang YM, Zhu XR, Li YB (2001). Postharvest control of litchi fruit rot by *Bacillus subtilis.*Lebensmittel-Wissenschaft Und-Technologie-Food Sci. Technol. 34(7):430-436.

Jones EE, Mead A and Whipps JM (2004). Effect of inoculums type and timing of application of *Coniothyrium minitans* on *Sclerotinia sclerotiorum*: control of sclerotinia disease in glasshouse lettuce. Plant Pathol. 53: 611–620

Kalita DJ, Bhagawati B, Gogoi BB (2012). *In vitro* Efficacy of *Trichoderma* spp. Against *Meloidogyne incognita* and *Rhizoctonia solani.* Indian J. Nemat. 42(1): 84-86.

Kapoor Shashi, Jaiswal Ashish, Shukla DN (2010). Antagonistic Effect of Trichoderma Strains Against*Fusarium Oxysporum* f. Sp. *udum* Buttler Causing Wilt of Pigeon Pea. Agric. Sci. Digest. 30(3):189-191.

Kaur S, Singh N, Sandhu PS (2010). *In vitro* evaluation of *Trichoderma viride* and *T. harzianum* against *Macrophomina phaseolina*, causing charcoal root rot of sunflower.Plant Disease Research. 25(1):79-79.

Kefialew Y, Ayalew A (2008). Postharvest biological control of anthracnose (*Colletotrichum gloeosporioides*) on mango (*Mangifera indica*). Posthar. Biol. Technol. 50(1): 8-11.

Ken A, Deer SV, Schroth MN and Bahmc JB (1990). Biological control. Methods in Phvtobacteriologv. Ed. Z. Klcment. K.Rudolph and b. c. Sands. cBudapest. AkademiaiKiado.

Khandelwal M, Datta S, MehtaJ, Naruka R, Makhijani K,Sharma G, Kumar R, Chandra S (2012). Isolation, characterization & biomass production of *Trichoderma viride* usingvarious agro products- A biocontrol agent. Advan. Appl. Sci. Res. 3(6):3950-3955.

Kim Han-Soo, Jiyong Park, Sung-Won Choi, Kee-Hyun Choi, Gung Pyo Lee,Soo Jung Ban, Chang Hoo Lee, and Chung Sun Kim (2003). Isolation and Characterization of *Bacillus* Strains for Biological Control. J. Microbiol. 41(3):196-201.

Kiss L (2003). A review of fungal antagonists of powdery mildews and their potential asbiocontrol agents. Pest Manag. Sci. 59:475-483.

Kloepper JW, Leong J, Teintze M, Schroth MN (1980). *Pseudomonas* siderophores: a mechanism explaining disease-suppressive soils. Curr. Microbiol. 4:317–320.

Kloepper JW, Schroth MN (1978). In Proceedings of the IVth International Conference on Plant Pathogenic Bacteria Vol. 2879–882 (Station de Pathologie Végétale et Phytobactériologie, INRA, Angers, France, 1978).

Korsten L (2006). Advances in control of postharvest diseases in tropical fresh produce.International J. Postharvest Technol. Innovation 1(1), 48–61.

Koumoutsi A, Chen XH, Henne A, Liesegang H, Gabriele H, Franke P,

Vater J, Borris R (2004). Structural and functional characterization of gene clusters directingnonribosomal synthesis of bioactive lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J. Bact. 186:1084-1096.

Krishnamurthy K and Gnanamanickam SS (1998). Biological control of rice blast by *Pseudomonas fluorescens* strain Pf7-14: Evaluation of a marker gene and formulations. Biol. Contr. 13:158-165.

Kucuk C, Kivank M (2003). Isolation of Trichoderma Spp. and Determination of Their Antifungal, Biochemical and Physiological Features. Turk J Biol. 27: 247-253

Kupper KC (2009). Controle biológico de doenças de flores e frutos jovens de citros, In:*Biocontrole de doenças de plantas: uso e perspectivas*. Bettiol, W., Morandi, M.A.B. (eds),pp. 289-297., Embrapa.Jaguariúna.

Lam ST, Gaffney TD (1993). Biological activities of bacteria used in plant pathogen control. In: Biotechnology in Plant Disease Control.I. Chet, ed., John Wiley, New York. pp. 291-320

Laville J, Blumer C, Schroetter CV, Gaia V, Défago G, Keel C, Haas D (1998). Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. The Journal of Bacteriology, 180(Suppl 12):3187–3196.

Leclere V, Bechet M, Adam A, Guez JS, Wathelet B, Ongena M, Thonart P, GancelF, Chollet-Imbert M, Jacques P (2005). Mycosubtilin overproduction by *Bacillussubtilis* BBG100 enhances the organism's antagonistic and biocontrol activities. Appl. Environ. Microbiol. 71:4577-4584.

Leelasuphakul W, Hemmanee P, Chuenchitt S (2008). Growth inhibitory properties of *Bacillus subtilis* strains and their metabolites against the green mold pathogen (*Penicillium digitatum* Sacc.) of citrus fruit. Posthar. Biol. Technol. 48(1):113-121.

Leeman M, Den Ouden FM, Van Pelt JA, Dirkx FPM, Steijl H, Bakker PAHM and Schippers B (1996). Iron availability affects induction of systemic resistance to *Fusarium* wilt of radish by *Pseudomonas fluorescens*. Phytopathology 86:149-155.

Lemessa F, Zeller W (2007) Screening rhizobacteria for biological control of *Ralstonia solanacearum* in Ethiopia. Biol. Cont. 42: 336–344.

Leong SA, Expert D (1989). Siderophores in plant pathogen interactions.Pages 62-83.in: Plant-Microbe Interactions, Molecular and Genetic Perspectives, T. Kosuge, and E. W. Nester, eds., McGraw-Hill, New York Vol. 3.

Lo CT (1997). Biological control of turfgrass diseases using *Trichoderma harzianum*. Plant Prot. Bull. 39: 207-225 .

Lorito M (2005). Molecular Biology of the Interactions between Trichoderma, Phytopathogenic Fungi and Plants: Opportunities for Developing Novel Disease Control Methods. Second Global Conference."Plant Health-Global Wealth". Abstracts, 162-163.

Lucy M, Reed E, Glick BR (2004). Applications of free living plant growth-promoting rhizobacteria. *Antonie vanLeeuwenhoek* 86, 1–25.

Maketon M, Apisitsantikul J, Siriraweekul C (2008). Greenhouse evaluation of *Bacillus subtilis* AP-01 and *Trichoderma harzianum* AP-001 in controlling tobacco diseases. Braz. J. Microbiol. 39(2):296-300.

Mandal G, Singh D, Sharma RR (2007).Effect of hot water treatment and biocontrol agent (*Debaryomyces hansenii*) on shelf life of peach. Indian J. Hortic. 64 (1): 25–28.

Manjula K and Podile AR (2001). Chitin supplemented formulations improve biocontrol and plant growth promoting efficiency of *Bacillus subtilis* AF1. Can. J. Microbiol. 47:618-625.

Mathew Sally K, Mary CF, Gleena Gopal K, Surendra Girija D (2011). Antagonistic activity of endophytic Trichoderma against *Phytophthora* rot of black pepper (*Piper nigrum* L.). J. Biol. Control. 25(1):48-50.

Milgroom MG, Cortesi P (2004) Biological control of chestnut blight with hypovirulense: a critical analysis. Ann. Rev. Phytopathol. 42: 331–638.

Monteiro L, Rosa de Lima Ramos Marianoand Ana Maria Souto-Maior (2005). Brazilian Archives of Biology and Technology. 48(1) : 23-29.

Morrissey JP, Cullinane M, Abbas A, Mark GL, O'Gara F (2004). In *Pseudomonas* (ed. Ramos J-L.) (Kluwer Academic/Plenum Publishers, New York. 3:637–670

Moyne AL, Shelby R, Cleveland TE, Tuzun S (2001). Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. J. Appl. Microbiol. 90:622-629.

Muthukumar A (2009). Trichoderma *viride* and *Pseudomonas fluorescens* Formulations Against *Pythium aphanidermatum* in *in vitro.* Indian J. Plant Protection. 37(1,2):204-206.

Nakkeeran S, Kavitha K, Chandrasekar G, Renukadevi P, Fernando WGD (2006). Induction of plant defence compounds by *Pseudomonas chlororaphis* PA23 and *Bacillus subtilis* BSCBE4 in controlling damping-off of hot pepper caused by *Pythium aphanidermatum.* Biocont. Sci. Technol., 16(4):403-416.

Nakkeeran S, Kavitha K, Mathiyazhagan S, Fernando WGD, Chandrasekar G and Renukadevi P (2004). Induced systemic resistance and plant growth promotion by *Pseudomonas chlororaphis* strain PA-23 and *Bacillus subtilis* strain CBE4 against rhizome rot of turmeric (*Curcuma longa* L.). Can. J. Plant Pathol. 26: 417-418.

Nandakumar R, Babu S, Viswanathan R, Raguchander T, Samiyappan R (2001). Induction of systemic resistance in riceagainst sheath blight disease by *Pseudomonas fluorescens*. Soil Biol. Biochem. 33:603–612.

Nofal MA, Haggag WM (2006). Integrated management of powdery mildew of mango in Egypt. Crop Protection, 25(5):480-486.

Nowak-Thompson B, Gould SJ, Kraus J, Loper JE (1994).Production of 2, 4-diacetylphloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Pf-5. Can. J. of Microbiol. 40 (Suppl 12): 1064–1066.

Ongena M, Duby F, Jourdan E, Beaudry T, Jadin V, Dommes J, Thonart P (2005). *Bacillus subtilis* M4 decreases plant susceptibility towards fungal pathogens by increasing host resistance associated with differential gene expression. Appl. Microbiol. Biotechnol. 67(5):692-698.

Pandit D and Kaushal RP (2011). *In vitro* evaluation of bio-control agents and biopesticides against *Colletotrichum truncatum.* Plant Dis. Res. 26(2):179.

Papavizas GC (1985). *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. Ann. Review of Phytopathol, 23: 23-54

Papavizas GC, Ayers WA (1974). Aphanomyces species and their root diseases in pea and sugarbeet.A Review, US Department of Agriculture, Washington DC.

Patale SS and Mukadam DS (2011). Management of plant pathogenic fungi by using *Trichoderma* Bioscience Discovery, 2(1): 36-37

Philipp WO (1988). Biologische Bekampfung von Pflanzenkrankheiten. Stuttgart, Eugen Ulmer.

Phoebe CH Jr, Combie J, Albert FG, Van Tran K, Cabrera J, Correira HJ, Guo Y, Lindermuth J, Rauert N, Galbraith W, Selitrennikoff CP (2001). Extremophilic organisms as an unexplored source of antifungal compounds. J. Antibiot. 54:56–65.

Qin GZ, Tian SP, Xu Y, Chan ZL, Li BQ (2006). Combination of antagonistic yeasts with two food additives for control of brown rot caused by *Monilinia fructicola* on sweet cherry fruit. J. Appl. Microbiol. 100 (3):508–515.

Raaijmakers JM, Vlami M, de Souza JT (2002). Antibiotic production by bacterial biocontrol agents.Antonie vanLeeuwenhoek 81, 537–547

Roberts PD, Momol MT, Ritchie L, Olson SM, Jones JB, Balogh B (2008). Evaluation of spray programs containing famoxadone plus cymoxanil, acibenzolar-S-methyl,and *Bacillus subtilis* compared to copper sprays for management of bacterial spot ontomato. Crop Protection, 27(12): 1519-1526.

Sadoma MT, El-Sayed ABB, El-Moghazy SM (2011). Biological control of downy mildew disease of maize caused by *Peronosclerospora sorghi* using certain biocontrol agents alone or in combination. J. Agric. Res. Kafer El-Sheikh Univ., 37(1):1-11.

Salerno CM, Sagardoy MA (2003). Antagonistic activity by *Bacillus subtilis* against *Xanthomonas campestris* pv. *Glycines* under controlled conditions. Spanish J. Agric. Res. 1(1):55-58.

Sangeetha A, Kumar P Suresh, Shankarganesh K (2011). *In vitro* Evaluation of Plant Products and Bio-control Agents against *Colletotrichum capsici* Causing Fruit Rot of Chilli (*Capsicum annum* L.). Pesticide Res. J. 23(2):164-167.

Saraf M, Thakker A, Patel BV (2008). Biocontrol activity of different species of *Pseudomonas* against phytopathogenic Fungi *In vivo* and *In vitro* conditions. Inter. J. Biotechnol. Biochem. 4 (Suppl 3&4).

Schirmbock M, Lorito M, Wang YL, Hayes CK, Arisan-Atac I, Scala F, Harman GE, Kubicek C (1994). Parallel formation and synergism of

hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. Applied Environ. Microbiol, 60: 4364-4370

Shanahan P, O'Sullivan DJ, Simpson P, Glennon JD, O'Gara F (1992). Isolation of 2,4-Diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiologicalparameters influencing its production. Appl. Environ. Microbiol. 58:353-358.

Singh D (2002). Bioefficacy of *Debaryomyces hansenii* on the incidence and growth of *Penicillium italicum* on Kinnow fruit in combination with oil and wax emulsions. Annals Plant Protection Sci. 10 (2): 272–276.

Singh D (2004). Effect of *Debaryomyces hansenii* and calcium salt on fruit rot of peach (*Rhizopus macrosporus*). Ann. Plant Prot. Sci. 12 (2): 310–313.

Singh D (2005). Interactive effect of *Debaryomyces hansenii* and calcium chloride to reduce Rhizopus rot of peaches. J. Mycol. Plant Pathol. 35 (1):118–121.

Singh V, Srivastava SN, Ram Ji Lal, Awasthi SK, Joshi BB (2008). Biological control of red rot disease of sugarcane through *Trichoderma harzianum* and *Trichoderma viride*. Indian Phytopath., 61(4): 486-491.

Sivakumar D, Wijeratnam RSW, Marikar FMMT, Abeyesekere M, Wijesundera RLC (2001). Antagonistic effect of *Trichoderma harzianum* on post harvest pathogens of rambutans. Acta Horticulturae. 553 (2):389–392.

Sivakumar D, Zeeman K, Korsten L (2007). Effect of a biocontrol agent (*Bacillus subtilis*) and modified atmosphere packaging on postharvest decay control and quality retention of litchi during storage. Phytoparasitica, 35(5):507-518.

Smith KP, Havey MJ, Handelsman J (1993). Suppression of cottony leak of cucumber with *Bacillus cereus* strain UW85. Plant Dis. 77:139-142.

Sriram S, Savitha MJ, Ramanujam B (2010). Trichoderma-enriched coco-peat for the management of *Phytophthora* and *Fusarium* diseases of chilli and tomato in nurseries. J. Biol. Control. 24(4):311-316.

Sumangala K, Patil MB, Nargund VB, Ramegowda G (2008). Evaluation of fungicides, botanicals and bio-agents against *Curvularia lunata*, a causal agent of gain discoloration in rice. J. Plant Dis. Sci. 3(2):159-164

Svetlana S, Zivkovic Zl, Stojanovic and Vanovic V (2010). "Gavrilovic Tatjana popovic and Lelica balaz: Screening of antagonistic activity of microorganisms against *Colletotrichum acutatum* and *Colletotrichum gloesporioides*," Archives of Biological Science Belgrade. 62(3): 611-623.

Swain MR, Ray RC, Nautiyal CS (2008). Biocontrol efficacy of *Bacillus subtilis* strains isolated from cow dung against postharvest Yam (*Dioscorea rotundata* L.) pathogens. Curr. Microbiol. 57(5):407-411.

Tian SP, Qin GZ, Xu Y (2004). Survival of antagonistic yeasts under field conditions and their biocontrol ability against postharvest diseases of sweet cherry. Postharvest Biology and Technology 33 (3): 327–331.

Toyoda H, Utsumi R (1991). Method for the prevention of *Fusarium* diseases and microorganisms used for the same. US Patent No. 4, 988, p. 586.

Triki MA, Prlou S (1997) Using chemical and biological treatments to reduce the potential of potato leak caused by *Pythium aphanidermatum* in Tunisia. Potato Res. 40, 391-398.

Tucci M, Ruocco M, De Masi L, De Palma M, Lorito M (2011) The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. Mol. Plant Pathol. 12:341-354.

Vanneste JL, Cornish DA, Yu J, Voyle MD (2002). P10c: a new biological control agent for control of fi re blight which can be sprayed or distributed using honey bees. Acta Horticulturae 590: 231-236.

Vasudevan P, Kavitha S, Priyadarisini VB, Babujee L and Gnanamanickam SS (2002). Biological control of rice diseases. Pp. 11-32 In: S.S. Gnanamanickam (ed.) Biological Control of Crop Diseases. Marcel Dekker Inc. New York, p. 468.

Vessey KJ (2003). Plant growth promoting rhizobacteria as biofertilizers. Plant Soil255, 571–586.

Vidhyasekaran P, Sethuraman K, Rajappan K and Vasumathi K (1997). Powder formulation of *Pseudomonas fluorescens* to control pigeonpea wilt. Biol. Contr. 8:166-171.

Voisard C, Keel C, Haas D, Defago G (1989). Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO J. 8 (Suppl 2): 351–358.

Weller DM, Raaijmakers JM, Gardener BBM & Thomashow LS (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens.Annu. Rev. Phytopathol. 40: 309–348.

Weller DM, Thomashow LS (1993).Microbial metabolites with biological activity against plant pathogens.in: Pest Management: Biologically Based Technologies. Lumsden RD, Vaughn JL, eds., American Chemical Society, Washington, D. C. 173-180

Wisniewski ME, Wilson CL (1992). Biological control of postharvest diseases of fruit and vegetables: recent advances. HortScience 27:94–98.

Wit JP, Kievitsbosh RA, Bettiol W (2009).Integração de métodos físicos e biológicospara o controle de doenças e pragas em Lírio e Espatifilo. , In: *Biocontrole de doençasde plantas: uso e perpectivas.* Bettiol W, Morandi, MAB (eds), Embrapa. Jaguariúna pp. 331-335

Wulff EG, Mguni CM, Mortensen CN, Keswani CL, Hockenhull J (2002). Biologicalcontrol of black rot (*Xanthomonas campestris* pv. *campestris*) of brassicas with anantagonistic strain of *Bacillus subtilis* in Zimbabwe.European J. Plant Pathol. 108(4):317-325.

Yadav PM, Rakholiya KB, Pawar DM (2013). Antagonistic Effect of Fungal Bioagents against *Alternaria porri* (Ellis) Under *In Vitro.* Trends Biosci. 6(3):303-304.

Yang DJ, Wang B, Wang JX, Chen Y, Zhou MG (2009). Activity and efficacy of *Bacillus subtilis* strain NJ-18 against rice sheath blight and Sclerotinia stem rot of rape. Biological Control, 51(1):61-65.

Yang DM, Bi Y, Chen XR, Ge YH, Zhao J (2006).Biological control of postharvest diseases with *Bacillus subtilis* (B1 strain) on muskmelons (*Cucumis melo* L. cv. Yindi). Acta Horticulturae 712 (2): 735–739.

Zhang JX, Xue AG, Tambong JT (2009). Evaluation of seed and soil treatments with novel *Bacillus subtilis* strains for control of soybean root rot caused by *Fusarium oxysporum* and *F. graminearums.* Plant Dis. 93(12):1317-1323.

Zhao Y, Shao XF, Tu K, Chen JK (2007). Inhibitory effect of *Bacillus subtilis* B10 on the diseases of postharvest strawberry. J. Fruit Sci. 24 (3):339–343.

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Phenotypic and symbiotic characterization of rhizobia isolated from *Medicago ciliaris* L. growing in Zerizer from Algeria

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Phenotypic characteristics of 37 rhizobia strains isolated from root nodules of *Medicago ciliaris* L. growing in soils collected from Zerizer (North Eastern Algeria) were studied. Tolerance to salinity, high temperatures, acid and alkaline pHs, drought and to antibiotics as well as symbiotic and cultural characteristics allowed the description of a wide physiological diversity among tested isolates. Thirteen (13) isolates from the total could grow at 45°C. Only six isolates grew at 4% NaCl. Furthermore, the isolates which showed tolerance to salinity stress also showed tolerance to water stress, indicating direct relationships between these two physiological pathways. High salt and water stress tolerant strains were isolated and tested for their ability to biological nitrogen fixation. However, seven isolates were categorized into *Agrobacterium.*

Key words: *Medica gociliaris*, stress tolerance, Algeria, symbiotic properties.

INTRODUCTION

Many species of the legume genus *Medicago* are native to the Mediterranean basin (Lesins and Lesins, 1979) and are important as agricultural crops (Irwin et al., 2001). Compared to *Medicago sativa*, the most important species for cultivation, and *Medicago truncatula*, the model chosen for studies in nitrogen fixation (Cook, 1999; de Billy et al., 2001; Ben Amor et al., 2003), investigations with *Medicago ciliaris* as the focus have been very limited (Laouar and Abdelguerfi, 2000) . The species *M. ciliaris* is an annual plant that is tolerat to salt stress (Ben Salah et al., 2009) and may show promise for cultivation in salt-affected soils. Due to the reason that this species grows in soils that are heavy with clay it has application as a cover crop, in pastures, or for producing forage (Laouar and Abdelguerfi, 2000).

Many species of this genus *Medicago* have significant and wide-ranging agricultural and environmental applications, such as the perennial species *M. sativa* L, alfalfa (Irwin et al., 2001). Alfalfa is one of the most important forage crops in the world because of its high nutritive quality, yield, drought-resistance and good adaptation to various climatic and soil conditions and, therefore, is reputed to be the “queen” of the forages, although it has been reported to be water use inefficient (Li et al.,

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2007). Furthermore, the annual species, collectively known as “medics”, are naturally distributed over a very wide range of environmental conditions in the Mediterranean basin, and the great importance in pastures in the Mediterranean and known to establish a nitrogen-fixing symbiosis with soil bacteria of the genus *Ensifer* (formerly *Sinorhizobium*) (Bena et al., 1998; Lesins and Lesins, 1979; Badri et al., 2008).

Nitrogen is a major limiting factor for plant productivity despite the inexhaustible reserve of atmosphere (78% N_2) (Foth, 1990). Biological fixation of molecular nitrogen (N_2) from the atmosphere is one of the main sources of nitrogen pool enhancement in agricultural soils (Bradic et al., 2003). The ability of legume species to establish nitrogen-fixing symbiosis with rhizobia makes them excellent candidates for use in sustainable agricultural systems (Howieson et al., 2000) .

Although most *Medicago* species form symbioses with the two species *Sinorhizobium meliloti* and *S. medicae* (Brunel et al., 1996; Rome et al., 1996), it is becoming evident that several different species of *Medicago* may have dissimilar affinities for infection by these 2 rhizobial species. For example, Garau et al. (2005) demonstrated that *S. medicae* frequently nodulated *Medicago* species that are adapted to acid soils, while *S. meliloti* formed symbioses with those growing in more alkaline to neutral soils. Bena et al. (2005) indicated that the geographic distribution of these rhizobial species appeared related to the incidence of the species of *Medicago* resulting from the characteristics of the soils.

In Algeria, diminution of pasture areas and deficit of forage production are major problems for development and extention of ovine and bovine breeding. Annual medics are grown as forage legume and regenerating pasture in the agro-pastoral Mediterranean systems or Australian ley-farming systems. However, the commercial Australian medic cultivars are not well adapted to most of agroecologicalzanes encountered in North Africa. Therefore, selection of better-adapted medics in association with appropriate symbiotic bacterial partners is agronomically important. Zerizer is an area of Algeria where most of *M. ciliaris* is represented, but less is known about their associated rhizobia. The advantages of this species are increased by the fact that, like most legumes, *Medicagociliarisis* able to form a symbiotic association with rhizobia and thus fix atmospheric nitrogen, which enriches the soil. The use of atmospheric nitrogen makes *M. ciliaris* a pioneer species capable of colonizing nitrogen poor soils.

This study is a preliminary step contributed to research efforts designed to uncover the biodiversity of rhizobia and, at the same time, select promising strains for the production of inoculants to improve *M. ciliaris* nitrogen fixation ability. We characterized 37 efficient rhizobia isolated from *M. ciliaris* L. collected from Zerizer area. The phenotypic characterization of these strains was conducted to evaluate their capacity to grow under abiotic stress such as severe temperatures, salinity, drought and high pH. Finally, the symbiotic properties of the representative strains were evaluated in terms of nodule numbers.

MATERIALS AND METHODS

Sampling zone

Thirty nodulated *M. ciliaris* plants were collected from from Zerizer (North Eastern Algeria). Healthy plants were uprooted carefully and those plants prossessing healthy nodules with pink colour were selected to isolate rhizobia.

Soil sample and isolation of rhizobia from *Medicago ciliaris*

Soil sample was collected from area Zerizer in 2010 and used for *M. ciliaris* cultivation as trap hosts (Soil samples were collected from the Zerizer area in 2010 and used for trapping rhizobia). Strains were isolated from naturally occurring root nodules collected on *M. ciliaris*. Nodules were washed several times with tap water and rinsed with sterile distilled water. They were surface sterilized by immersion for 30 s in ethanol (96% v/v), 3 min in 3% sodium hypochlorite and then washed ten times with sterile distilled water.

A single surface-sterilized nodule was placed into a Petri dish and crushed with a sterile glass rod in the presence of a sterile solution of sterile distilled water. A loopful of the resulting suspension was then streaked on Tryptone Agar medium surface containing 25 µg/ml Congo red (TA) in a Petri dish and incubated at 28°C.

Bromothymol blue (BTB) agar medium was used for differentiating of the isolates. The cultures were streaked on BTB agar plates. BTB agar was made by adding 5 ml of (0.5% BTB in ethanol) to 1 L of YEMA medium. The plates were incubated at 28°C for 4 days. The change in color of medium was observed. The isolates were classified as slow growers (medium turns blue) or fast growers (medium turns yellow) on their reaction on YEMA supplemented with BTB (Table 1) (Somasegaran and Hoben, 1994). Isolates were purified by repeated streaking of a single colony on TA medium and were checked for purity by light microscopic examination of living cells and Gram staining (Vincent, 1970). They were then stored at 4°C on TA slants and at -16°C in Tryptone yeast extract (TY) liquid culture aliquots in the presence of 20 and 50% glycerol (v/v).

Purification of isolates

a)-Growth on congo red medium

Rhizobia colonies appeared white, translucent, gummy, glistening elevated and comparatively small withenremargine were selected in contrast to of *Agrobacterium* on congo red medium which were red in color.

b)- Gram staining

Gram staining was done to ensure purity and freedom from Gram +ve bacteria. Gram-staining reaction was carried out by using a loopful of pure culture grown on Tryptone agar and stained as per the standard Gram’s procedure (Somasegaran and Hoben, 1994).

Table 1. Colony morphology of rhizobial isolates from *Medicago ciliaris*.

Isolate	Age of culture (hour)	Color and opacity	Size
MedS01	72	Slightly pink colony	Less than 2 mm Ø
MedS02	72	Whitish colony	1 mm Ø
MedS04	72	Milky translucent colony	1 mm Ø
MedS07	72	White circular colony	Less than 1 mm Ø
MedS08	72	Slightly pink mucilaginous colony	1 mm Ø
MedS09	72	Translucent colony	Less than 1 mm Ø
MedS10	72	White colony	Less than 1 mm Ø
MedS11	72	Pink slightly mucilaginous colony	Less than 2 mm Ø
MedS12	72	Pink mucilaginous colony	2 mm de Ø
MedS13	72	Transparent colony	1 mm Ø
MedS14	72	Whitish colony	1 mm Ø
MedS15	72	Milky gummy colony	Less than 2 mm Ø
MedS16	72	Pink slightly mucilaginous colony	1 mm Ø
MedS20	72	Milky mucilaginous colony	Less than 2 mm Ø
MedS23	72	Pink mucilaginous colony	1 mm Ø
MedS24	48	Whitish opaque colony	Less than 2 mm Ø
MedS25	48	Milky translucent colony	1 mm Ø
MedS26	48	Slightly pink opaque colony	1 mm Ø
MedS28	72	White plate colony	Less than 2 mm Ø
MedS29	48	White opaque colony	2 mm de Ø
MedS30	72	Translucent colony	Less than 1 mm Ø
MedS31	48	Pink gummy colony	Less than 2 mm Ø
MedS32	72	Transparent mucilaginous colony	2 mm de Ø
MedP01	72	Slightly pink opaque colony	Less than 3 mm Ø
MedP03	72	Transparent mucilaginous colony	Less than 2 mm Ø
MedP04	72	Pink slightly gummy colony	2 mm de Ø
MedP05	72	White opaque colony	1 mm Ø
MedP06	72	Translucent colony	1 mm Ø
MedP07	72	Slightly pink mucilaginous colony	1 mm Ø
MedP09	72	Whitish opaque colony	1 mm Ø
MedP10	72	Pink slightly gummy colony	Less than 3 mm Ø
MedP12	72	Pink slightly opaque colony	Less than 2 mm Ø
MedP13	72	slightly pink mucilaginous colony	2 mm de Ø
MedP14	72	Milky colony	1 mm Ø
MedP17	72	Translucent colony	1 mm Ø
MedP18	72	Milky opaque colony	Less than 2 mm Ø
MedP19	72	Translucent colony	Less than 1 mm Ø
S. meliloti	72	Slightly pink colony	Less than 1 mm Ø
S. meliloti	72	Whitish colony	Less than 2 mm Ø
S. fredii	72	Milky translucent colony	1 mm Ø
S. medicae	72	White circular colony	Less than 2 mm Ø
A. tumefaciens	72	Slightly pink mucilaginous colony	1 mm Ø
Rhizobium sp.	72	Translucent colony	Less than 1 mm Ø

c)*-Distinguishing test between *Rhizobium* and *Agrobacterium

The *A. tumefaciens* species complex (biovar 1) has the enzymatic ability to aerobically convert lactose to 3-ketolactose. This is tested by streaking on medium containing lactose. After 2 days of growth at 28°C, the plates are flooded with a layer of Benedict's reagents (17.3 g of sodium citrate and 10.0 g of Na_2CO_3, are dissolved by heating in 80 ml of distilled water; after filtration, the filtrate is added

to a 10 ml solution containing 17.3 g of $CuS0_4.5H_2O$, and the mixture is diluted to 100 ml). The presence of 3-ketolactose in the medium is indicated by the formation of a yellow ring around the growth of a positive strain (Table 1, Figure 3). Maximum intensity of the yellow ring (2-3 cm in diameter) of cuprous oxide around the bacterial spot. Around 3-ketolactose positive strains is reached in about 1-2 h after flooding with Benedict's reagent. Biovar 1 strains have the unique ability to oxidize lactose into 3-ketolactose (Bernaerts and Deley, 1963).

Morphological studies

The thick bacterial smear of all the isolates was Gram stained and morphological characterization was done on the basis of colony morphology including shape,color and surface margin (Table 2).

Biochemical studies

Biochemical characterization was done on the basis of oxidase, catalase.

Stress tolerance screening

The isolates characterized in this study were examined for growth under different stress conditions of high temperature, high salinity, alkaline pH and extreme drought. In the case of temperature tolerance, isolates were kept at 28 (as a control), 37, 40, 42 or 45°C on YMA plates for four to five days. To check the ability of isolates to grow under different concentrations of NaCl, the medium was supplemented with 0 (control), 1, 2, 3, or 4% NaCl. To test the tolerance to acid and alkaline pH, the pH of the medium was adjusted with 0.5 M HCl or 0.5 M NaOH to 4.5, 5.5, 6.8 (as a control), 8, and 9.

The salinity and pH test were performed on YMA plates kept at 28°C for 4-5 days. To test drought resistance, different concentrations of polyethylene glycol (PEG 4000) were applied to the sterile distilled water at 10, 15, 20, 25%. In this experiment, isolates were first grown in TA medium for 3 days at 28°C and the resulting bacterial suspensions containing approxi-mately 10^9 cells ml^{-1}, were transferred to YMA plates as previously indicated. The screening for stress tolerance was performed in Petri dishes divided into equal squares. Each square was spot inoculated with 10 µL of the cell suspensions at 10^9 cell ml^{-1} grown in Tryptone agar at an exponential phase. After incubation under different stressful conditions, the growth of isolates was estimated in comparison with that following control treatment, as follows: −, no growth; + weak growth (10-30% in relation to the control); ++, good growth (30-80% in relation to the control); and +++, very good growth (similar to the control).

Antibiotic susceptibility

Antibiotic resistance tests were performed by measuring the diameters of inhibition zones on YEM agar plates containing the following antibiotic discs: streptomycine (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), nalidicic acid (30 µg); kanamycin (30 µg); ampicillin (10 µg). The antibiotic resistance was detected by an inhibition zone measured over a seven day period for each disc. Determination of intrinsic antibiotic resistance was evaluated in plates of YEM with different concentrations of antibiotics (rifampicine, erythromycin and neomycin). Filter-sterilized aliquots of each antibiotic were added aseptically to sterile YEM medium at 50°C to give the final concentrations. Control plates contained no antibiotic (Van Berkum et al., 1998).

Plant test

To assess their abilities to generate root nodules on their original hosts, the isolates were grown on Tryptone agar for 3 days at 28°C and the resulting bacterial suspensions containing approximately 10^9 cells mL^{-1} were inoculated on aseptic *M. ciliaris* seeds. Seeds were surface sterilized in 3% sodium hypochlorite for 10 min, rinsed with sterile distilled water, and then scarified. These seeds were germinated for 72 h on water agar (0.7 w/v) and planted at the rate of two seedlings in plastic pots containing sterilized sand. As controls, two pots (T0) with non inoculated seedlings were tested. Plants were sprayed with sterile distilled water every two days, in addition to being provided once a week with a nitrogen-free nutrient solution.

Plants were inoculated with 1 ml of early stationary phaserhizobial culture (10^8-10^9 cells mL^{-1}) cultivated at 28°C in TA medium. Two replications were carried out for both inoculated and non-inoculated plants (negative controls). Plantlets were harvested six weeks growth. Nodulation was recorded by the existence of nodules and the efficiency was estimated by the presence of red coloring (leghemoglobin) inside the nodules (Vincent, 1970). Shoot weight and root nodule numbers in each plant were also determined. For each isolate, the inoculation effect was estimated by determining the relative index of dry weight increase according to the following formula: relative index of dry weight increase = (inoculated plant dry weight) / (control plant dry weight).

RESULTS

Isolation of rhizobia from *Medicago ciliaris*

A total of 37 isolates were isolated from root nodules of *M. ciliaris* . These rhizobia are Gramnegative, nonspore forming, rod-shaped, with circular and convex colonies having 1-3 mm in diameter when grown on TA plates at 28°C. Most strains are translucent and gummy, white coloured except the pink pigmented (Meds1, Meds8, Meds11, Meds12, Meds16, Meds23, Meds26, Meds31, Meds32, Medp1, Medp4, Medp7, Medp10, Medp12, and Medp13). Thirty two (32) strains can be regarded as fast-growers (Jordan, 1984) (visible colonies develop within (2-3 days). in contrast (Meds24, Meds25, Meds26, Meds29, Meds31) are very fast growers (visible colonies develop within (1-2 days).

Stress tolerance

The 37 isolates obtained from *M. ciliaris* and 6 reference strains of laboratory collection were first screened for resistance to high salinity, alkaline pH, high drought and high temperature conditions. According to this preliminary characterization, high diversity in stress resistance was observed. The data in Figure 1 show that *M. ciliaris* rhizobia exhibited a wide diversity in their salt tolerance. The salt inhibitory concentrations varied among strains

Table 1. Characterization of isolates.

Isolate	Gram's nature	Color produced on BTB agar	Fast/Slow grower	3-ketolactose test	Catalase test	Oxidase test
MedS01	Gram -	Yellow	Fast	Negative reaction	+	+
MedS02	Gram -	slightlyyellow	Fast	Negative reaction	+	+
MedS04	Gram -	Yellow	Fast	Negative reaction	+	-
MedS07	Gram -	slightlyyellow	Fast	Negative reaction	+	-
MedS08	Gram -	Yellow	Fast	Negative reaction	+	-
MedS09	Gram -	Yellow	Fast	Negative reaction	+	+
MedS10	Gram -	Yellow	Fast	Negative reaction	+	+
MedS11	Gram -	Yellow	Fast	Negative reaction	+	+
MedS12	Gram -	slightlyyellow	Fast	Negative reaction	+	+
MedS13	Gram -	Yellow	Fast	Negative reaction	+	+
MedS14	Gram -	Yellow	Fast	positive reaction	+	+
MedS15	Gram -	slightlyyellow	Fast	Negative reaction	+	+
MedS16	Gram -	slightlyyellow	Fast	Negative reaction	+	+
MedS20	Gram -	slightlyyellow	Fast	Negative reaction	+	+
MedS23	Gram -	Yellow	Fast	Negative reaction	+	-
MedS24	Gram -	Yellow	Fast	positive reaction	+	-
MedS25	Gram -	Yellow	Fast	positive reaction	+	+
MedS26	Gram -	Yellow	Fast	positive reaction	+	+
MedS28	Gram -	Yellow	Fast	Negative reaction	+	+
MedS29	Gram -	Yellow	Fast	positive reaction	+	+
MedS30	Gram -	Yellow	Fast	Negative reaction	+	+
MedS31	Gram -	Yellow	Fast	positive reaction	+	+
MedS32	Gram -	Yellow	Fast	Negative reaction	+	+
MedP01	Gram -	Slightly yellow	Fast	Negative reaction	+	+
MedP03	Gram -	Slightly yellow	Fast	Negative reaction	+	+
MedP04	Gram -	Slightly yellow	Fast	Negative reaction	+	+
MedP05	Gram -	Yellow	Fast	Negative reaction	+	-
MedP06	Gram -	Yellow	Fast	Negative reaction	+	+
MedP07	Gram -	Yellow	Fast	positive reaction	+	-
MedP09	Gram -	Yellow	Fast	Negative reaction	+	+
MedP10	Gram -	Yellow	Fast	Negative reaction	+	+
MedP12	Gram -	Slightly yellow	Fast	Negative reaction	+	+
MedP13	Gram -	Slightly yellow	Fast	Negative reaction	+	+
MedP14	Gram -	Yellow	Fast	Negative reaction	+	-
MedP17	Gram -	Yellow	Fast	Negative reaction	+	+
MedP18	Gram -	Yellow	Fast	Negative reaction	+	+
MedP19	Gram -	Yellow	Fast	Negative reaction	+	+
S. meliloti	Gram -	Yellow	Fast	Negative reaction	+	+
S. meliloti	Gram -	Yellow	Fast	Negative reaction	+	+
S. fredii	Gram -	Yellow	Fast	Negative reaction	+	+
S. medicae	Gram -	Yellow	Fast	Negative reaction	+	+
A. tumefaciens	Gram -	Yellow	Fast	Positive reaction	+	+
Rhizobium sp.	Gram -	Yellow	Fast	Negative reaction	+	+

and salt nature. Indeed, tolerance to sodium chloride (NaCl) was found since than 100% of the tested rhizobia continued to grow with 1% NaCl. However, at higher concentrations, the percentage of tolerant strains decreased rapidly and only two isolates (4.65% of all isolates) were able to grow at 4% NaCl, while at 3% NaCl. All the isolates were sensitive to the high salinity level of 3 and 4% NaCl, however, they showed relatively

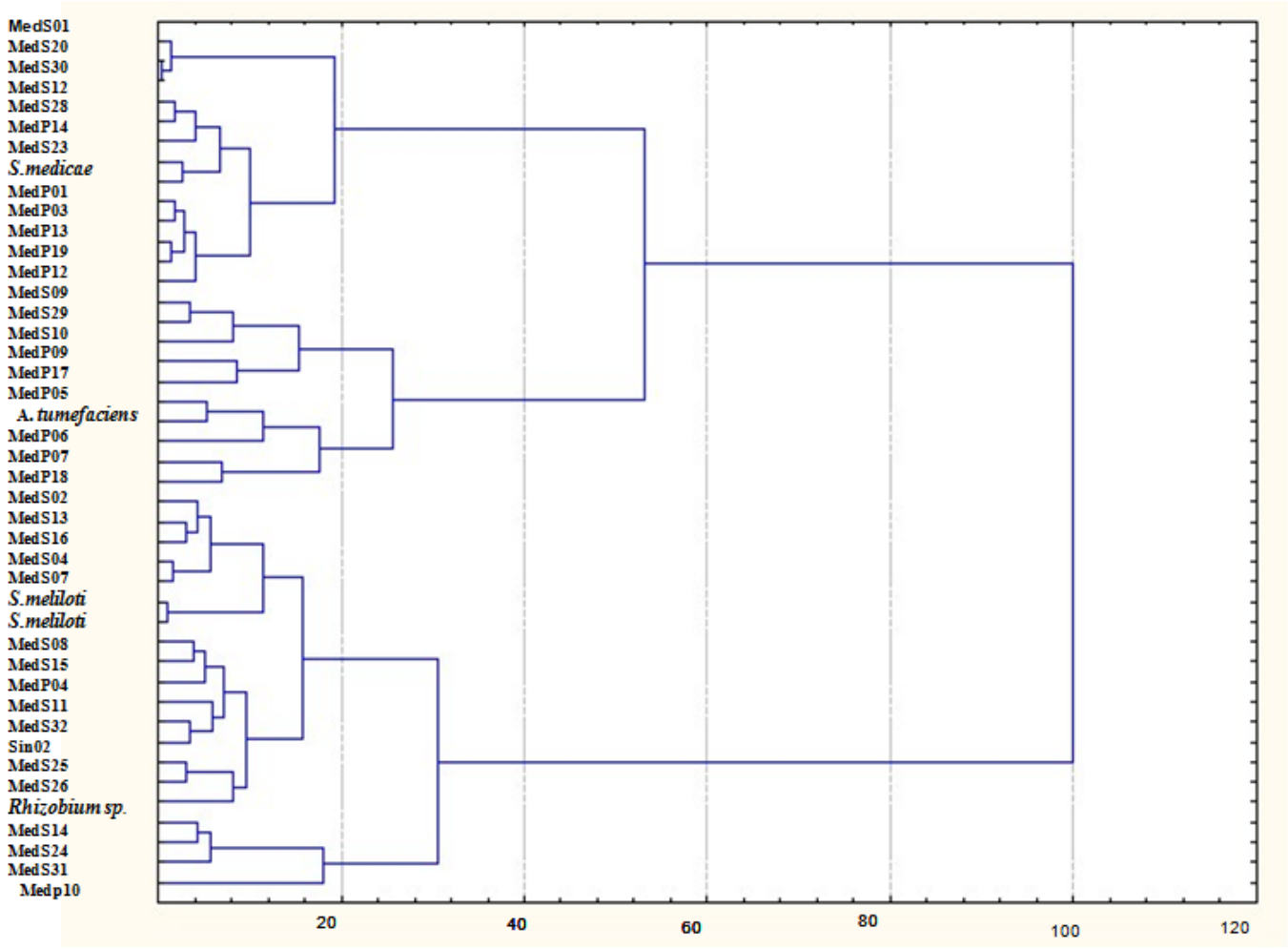

Figure 1. Dendrogram showing effect of different concentrations of Nacl and temperature on growth of *Medicago ciliaris* rhizobia.

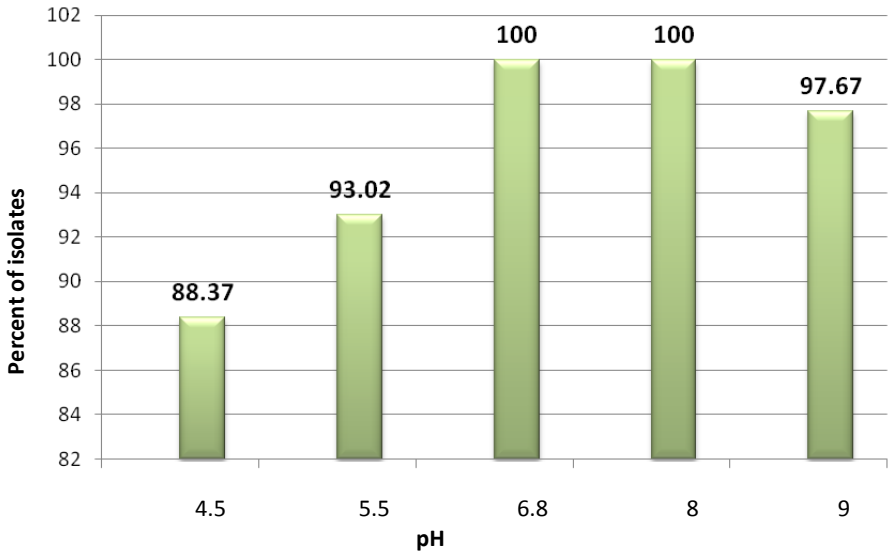

Figure 2. Effect of different concentrations of pH on growth of *Medicago ciliaris* rhizobia.

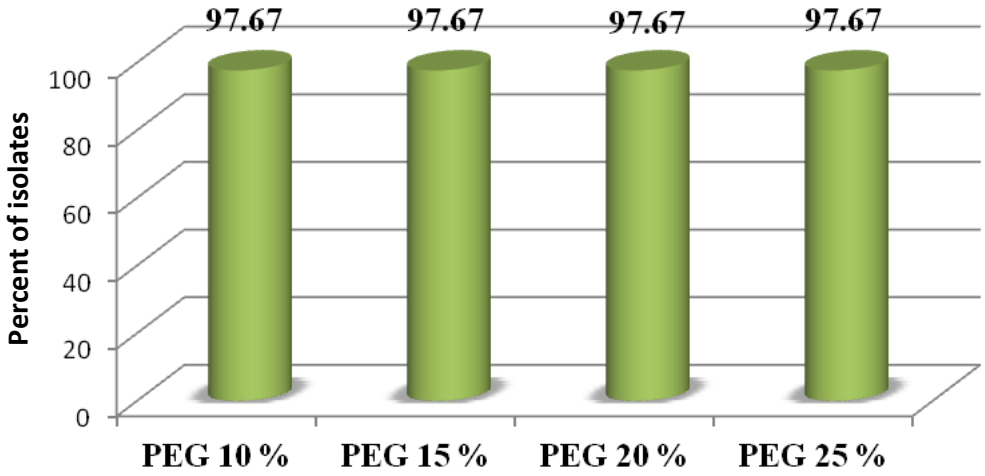

Figure 3. Effect of different concentrations of PEG 4000 on growth of *Medicago ciliaris* rhizobia.

good growth a 2% NaCl. Regarding high temperature resistance, response to extreme temperatures was positive for all the strains. Optimum temperature range for growth of culture is 28-30^{0}C. 100 % of the isolates were able to grow at 37°C. Above those values, the percentage of isolates that grew decreased to reach 69.76 % at 42°C and 27.90% at 45°C.

In the case of the pH test (Table 3, Figure 2), showed a wide diversity in their pH tolerance. From 93.02 to 100% of the isolates grew in lightly acid and neutral pH. At low pH, some isolates exhibited anacido-tolerant character. Above pH 8, 100% of the isolates grew in alkaline pHs. Osmotolerance of all isolates was tested in minimal medium YMA supplemented with increasing concentrations PEG 4000 (drought), as described in the Materials and methods. All strains were able to grow in YMA with PEG added. Nearly all isolates survived at 25% PEG 4000 with the exception of Medp01.

Distinguishing test between *Rhizobium* and *Agrobacterium*

Agrobacterium is common in soil and in the plant rhizosphere, but was never described inside root nodules. Distinguished *Agrobacterium* from *Rhizobia* by 3-ketolactose test, whereas the *Agrobacterium* produced yellow ring of precipitate of CuO_2 around the colonies of the bacterium when plates were flooded with Benedict's reagent. In the present study six isolates showed positive results for 3-ketolactose test (Table 1 and Figure 4).

Antibiotic susceptibility

Intrinsic resistance to antibiotics showed a general resistance to erythromycin, and 69.67% of the strains were also resistant to 10 µg ml^{-1} ampicillin. 18.60% of strains were scored resistant to 30 µg m^{-1} chloramphe-nicol and 55.81% were also resistant to 30 µg ml^{-1} nalidixic acid; 51.16 and 93.02% were also resistant to 10 and 30 µg ml^{-1} of streptomycin and neomycin, respectively. Nevertheless, all strains were highly sensitive to tetracycline and kanamycin; rifampicin the concen-tration of 30 µg ml^{-1} (Table 5 and Figure 5).

Plant tests

The 37 isolates and reference strains of laboratory collection were tested for their capacity to form root nodules on their original host plants under controlled laboratory conditions of temperature and relative humidity. All the isolates induced root nodules to form on their original hosts, and the uninoculated plants used as negative controls were not nodulated - root nodule numbers in the original host plants.

The mean number varied from 2.25 in *Agrobacterium tumefacians* (reference strain)to 19.50 in *S. meliloti* (reference strain). Seven isolates MedS14, MedS24, MedS25, Meds26, Meds29, and Meds31 and Medp07 have the enzymatic ability to aerobically convert lactose to 3-ketolactose, Strains Medp09 and *A. tumefaciens* were the less infective, with a respective average of 2 and 0 formed per plant. While strains MedS32 and *S. meliloti* were the most infective with 22 and 28 nodules formed per plant respectively. Relative indexes expressed as a shoot dry weight of the inoculated plants compared to the positive control plants, was largely variable (Table 4 and Figure 7). The most infective strains were also the most effective (Table 4 and Figure 6).

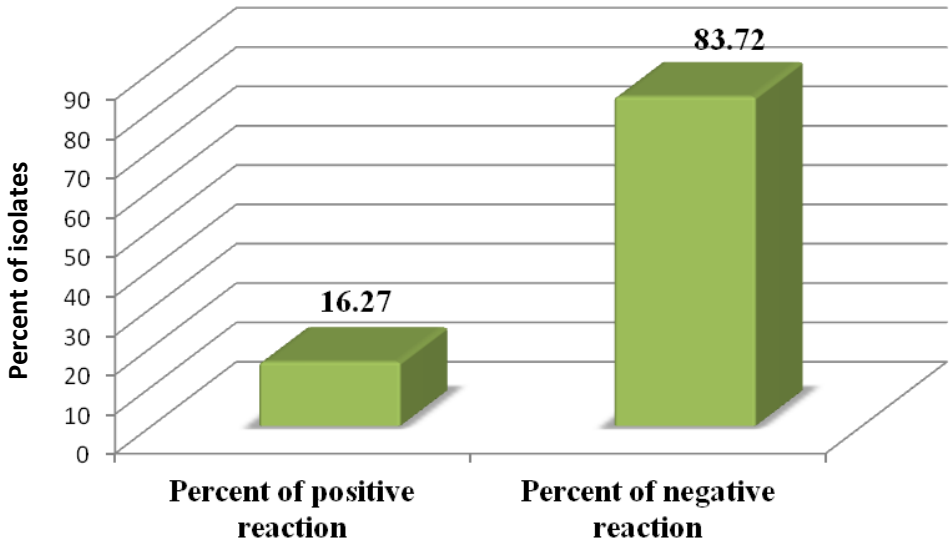

Figure 4. 3-ketolactose test reaction of *Medicago ciliaris* rhizobia.

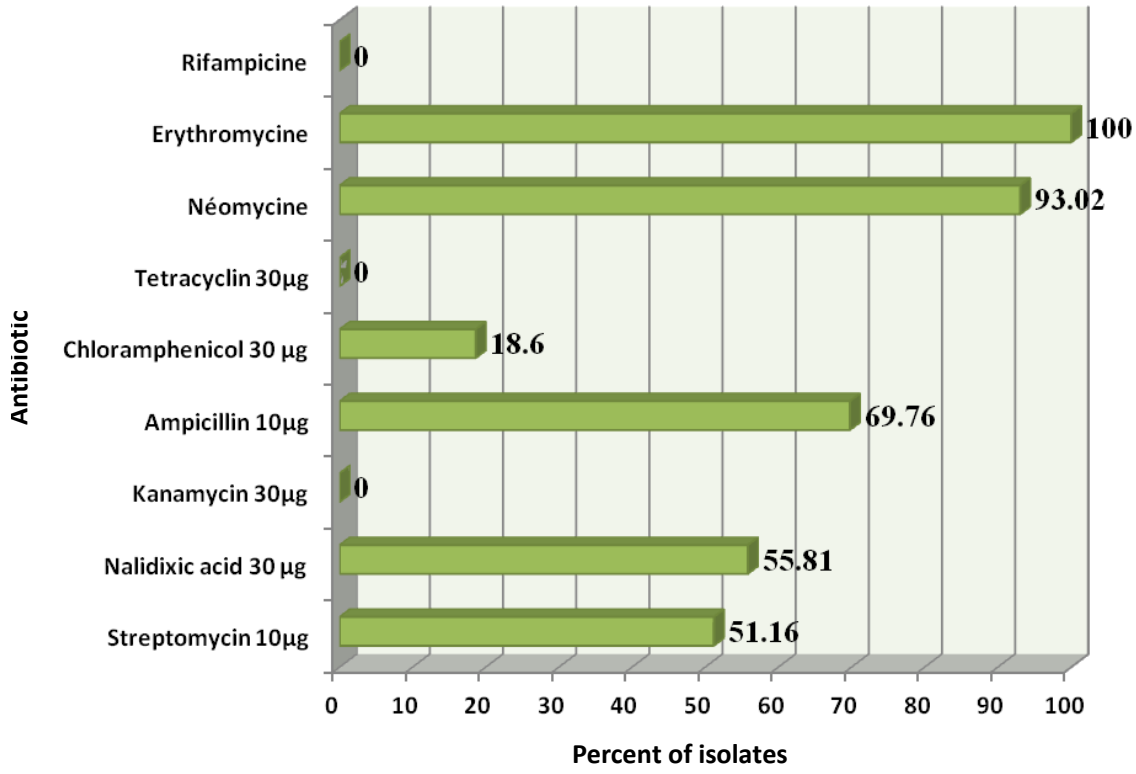

Figure 5. Effect of different antibiotics on growth of *Medicago ciliaris* rhizobia.

DISCUSSION

Although phenotypic and genotypic approaches provided very different information on the *M. ciliaris* rhizobia strains, they were similarly sensitive in demonstrating the large diversity found amongst these bacteria. The phenotypic characterization of the sampled 37 isolates and six reference strains of laboratory collection for above

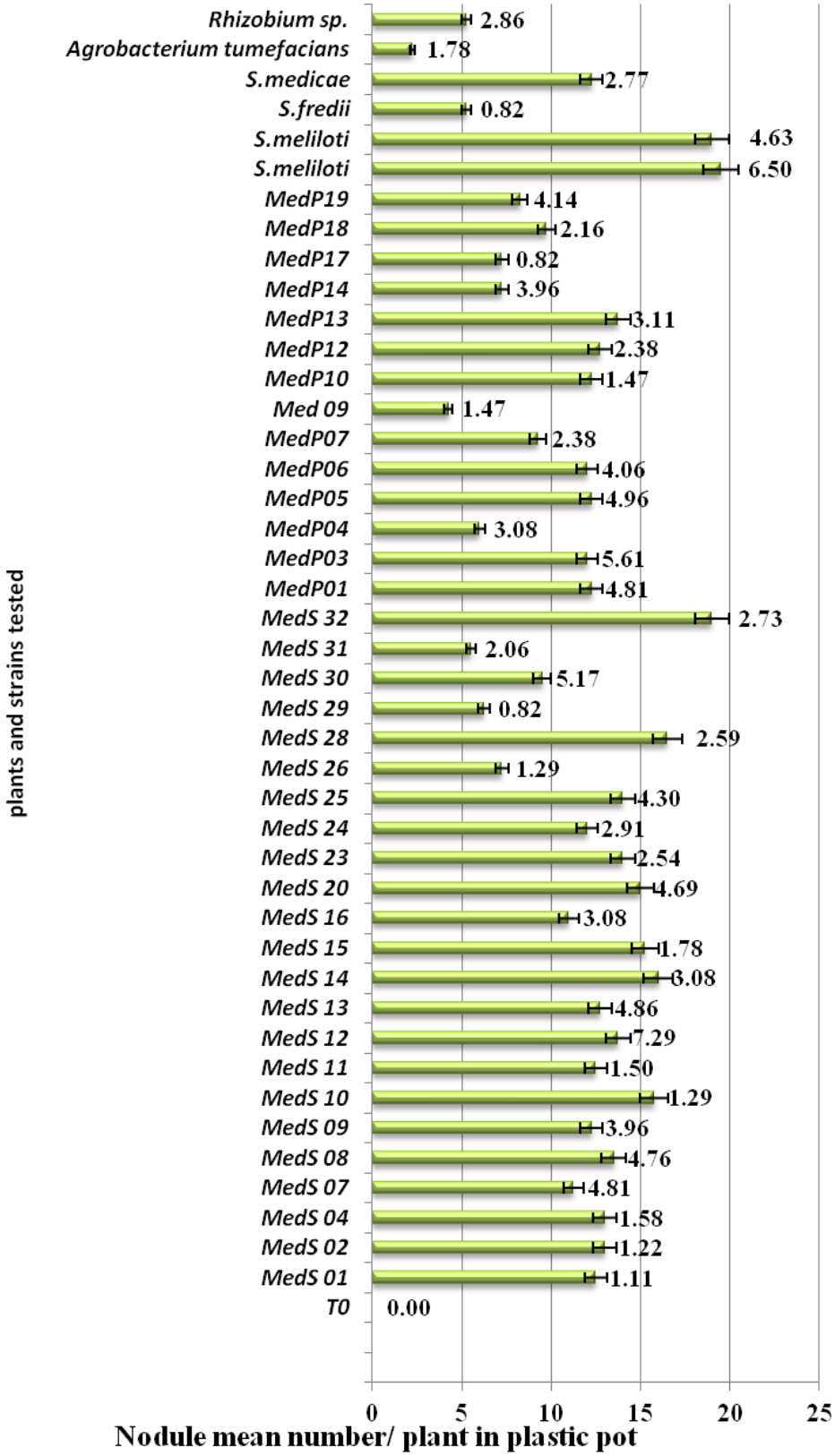

Figure 6. Infectivity of *Medicago ciliaris* rhizobia of nodulation test in plastic pot.

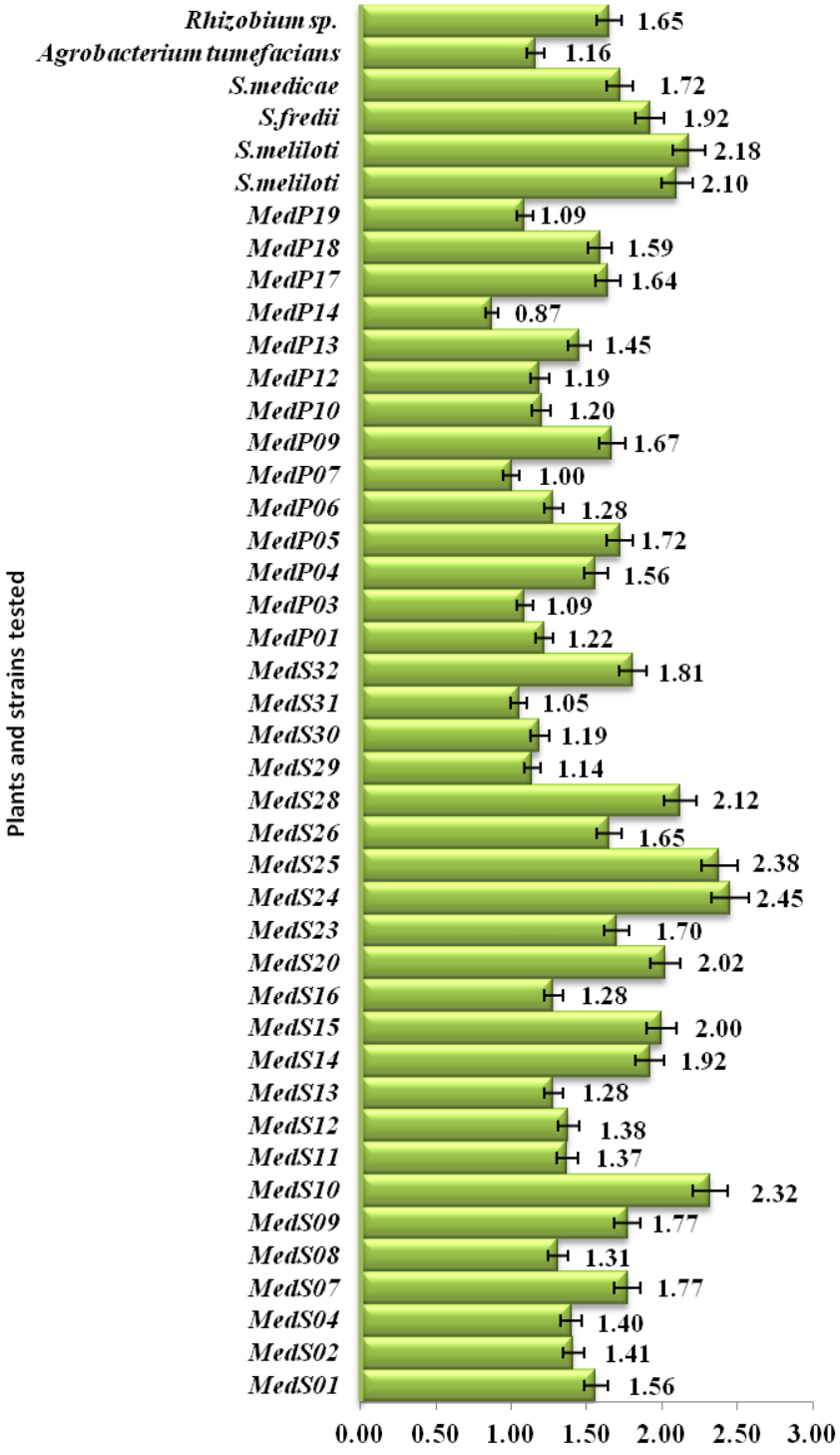

Figure 7. Relative index of dry weight increase of nodulation test in plastic pot.

characters revealed a large degree of variation. Consequently, a total of the strains was isolated from root nodules. Based on differences in high salinity, alkaline pH, high temperature and drought stress resistance. Concerning the symbiotic partner of *M. ciliaris* has a preference for *E. medicae*. Out of thirty seven isolates and six reference strains, seven shown a positive reaction3-ketolactose test. In the case of high salt resis-

tance, Meds09, Meds10, Meds29, Medp06 and Medp10 survived at 4% NaCl. Shamseldin et al. (2009) reported that *E. meliloti* strains from faba bean root nodules survived at 3% NaCl. Payakapong et al. (2006) also reported that an Ensifer strain of BL3 obtained from root nodules of *Phaseolus lathyroides* could survive at 3.5% NaCl. Shamseldin et al. (2006) reported the proteomic characterization of *Rhizobium etli* at 4% NaCl. In the case of high temperature resistance, out of 37 isolates, and six of reference strains thirteen isolates and two of reference strains survived at 45°C as shown in Table 3. Concerning high temperature resistance, some tolerant rhizobial isolates have been described. For instance, Fall et al. (2008) isolated rhizobia from *Acacia Senegal* that showed good growth at 45°C. Furthermore, Ge-Hong et al. (2008) reported a temperature tolerant strain of *Mesorhizobium* at 35°C. However, there is no report about *E. meliloti* surviving at 45°C.

Moreover, two isolates, Meds13 and Meds14 survived at both 45°C and 2% NaCl. Nonetheless, we could not find an isolate able to survive abilities at 45°C and 3% NaCl. Out of these 37 isolates, only 2 (5.40% of the total) could survive at both 45°C and 2% NaCl as shown in Figure 1 and Table 3**.** This shows that the frequency of isolates having both high temperature resistance and high salt resistance is low. For salinity tolerance, we observed a wide variability for tolerance (0-4%). The isolates showed variation for NaCl tolerance, indicating that the rhizobia nodulating *Medicago* spp. are more tolerant compared to other rhizobia species (Struffi et al., 1998; Zahran, 1999). However, as suggested by El Sheikh and Wood (1989) and Odee et al. (1997), we found that fast growing strains were generally more tolerant to high salt concentrations than slow-growing strains. Salinity imposes both ionic and osmotic stresses. Indeed, the imposition of any stress to rhizobia results in adaptive responses, which lead to changes in the regular metabolic processes that are then reflected in protein profiles. The tolerant rhizobia to osmotic stress accumulate the osmolytes, and changes their morphology and dehydration of cells (Buss and Bottomley, 1989). There was a good correlation between the tolerances to both stressors; strains that were halotolerant were in general also tolerant to PEG, suggesting that common osmo-adaptation mechanisms were operating. Hypersaline stress, most bacteria synthesize and accumulate small organic molecules called compatible solutes, as they compensate for hyperosmotic stress without interfering with cellular metabolism. As drought also imposes osmotic stress, it is plausible that at least part of the cell response to drought involves the synthesis and accumulation of compatible solutes. Our results showed that all the isolates grew at 25% of PEG 4000. This result showed that Zerizer soils contained rhizobial strains well adapted to dry conditions. For the most rhizobia, optimum temperature range for growth of culture is 28-31°C, and many cannot grow even at 37°C (Graham, 1992). At 28, 37 and 40°C, the isolates grew well like most *Sinorhizobium* species (Lindstrom and Lehtomaki, 1988; De Lajudie et al., 1994), could grow above 40°C. There was a varied response of the isolates tested to pH. All the isolates tested grew in alkaline pH (pH 8 and 9). At very low pH (pH 4.5), isolates grew normally with the exception of Meds09, Medp01, Medp04 and Medp05 *S. fredii.* According to Jordan (1984), slow-growing strains appear to be more tolerant to low pH than fast-growing strains. Some fast-growing strains such as *Rhizobium tropici* and *Mesorhizobium loti* can grow at a pH as low as 4 (Cooper, 1982; Cunningham and Munns, 1984; Graham, 1992; Gao et al., 1994). It has been reported that *S. melilot*i grows at a pH range of 5.0-9.5 (Jordan, 1984) and is tolerant to 2.0% (w/v) NaCl. Another *Medicago*-nodulating species, *S. medicae*, can grow at pH 5.0-10.0 and is resistant to 2.0% NaCl (Rome et al., 1996). Growth at pH 5.0 has been recorded for only some strains (Jordan, 1984) from acidic soils. Regarding the intrinsic resistance to antibiotics, it has been reported that fast-growing strains are more sensitive to antibiotics (Jordan, 1984) than slow-growing rhizobia. The evaluation of intrinsic resistance to antibiotics showed that most tested isolates had high resistance to erythromycin and nalidixic acid, chloramphenicol, and streptomycin. However, the degree of resistance to antibiotics was higher than in other species of rhizobia (Wei et al., 2003), indicating that *S. meliloti* and *S. medicae* had higher levels of tolerance to these antibiotics.

All tested strains were able to infect their host plant and to fix atmospheric nitrogen leading to plant shoot production above the noninoculated controls. Strains Meds10, Meds14, Meds15, Meds28, Meds32 developed 15.75, 16, 15.25, 16.50 and 19 mean number of root nodules respectively, while *S. meliloti* developed 19.50 mean number of root nodules with the same host plant. This result shows that the root nodule forming ability of the symbiotic *Agrobacterium* is significantly lower than that of the reference strain *S. meliloti.* The root nodule number inoculated with trap host isolates is clearly lower than that of host plants inoculated with the remaining isolates. Sullivan and Ronson (1998) reported that a symbiotic element of *M. loti* was transferred into three non symbiotic species. Bailly et al. (2007) reported that several interspecific horizontal gene transfers occurred during the diversification of *Medicago* symbionts. Similarly, Wong and Golding (2003) reported that a large portion of pSym B genes in *E. meliloti* are most closely related to genes in *A. tumefaciens* linear chromosomes. These reports support the existence of symbiotic *Agrobacterium* isolates produced by horizontal transfer of symbiotic genes.

We conclude that rhizobia strains isolated from *M. ciliaris* nodules in Algerian soil are both phenotypically diverse. To verify this suggestion, we need to complete

Table 3. Stress tolerance screening.

Isolate	Temperature range (°C)	NaCl (%)*	pH*	Drought PEG*
MedS01	28-42	1%(++)	9 (++)	25% (++)
MedS02	28-45	1%(++)	9 (++)	25% (++)
MedS04	28-45	0.01%(++)	9 (++)	25% (++)
MedS07	28-45	0.01%(++)	9 (++)	25% (++)
MedS08	28-42	1%(++)	9 (++)	25% (++)
MedS09	28-42	4%(++)	9 (++)	25% (++)
MedS10	28-42	4%(++)	9 (++)	25% (++)
MedS11	28-45	1%(++)	9 (++)	25% (++)
MedS12	28-40	0.01%(++)	9 (++)	25% (++)
MedS13	28-45	2%(++)	9 (++)	25% (++)
MedS14	28-45	2%(++)	9 (++)	25% (++)
MedS15	28-42	2%(++)	9 (++)	25% (++)
MedS16	28-45	0.01%(+)	9 (++)	25% (++)
MedS20	28-40	1%(++)	9 (++)	25% (++)
MedS23	28-45	1%(+)	9 (++)	25% (++)
MedS24	28-45	0%(+)	9 (++)	25% (++)
MedS25	28-45	0.01%(+)	9 (++)	25% (++)
MedS26	28-40	1%(++)	9 (++)	25% (++)
MedS28	28-42	4%(+)	9 (++)	25% (++)
MedS29	28-40	1%(++)	9 (++)	25% (++)
MedS30	28-45	1%(+)	9 (++)	25% (++)
MedS31	28-42	2%(++)	9 (++)	25% (++)
MedS32	28-42	2%(++)	9 (++)	25% (++)
MedP01	28-40	2%(++)	9 (++)	25% (+)
MedP03	28-40	2%(++)	9 (++)	25% (++)
MedP04	28-42	2%(++)	9 (++)	25% (++)
MedP05	28-42	1%(++)	9 (++)	25% (++)
MedP06	28-45	4%(+)	9 (++)	25% (++)
MedP07	28-42	2%(++)	9 (++)	25% (++)
MedP09	28-42	4%(++)	9 (++)	25% (++)
MedP10	28-45	4%(+)	9 (++)	25% (++)
MedP12	28-42	2%(++)	9 (++)	25% (++)
MedP13	28-42	2%(++)	9 (++)	25% (++)
MedP14	28-37	3%(++)	9 (++)	25% (++)
MedP17	28-40	3%(++)	9 (++)	25% (++)
MedP18	28-42	2%(++)	9 (++)	25% (++)
MedP19	28-42	1%(++)	9 (++)	25% (++)
S.meliloti	28-37	3%(++)	9 (2+)	25% (2+)
S.meliloti	28-45	1%(+)	9 (2+)	25% (2+)
S.fredii	28-45	1%(++)	9 (2+)	25% (2+)
S.medicae	28-40	1%(++)	9 (2+)	25% (2+)
A.tumefaciens	28-42	2%(++)	9 (2+)	25% (2+)
Rhizobium sp.	28-42	3%(++)	9 (2+)	25% (2+)

*Growth scores were recorded as follows: −, no growth; +, weak growth (10-30% in relation to the control); ++, good growth (30-80% in relationto the control); and +++, very good growth (similar to the control).

this study using molecular techniques such as REP/PCR or RFLP/PCR, sequencing of 16S rDNA genes and

Table 4. Symbiotic properties of isolates on *M. ciliaris* nodulation test in plastic pots.

Isolate	Mean No. of nodules/ plant * nodulation test in plastic pot	Plant dry weight (mg)	Relative indexes
Control	00	37.34 ± 17.94 [a]	/
MedS01	12.5 ± 1.11[a]	58.61 ± 16.81	1.56
MedS02	13 ± 1.22	52.79 ± 9.27	1.41
MedS04	13 ± 1.58	52.44 ± 16.73	1.40
MedS07	11.25 ± 4.81	66.13 ± 18.49	1.77
MedS08	13.5 ± 4.76	49.25 ± 9.37	1.31
MedS09	12.25 ± 3.96	66.33 ± 7.12	1.77
MedS10	15.75 ± 1.29	86.67 ± 33.39	2.32
MedS11	12.5 ± 1.50	51.35 ± 2.43	1.37
MedS12	13.75 ± 7.29	51.71 ± 16.76	1.38
MedS13	12.75 ± 4.86	47.80 ± 8.51	1.28
MedS14	16 ± 3.08	72.02 ± 24.77	1.92
MedS15	15.25 ± 1.78	74.75 ± 12.4	2.00
MedS16	11 ± 3.08	47.91 ± 8.76	1.28
MedS20	14 ± 2.54	63.51 ± 10.78	1.70
MedS23	12 ± 2.91	91.77 ± 30.88	2.45
MedS24	14 ± 4.30	88.90 ± 39.91	2.38
MedS25	7.25 ± 1.29	61.85 ± 10.61	1.65
MedS26	16.5 ± 2.59	79.45 ± 9.24	2.12
MedS28	6.25 ± 0.82	42.69 ± 5.43	1.14
MedS29	9.5 ± 5.17	44.73 ± 7.87	1.19
MedS30	5.50 ± 2.06	39.57 ± 2.75	1.05
MedS31	19 ±2.73	67.65 ± 18.15	1.81
MedS32	19 ±2.73	67.65 ± 18.15	1.81
MedP01	12.25 ± 4.81	45.73 ± 16.19	1.22
MedP03	12 ± 5.61	40.96 ± 12.11	1.09
MedP04	6 ± 3.08	58.61 ± 3.81	1.56
MedP05	12.25 ± 4.96	64.44 ± 11.82	1.72
MedP06	12 ± 4.06	48.13 ± 26.49	1.28
MedP07	9.25 ± 2.38	37.57 ± 7.32	1.00
MedP09	4.25 ± 1.47	62.73 ± 5.19	1.67
MedP10	12.25 ± 1.47	44.98 ± 4.03	1.20
MedP12	12.75 ± 2.38	44.62 ± 6.49	1.19
MedP13	13.75 ± 3.11	54.38 ± 5.26	1.45
MedP14	7.25 ± 3.96	32.77 ±11.23	0.87
MedP17	7.25 ± 0.82	61.52 ± 15.86	1.64
MedP18	9.75 ± 2.16	59.55 ± 13.61	1.59
MedP19	8.25 ± 4.14	40.93 ±11.49	1.09
S. meliloti	19.5 ± 6.5	78.51 ±17.61	2.10
S. meliloti	19 ± 4.63	81.75 ± 13	2.18
S. fredii	5.25 ± 0.82	71.95 ± 27.91	1.92
S. medicae	12.25 ± 2.77	64.40 ± 20.37	1.72
A. tumefaciens	2.25 ± 1.78	43.61 ± 8.35	1.16
Rhizobium sp.	5.25 ± 2.86	61.70 ± 11.49	1.65

[a]Average ± standard deviation.

Table 5. Effect of different antibiotics on growth of *Medicago ciliaris*L. rhizobia.

Strain	Str 10 µg	Nal. ac 30 µg	Km 30 µg	Amp 10 µg	Chl 30 µg	Tet 30 µg	Rif	Neo	Ery
MedS01	R	R	S	R	R	S	S	R	R
MedS02	R	R	S	R	R	S	S	R	R
MedS04	R	R	S	S	R	S	S	R	R
MedS07	R	R	S	R	R	S	S	R	R
MedS08	R	R	S	R	R	S	S	R	R
MedS09	S	S	S	R	S	S	S	R	R
MedS10	S	R	S	R	R	S	S	R	R
MedS11	R	R	S	R	S	S	S	R	R
MedS12	R	R	S	R	S	S	S	S	R
MedS13	S	R	S	R	S	S	S	R	R
MedS14	R	S	S	R	S	S	S	R	R
MedS15	S	R	S	R	R	S	S	R	R
MedS16	S	R	S	S	S	S	S	R	R
MedS20	S	R	S	S	S	S	S	R	R
MedS23	R	S	S	R	S	S	S	R	R
MedS24	R	S	S	R	S	S	S	R	R
MedS25	R	S	S	R	S	S	S	S	R
MedS26	R	S	S	R	S	S	S	R	R
MedS28	S	S	S	S	S	S	S	R	R
MedS29	R	S	S	R	S	S	S	R	R
MedS30	S	R	S	R	S	S	S	R	R
MedS31	R	S	S	R	S	S	S	R	R
MedS32	R	S	S	S	S	S	S	R	R
MedP01	S	R	S	R	S	S	S	R	R
MedP03	R	S	S	S	S	S	S	R	R
MedP04	R	R	S	R	S	S	S	R	R
MedP05	S	S	S	S	S	S	S	R	R
MedP06	S	S	S	R	R	S	S	R	R
MedP07	S	S	S	S	S	S	S	S	R
MedP09	S	S	S	S	S	S	S	R	R
MedP10	R	R	S	R	S	S	S	R	R
MedP12	S	R	S	R	S	S	S	R	R
MedP13	R	R	S	R	S	S	S	R	R
MedP14	S	R	S	R	S	S	S	R	R
MedP17	S	R	S	S	S	S	S	R	R
MedP18	S	R	S	S	S	S	S	R	R
MedP19	S	S	S	R	S	S	S	R	R
S. meliloti	S	R	S	R	S	S	S	R	R
S. meliloti	R	S	S	S	S	S	S	R	R
S. fredii	S	R	S	S	S	S	S	R	R
S. medicae	S	R	S	R	S	S	S	R	R
A. tumefaciens	R	S	S	R	S	S	S	R	R
Rhizobium sp.	R	S	S	R	S	S	S	R	R

R, Resistant to antibiotic; S, sensivite to antibiotic; Str: Streptomycin; Nal. ac: nalidicic acid; Km: kanamycin; Amp: Ampicillin; Chl: chloramphenicol; Tet: tetracycline; Rif: Rifampicin; Neo: Neomycin; Ery: Erythromycin.

DNA/DNA hybridization.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Badri M, Zitoun A, Soula S, Ilahi H, Huguet T, Aouani ME (2008). Low levels of quantitative and molecular genetic differentiation among natural populations of *Medicago ciliaris* Kroch. (Fabaceae) of different Tunisian eco-geographical origin. Conserv. Genet. 9(6):1509-1520.

Bailly X, Olivieri B, Brunel JC, Cleyet-Marel, Bena G (2007). Horizontal gene transfer and homologous recombination drive the evolution of the nitrogen-fixing symbionts of Medicago species. J. Bacteriol. 189:5223-5236.

Ben Amor B, Shaw SL, Oldroyd GED, Maillet F, Penmetsa RV, Cook D, Long SR, Denarie J, Gough C (2003). The NFP locus of Medicagotruncatula controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. Plant J. 34:495-506.

Ben Salah I, Albacete A, Martinez Andujar C, Haouala R , Labidi N, Zribi F, Martinez V, Perez-Alfocea F, Abdelly C (2009). Response of nitrogen fixation in relation to nodule carbohydrate metabolism in *Medicago ciliaris* lines subjected to salt stress. J. Plant Physiol. 166:477-488.

Bena G, Lyet A, Huguet T, Olivieri I (2005). Medicago-Sinorhizobium symbiotic specificity evolution and the geographic expansion of Medicago. J. Evol. Biol. 18:1547-1559.

Bena G, ProsperiJM , Lejeune B,Olivieri I (1998). Evolution of annual species of the genus *Medicago*: a molecular phylogenetic approach. J. Mol. Evol. 9:552-559.

Bernaerts MJ, De Ley J (1963). A biochemical test for crown gall bacteria. Nature 197:406-407.

Bradic M, Sikora S, Redzepovic S, Stafa Z (2003). Genetic identification and symbiotic efficiency of an indigenous *Sinorhizobium meliloti* field population. Food Technol. Biotech. 41(1):69-75.

Brunel B, Rome S, Ziani R, Cleyet-Marel JC (1996) Comparison of nucleotide diversity and symbiotic properties of Rhizobium meliloti populations from annual Medicago species. FEMS Microbiol. Ecol. 19:71-82.

Buss MD, Bottomley PJ (1989). Growth and nodulation responses of *Rhizobium meliloti*to water stress induced by permeating and nonpermeating solutes. Appl. Environ. Microbiol. 55:2431- 2436.

Cook D (1999).Medicagotruncatula: a model in the making. Curr. Opin. Plant Biol. 2: 301-304.

Cooper JE (1982). Acid production, acid tolerance and growth rate of lotus rhizobia in laboratory media. Soil Biol. Biochem. 14:127-131.

Cunningham SD, Munns DN (1984). The correlation between extra cellular polysaccharide production and acid tolerance in Rhizobium. Soil Biol. Biochem. 14:127-131.

De Billy F, Grosjean C, May S, Bennett M, Cullimore JV (2001). Expression studies on Auxl-like genes in Medicagotruncatula suggest that auxin is required at two steps in early nodule development. Mol. Plant Microbe Interact. 14:267-277.

De Lajudie P, Willems A, Pot B, Dewettinck D, Maestrojuan G, Neyra M, Collins M.D, Dreyfus B, Kersters K, Gillis M (1994). Polyphasic taxonomy of rhizobia: emendation of the genus Sinorhizobium and description of Sinorhizobiummeliloti comb. nov., Sinorhizobiumsaheli sp. nov., and Sinorhizobiumteranga sp. nov. Int. J. Sys. Bacteriol. 44:715-733

El Sheikh EAE, Wood M. (1989). Salt effects on survival and multiplication of chickpea and soybean rhizobia. Soil Biol. Biochem. 22:343-347.

Fall D, Diouf D, Ourarhi M, Faye A, Abdelmounen H, Neyra M, Sylla SN, El Idrissi MM (2008). Phenotypic and genotypic characteristics of *Acacia senegal* (L.) Wild.root-nodulating bacteria isolated from soils in the dryland part of Senegal. Lett. Appl. Microbiol. 47:85-97.

Foth HD (1990). Fundamentals of soil science (eighth ed.), Wiley, New York, 384. Gagnon, M., W. Hunting and W. B. Esselen. 1959. A new method for catalase determination. Anal. Chem. 31:144.

Gao JL, Sun JG, Li Y, Wang ET, Chen WX (1994). Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan Province. China. Int. J. Syst. Bacteriol. 44:151-158.

Garau G, Reeve WG, Brau L, Deiana P, Yates RJ, James D, Tiwari R, O'Hara GW, Howieson JG (2005). The symbiotic requirements of different Medicago spp. suggest the evolution of *Sinorhizobium meliloti* and *S. medicae* with hosts differentially adapted to soil pH. Plant Soil 276:263-277.

Ge-Hong, WXue-Ying Y, Zhi-Xin Z, Ya-Zhen Y, Lindsrom K (2008). Strain *Mesorhizobium* sp. CCNWGX035: A stress stolerant isolate from Glycyrrhiza glabra displaying a wide host range of nodulation. Pedosphere 18:102-112.

Graham PH(1992). Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. Can. J. Microbiol. 38: 475-484.

Howieson JG, O'Hara GW, Carr SJ (2000).Changing roles of legumes in Mediterranean agriculture; developments from an Australian perspective. Field Crops Res. 65:107-122.

Irwin JAG, Lloyd DL, Lowe KF (2001). Lucerne biology and improvement - an analysis of past activities and future goals in Australia. Aust. J. Agric. Res. 52:699-712.

Jordan DC (1984). Rhizobiaceae. In: Bergey's Manual of Systematic Bacteriology ed. Krieg NR , Holt JG, pp. 234-242. Baltimore:The Williams & Wilkins.

Laouar M, Abdelguerfi A (2000). Study of complex species Medicago Medicago ciliaris - intertexta: characterizations of different types of seed heads. Cahiers Options Mediterraneennes 45: 39-41.

Lesins KA, Lesins I (1979). Genus *Medicago*(Leguminosae): A Taxogenetic Study. The Hague, The Netherlands.

Li X, Su D, Yuan Q (2007). Ridge-furrow planting alfalfa (Medicago sativa L.) for improved rainwater harvest in rainfed semiarid areas in Northwest China. Soil Till. Res. 93:117-125.

Lindstrom K, Lehtomaki S (1988). Metabolic properties, maximum growth temperature and phage sensitivity of Rhizobium sp. (Galega) compared with other fast-growing rhizobia. FEMS Microbiol. Lett. 50:277-287.

Odee DW, Sutherland JM ,Makatiani ET, McInroy SG, Sprent JI (1997). Phenotypic characteristics and composition of rhizobia associated with woody legumes growing in diverse Kenyan conditions. Plant Soil 188:65-75.

Payakapong W, Tittabutr P, Teaumroong N, Boonkerd N, Singleton PW, Borthakur D (2006). Identification of two clusters of genes involved in salt tolerance in Sinorhizobium sp. strain BL3. Symbiosis 41:47-51.

Rome S, Fernandez MA, Brunel B, Normand P, Cleyet-Marel JC (1996). Sinorhizobiummedicae sp. nov., isolated from annual Medicago spp. Int. J. Syst. Bacteriol. 4:972-980.

Shamseldin A, El-Saadani M, Sadowsky MJ, An CS (2009). Rapid identification and discrimination among Egyptian genotypes of *Rhizobium leguminosarum* bv. viciae and *Sinorhizobium meliloti* nodulating faba bean (*Vicia faba* L.) by analysis of nodC, ARDRA, and rDNA sequence analysis. Soil Biol. Biochem. 41:45-53.

Shamseldin A, Nyalwidhe J, Werner D (2006). A proteomic approach towards the analysis of salt tolerance in *Rhizobium etli* and *Sinorhizobium meliloti* strains. Curr. Microbiol. 52:333-339.

Struffi P, Corich V, Giacomini A, Benguedouar A, Squartini A, Casella S, Nuti MP (1998). Metabolic proprieties, stress tolerance and macromolecular profiles of rhizobia nodulating *Hedysarum coronarium*. J. Appl. Microbiol. 84:81-89.

Sullivan GT, Ronson CW(1998). Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. Proc. Natl. Acad. Sci. USA. 95:5145-5149.

Van Berkum P, Beyene D, Bao G, Campbell TA, Eardly BD (1998). Rhizobium mongolense sp. nov. is one of the three rhizobial genotypes identified which nodulate and form nitrogen fixing symbiosis with *Medicago ruthenica* [(L.) Ledebour]. Int. J. Syst.

Bacteriol. 48:13-22.
Vincent JM (1970). A Manual for the Practical Study of Root Nodule Bacteria. IBP Handbook, No. 15. Oxford: Blackwell Scientific Publications Ltd.
Wei GH, Tan ZY, Zhu ME, Wang ET, Han SZ, Chen WX (2003). Characterization of rhizobia isolated from legume species within the genera *Astragalus* and *Lespedeza* grown in the Loess Plateau of China and description of Rhizobium loessense sp. nov. Int. J. Syst. Evol. Microbiol. 53(Pt 5):1575-83.
Wong K, Golding GB (2003). A phylogenetic analysis of the pSymB replicon from the Sinorhizobiummeliloti genome reveals a complex evolutionary history. Can. J. Microbiol. 49:269-280.
Zahran HH (1999). Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in arid climate. Micobiol. Mol. Biol. Rev. 63:968-989.

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Full Length Research Paper

Isolation and characterization of endophytic bacteria colonizing halophyte and other salt tolerant plant species from coastal Gujarat

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Endophytic bacteria were isolated from leaves of four dominant halophyte and salt tolerant plant species of coastal Gujarat. The bacterial counts on nutrient agar were found to be maximum in *Spharanthus indicus* (40%) and were minimum in *Salicornia brachiata* (10%). Twenty (20) bacterial isolates were selected and were characterized through morphological characters and biochemical tests. Three were pigmented and 17 were non-pigmented and 50% isolates exhibited amylase activity and only 15% isolates showed urease activity. Six (30%) and two (10%) isolates showed positive results for ammonia production and phosphate solubilization activity. Salt tolerance of the endophytes was also tested. Of the 20 endophytic bacteria, seventeen (85%) isolates showed growth at 7.5% NaCl and fifteen (75%) tolerated upto 10% NaCl concentration. Overall, the growth rate of endophytes decreased with increasing concentration of NaCl in media. The endophytic bacteria were identified through 16S rRNA sequencing and mostly the isolated endophytic bacteria belong to genera *Bacillus* spp.

Key words: Endophyte, halophyte, halophilic bacteria, coastal region, salt tolerance.

INTRODUCTION

About 1% of the species of the land plants can grow and reproduce in coastal or inland saline soils (Manousaki and Kalogerakis, 2011). These remarkable plants, halophytes, are able to survive and reproduce in environments where the salt (NaCl) concentration is around 200 mM or more and tolerate salt concentrations that kill 99% of other species (Flowers and Colmer, 2008).

Among these salt-adapted halophytes are annuals and perennials, monocotyledonous and dicotyledonous species, shrubs, and some trees. Halophytes are highly adaptable plants, which can accrue relatively large amounts of salts.

The halophytes may be productive under harsh conditions of high salt contents of soil, which they manage by balancing their internal osmotic potential through salt accumulation in foliage. So far, over 2,000 halophytic plant species from more than 550 genera in over 100 families have been identified.

Various studies indicate that more than fifty salt-tolerant

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Figure 1. Map showing the study area.

economically useful roots, trunks, bark, stems, leaves, flowers, fruit, and seeds. The vegetative yields of halophytes and other salt tolerant species could have great potentials particularly as a source of livestock fodders (El Shaer, 2010). The sustainable cultivation of halophytes and other salt-tolerant crops on appropriate lands can serve commercial purposes without the degradation associated with large-scale annual mono-cultures and modern industrial agriculture in general.

Microbes that colonize living, internal tissues of plants such as leaf, root, stem and seeds without causing any immediate, over-negative effects are termed as endophytes (Bacon and White, 2000). It is noteworthy that, of the nearly 3, 00,000 plants species that exist on the earth, each individual is a host to endophytes (Petrini, 1991). Each plant has been reported to harbor one or more endophytes (Verma et al., 2007; Kharwar et al., 2008). Endophytes are viewed as outstanding source of secondary metabolites bioactive antimicrobial natural products.

The endophytic microbes were studied in terrestrial plants (Petrini, 1991; Saikkonen et al., 1998; Tan and Zou, 2001) which are found to possess antibacterial (Sessitsch et al., 2004; Wiyakrutta et al., 2004; Long et al., 2003), antifungal (Sessitsch et al., 2004), anticancer (Wiyakrutta et al., 2004; Strobel et al., 1993), anti-malarial (Wiyakrutta et al., 2004), antiviral (Guo et al., 2000), antioxidant (Harper et al., 2003; Strobel et al., 2002) and anti-diabetic (Zhang et al., 1999) activities. In fact, endophyte carrying plants grow more vigorously and are toxic to herbivores. Furthermore, such plants are more drought tolerant than non-infected plants. Also salt tolerance is observed in plants infected with endophytes (Waller et al., 2005). Endophytes acts as biological triggers to activate the stress response more rapidly and strongly than non symbiotic plants. An endophyte with near negligible biomass relative to plants, possesses the the capacity to alter plant community structure, and this process would have been in operation throughout its expanding range (Johri, 2006).

Endophytes are largely unexplored component of biodiversity, especially in the tropics. Endophytes are constantly exposed to intergeneric-genetic exchange with the host plant. Isolation of a potent anticancer agent, taxol from *Pestalotiopsis microspora*, an endophyte of the Yew tree and the phytohormone producing fungus from rice plant, *Gibberella fujikuroi* suggests the potential of endophytes as a source of useful metabolities (Strobel and Long, 1998; Stierle et al., 1993).

Although the presence of endophytic fungi in leaves of some of the halophytes from coastal region is known but endophytic bacteria and their bioprospecting potential from dominant halophytes and/or salt tolerant plant species like *Salicornia brachiata*, *Spharanthus indicus*, *Cressa cretica* and *Suaeda nudiflora* is largely unknown. Hence, the present attempt was to isolate, characterize and explore the biological activity of endophytic bacteria from the leaves of 4 different halophyte and salt tolerant plant species dominant in coastal region of Gujarat. The study was undertaken to test the salt tolerance of the enophytic bacteria and screen them for their plant growth promoting characters.

MATERIALS AND METHODS

Study area and sample collection

The Bara tract that lies between Gulf of Khambhat in Gujarat state covers 3 Tehsils namely: Vagra, Jambusar and Amod of Bharuch district. It lies between 21° 40' to 22° 13'N latitude and 72° 32' to 72° 55' E longitude at level of 5-9 m above mean sea level. The Bara tract experiences a tropical climate. The annual rainfall ranges from 275 to 1484 mm with an average of 737 mm (cv = 37.2%). The onset of monsoon is erratic which normally affects crop seeding operations, germination and seedling establishment. There is at least one critical dry spell of three to four weeks during the months of July-September. The land is having a gentle slope towards the coastal side. The region is also affected by poor quality of ground water which can be used for irrigation in conjunction with surface water.

The coastline of Gujarat is 1,663 km long with total coastal area of 30,022.25 km^2 stretching upto 20 km from the shoreline. The Bara Tract of Bharuch district falls near the Gulf of Cambay (Figure 1). Healthy leaf samples of four different species of halophytic plants namely: *S. brachiata*, *S. indicus*, *C. cretica* and *S. nudiflora* were collected from coastal salt affected soils of Gujarat, India. The details of location of each sampling site are presented in Table 1. Five sub-samples of each plant species growing in different locations were collected. Also, the rhizospheric soils (0-30 cm) of these plant species were collected for estimating soil properties and nutrient status.

All the samples were collected in sealed sterile plastic bags and transported aseptically to the laboratory. A portion of collected leaf samples of each plant species was separated and after washing, air dried followed by oven drying at 65°C for 24 h. The dried samples were grinded through Wiley mill and passed through sieve. The grinded samples were digested in di-acid mixture and the extract was analyzed for Na and K content through flame photometer and Ca and Mg content in the acid extract was determined through

Table 1. Details and locations of the halophyte and salt tolerant plant samples collected from halophyte and salt tolerant plant species.

Plant species	Common name	Family	Type	Location	Site
Cressa cretica	Luni	*Convolvulacea*	Herb	Inland	Occhan, Pahaj
Salicornia brachiata	Marchar	*Chenopodiaceae*	Herb	Coastal	Hatab, Bhavnagar
Suadea nudiflora	Moras	*Chenopodiaceae*	Herb	Coastal	Aladar
Sphaeranthus indicus	Gorakh mundi	*Asteraceae*	Herb	Coastal	Gandhar

titration method (Singh et al., 1999).

Isolation and characterization of endophytic bacteria

For the isolation of endophytic bacteria, the fresh leaf samples were subjected to pretreatment as per the method described by Sun et al. (2006). Fresh leaf samples were washed in running tap water, followed by 2 min wash in 70% ethanol. Then the leaf samples were washed in 2% sodium hypochlorite for 1 min. Finally, leaf were washed in sterile distilled water for 2 min and dried. After pretreatment, leaves were crushed in sterile distilled water using mortar and pestle.

About 1 ml of crushed samples was serially diluted and 0.1 ml of aliquot from 10^{-2} to 10^{-4} dilutions were taken and spread onto nutrient agar medium using sterilized glass L-rod. Plating was done in duplicates and all the plates were incubated at 28°C for 5 days. After incubation morphologically different bacterial colonies were selected and streaked on nutrient agar plants and incubated at 28°C for 48 h. From the total isolates, based on the difference in cultural morphology such as colour, texture, consistency and size limited numbers of representative isolates were selected from all the samples for further investigations. All the selected isolates were sub-cultured in nutrient agar slants and preserved in a refrigerator at 4°C. Phenotypic characteristics, such as Grams' reaction, motility, catalase and oxidase activity of all the isolates were performed following standard procedures.

Screening of endophytic bacteria for enzymatic activity

All the endophytic bacterial isolates were screened for 2 enzymes, amylase and urease as per the method described by Sahu et al. (2005). For the screening of amylase activity all the isolates were spot inoculated on starch agar plates and incubated at 28°C for 5 days. After incubation, plates were flooded with Lugol's iodine. Clear colourless zone around the growth indicates amylase production. Urease activity was determined by inoculating 0.1 ml of each culture into 5 ml urea broth and incubating at 28°C for five days. Purple red colour throughout the medium indicates alkalinezation and urea hydrolysis.

Screening of endophytic bacteria for production of plant growth promoting substances

Ammonia production by endophytic bacteria was studied by inoculating culture into 5 ml Peptone Nitrate Broth and incubating at 28°C for 48 h. The change of red litmus to purple or blue indicates ammonia production. Phosphate solubilizing activity of endophytic bacteria was studied by the method described by Pandey et al. (2008) using Pikovskaya's agar medium. After incubation, the presence of clear halo around the growth indicates phosphate solubilization.

The Methyl red test was used to detect mixed acid fermentation by endophytic bacteria. Endophytic cultures were inoculated into 5 ml of GPB broth individually and incubated at 28°C for 48 h. After incubation, 5 drops of methyl red indicator was added to the medium. Only mixed acid fermenters produce sufficient quantities of acids during the initial phase of incubation which was detected by methyl red indicator.

The Voges-Proskauer test was used as a qualitative method for the detection of acetoin. Endophytic cultures were inoculated into 5 ml of MR-VP broth individually and incubated at 28°C for five days. After incubation, to 1 ml of bacterial culture, 3 ml of freshly prepared 5% α-naphthol in absolute ethanol and 1 ml of 40% KOH were added and the mixture was stirred vigorously. The formation of red colour was indicative of the presence of acetoin. For the screening of indole acetic acid (IAA), about 0.1 ml of 24 h old culture was inoculated into each 5 ml of 1% Tryptone broth and incubated at 28°C for five days. After incubation, three to four drops of xylene was added and mixed vigorously. Two layers were allowed to separate followed by slow addition of 1 ml Ehrlich's reagent so as to form the layer on the surface of xylene. The formation of pink coloured ring at the lower surface of xylene layer indicated the production of IAA.

Screening of endophytic bacteria for salt (NaCl) tolerance

Endophytic bacteria were inoculated onto Nutrient agar medium supplemented with different concentrations of NaCl (2.5, 5.0, 7.5 and 10%). All the plates were incubated at 28°C for 5 days and bacterial growth was observed at every 24 h.

Identification of endophytic bacteria

The twenty endophytic bacterial isolates were submitted for molecular identification where Fast MicroSeq 500 16S rDNA Bacterial identification kit was used for extraction. Sequencing of the 16S rRNA gene was carried out using primers in 3130 Genetic analyzer and submitted to NCBI Genebank database.

RESULTS

Properties of rhizosphere soil

The rhizosphere soils of the 4 different dominant halophytes and salt tolerant plant species were found to be medium black to coastal alluvium and moderate to highly saline. The soils are clayey in texture with swell-shrink properties thus high water holding capacity. The rhizosphere soils are dominated by high soluble salt content where Na and Ca ions were present in high concentrations. The soils are found to be low in N and P while sufficient to high in sulphate content. The details of the soil properties are presented in Table 2.

Table 2. Properties of rhizosphere soil of halophyte and salt tolerant plant species.

Soil properties	Range	Mean
pH (1:2 w/v)	7.85-9.25	8.40
EC (dS/m)	2.05-35.50	17.05
Org. C. (g/kg)	1.3-6.7	4.1
Available P (mg/kg)	1.41-2.62	1.98
Available S (mg/kg)	24.4-302.10	124.5
NH_4-N (mg/kg)	3.10-10.08	7.91
NO_3-N (mg/kg)	2.64-7.06	4.17
Exch. Na (mg/kg)	968.7-5171.8	2568.5
Exch. K (mg/kg)	387.5-1775.0	935.2
Exch. Ca (mg/kg)	3400-7100	5600
Exch. Mg (mg/kg)	240-2160	1080
Water holding capacity (%)	43.7-59.4	51.6

EC = Electrical conductivity; Org. C. = organic carbon content; Exch. = exchangeable ion.

Table 3. Ionic content in leaves of halophyte and salt tolerant plant species.

Plant species	K (mg/g)	Na (mg/g)	Ca (mg/g)	Mg (mg/g)	S content (mg/g)
Cressa cretica	5.91	50.50	20.0	16.8	3.97
Salicornia brachiata	11.92	17.76	24.0	16.8	7.33
Suadea nudiflora	10.32	21.42	12.0	4.8	0.53
Spharanthus indicus	7.66	15.30	14.0	4.8	2.42

Ionic content of leaf samples

Plant leaf samples were analysed for various ionic elemental contents to get an idea about the uptake of these elements. By the analysis of the leaf samples of various halophyte and salt tolerant plant species, it was found that *S. brachiata* contain maximum amount of potassium content in their leaves. *S. nudiflora* leaves contain higher K than plant species studied (Table 3). The highest Na (50.50 mg/g) content was found in the leaves of *C. cretica* and the lowest amount (15.30 mg/g) was found in the leaf samples of *S. indicus*. It was found that the leaf samples of all the plant species contain calcium in the range of 12.0 to 24.0 mg/g, in which the highest Ca (24.0 mg/g) was found in the leaves of *S. brachiata* (24.0 mg/g) (Table 3). The highest content of magnesium (16.8 mg/g) was found in the leaves of *C. cretica* and *S. brachiata* while the lowest value of Mg (4.80 mg/g) was observed in the leaves of *S. indicus*. The sulphur content of leaf samples of different plant species varied from 0.53 to 7.33 mg/g on dry weight basis (Table 3). The leaves of *S. brachiata* contain the highest sulphur (7.33 mg/g), while the leaf samples of *Suadea nudiflora* contain the lowest sulphur (0.53 mg/g).

Isolation of endophytic bacteria

Nutrient agar plates inoculated with leaf extracts of four dominant halophytes or salt tolerant plants showed morphologically different bacterial colonies. Twenty isolates were selected for further investigations based on their fast growth. The bacterial counts were found maximum in *S. indicus* (40%) and were minimum in *S. brachiata* (10%) (Figures 2 and 3).

Characterization of endophytic bacteria

Morphological characteristics of endophytic bacteria isolated from leaves of different halophyte and salt tolerant plant species are shown in Table 4.

Of the 20 isolates selected, 3 were pigmented and 17 were non-pigmented isolates. Regarding cell shape and Gram's staining, 7 were Gram-negative cocci, 2 Gram-positive cocci, 4 Gram-negative bacilli and 7 Gram-positive bacilli. Motility test results depicted that 18 isolates were motile while only 2 isolates were non-motile. In total, 11 isolates showed positive results for oxidase test whereas all endophytic bacterial cultures showed negative catalase test.

Enzymatic activity

The enzymatic activity of endophytic isolates revealed that 50% isolates exhibited amylase activity and only

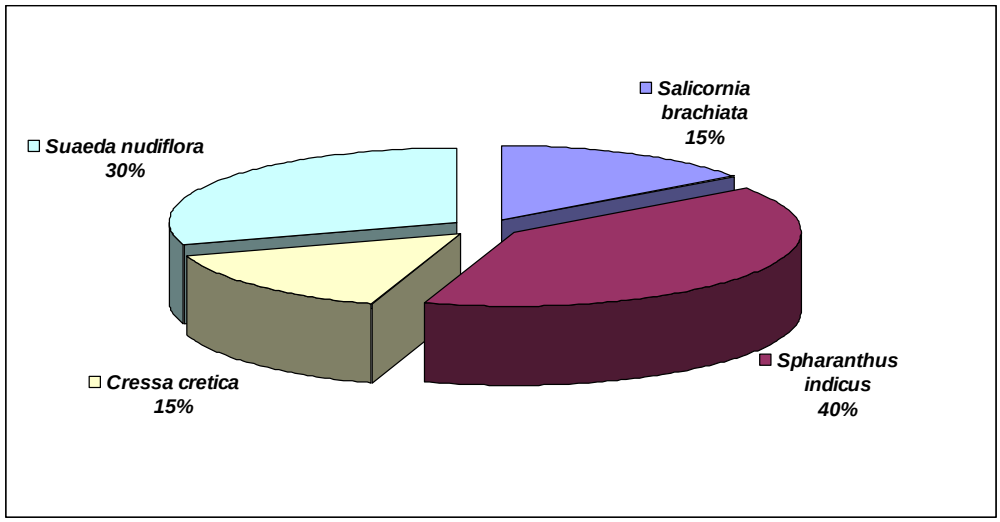

Figure 2. Percent distribution of endophytic bacteria in leaves of halophytes and salt tolerant plant species from Coastal Gujarat.

Figure 3. Dominant halophytes of coastal Gujarat.

15% isolates showed urease activity. Detailed results are shown in Table 5.

Production of plant growth promoters

Of 20 endophytic bacterial isolates screened for plant growth promoting substances, 6 (30%) and 2 (10%) isolates showed positive results for ammonia production and phosphate solubilization activity. Only 4 (20%) were mixed acid fermenters, 5 (25%) showed the production of acetoin and none of the isolates exhibited IAA production (Table 6).

Molecular identification of endophytic bacteria

The selected bacterial isolates were submitted for 16S rRNA gene sequencing and it was observed that *Acinetobacter baumannii, Bacillus cereus*, *Bacillus firmus, Bacillus aerius*, *Pseudomonas fluorescens* and *Bacillus subtilis* were positive for ammonia production while phosphate solubilization was positive for *A. baumannii* and *P. fluorescens* (Table 7).

Tolerance to sodium chloride

All the 20 endophytic bacteria showed good growth at 2.5% NaCl concentration while 18 (90%) isolates grow upto 5% NaCl, seventeen (85%) isolates showed growth at 7.5% NaCl and fifteen (75%) tolerated upto 10% NaCl concentration. *Bacillus foraminis* and *Bacillus gibsonii* could tolerate upto 7.5% NaCl while *A. baumannii* and *Paenibacillus xylanisolvens* tolerated only upto 2.5% NaCl concentration and *P. fluorescens* upto 5% NaCl. All

Table 4. Morphological characteristics of endophytic bacteria isolated from leaves of different salt tolerant plant species.

Isolate no.	Colony morphology				Grams' staining	Shape	Motility
	Form	Elevation	Margin	Colour			
EB1	Circular	Pulvunate	Entire	White	-	Cocci	M
EB2	Circular	Convex	Entire	Yellow	-	Cocci	HM
EB3	Irregular	Raised	Wavy	White	+	Bacilli	M
EB4	Irregular	Effused	Wavy	Off- white	-	Cocci	HM
EB5	Circular	Convex	Entire	White	-	Cocci	HM
EB6	Circular	Convex	Entire	Off- white	+	Bacilli	M
EB7	Irregular	Flat	Wavy	White	-	Cocci	M
EB8	Circular	Pulvunate	Entire	White	-	Cocci	HM
EB9	Irregular	Flat	Wavy	Off- white	+	Bacilli	M
EB10	Circular	Convex	Entire	Off- white	+	Cocci	HM
EB11	Circular	Flat	Entire	Dew drop	+	Bacilli	HM
EB12	Irregular	Effused	Wavy	Off- white	+	Bacilli	NM
EB13	Circular	Capitate	Entire	Dew drop	+	Bacilli	M
EB14	Circular	Effused	Wavy	Off-white	-	Bacilli	HM
EB15	Circular	Umbonate	Entire	White	+	Cocci	HM
EB16	Irregular	Flat	Wavy	White	+	Bacilli	M
EB17	Circular	Convex	Entire	Off-white	-	Bacilli	NM
EB18	Circular	Convex	Entire	Orange	-	Bacilli	M
EB19	Round	Convex	Entire	Light yellow	-	Cocci	HM
EB20	Round	Flat	Erose	Off-white	-	Bacilli	M

- Negative; + positive; M motile; HM highly motile; NM non-motile.

Table 5. Enzyme activity of endophytic bacteria isolated from leaves of halophyte and salt tolerant plant species.

Isolate no.	Oxidase test	Catalase test	Amylase activity	Urease activity
EB1	-	-	+	-
EB2	-	-	-	-
EB3	+	-	+	-
EB4	+	-	+	-
EB5	-	-	-	+
EB6	+	-	-	-
EB7	+	-	-	+
EB8	-	-	-	-
EB9	+	-	-	-
EB10	-	-	+	-
EB11	+	-	+	-
EB12	+	-	+	-
EB13	+	-	+	-
EB14	-	-	+	+
EB15	+	-	-	-
EB16	+	-	+	-
EB17	-	-	+	-
EB18	-	-	-	-
EB19	-	-	-	-
EB20	+	-	-	-

Table 6. Plant growth promotion properties of endophytic bacteria isolated from leaves of halophytes and salt tolerant plant species.

Isolate no.	MR test	VP test	Ammonia production	Indole production	Phosphate solubilization
EB1	-	-	+	-	+
EB2	+	-	-	-	-
EB3	+	+	+	-	-
EB4	-	-	+	-	-
EB5	+	+	-	-	-
EB6	-	-	-	-	-
EB7	-	-	-	-	-
EB8	+	+	-	-	-
EB9	-	+	-	-	-
EB10	-	-	-	-	-
EB11	-	-	-	-	-
EB12	-	-	-	-	-
EB13	-	-	-	-	-
EB14	-	+	+	-	-
EB15	-	-	+	-	+
EB16	-	-	+	-	-
EB17	-	-	-	-	-
EB18	-	-	-	-	-
EB19	-	-	-	-	-
EB20	-	-	-	-	-

+ Positive; - negative

Table 7. Molecular characterization of endophytic bacteria.

Isolate ID	Endophytic bacteria
EB1	*Acinetobacter baumannii*
EB2	*Kocuria flavus*
EB3	*Bacillus cereus*
EB4	*Bacillus firmus*
EB5	*Staphylococcus pasteuri*
EB6	*Paenibacillus xylanisolvens*
EB7	*Bacillus horneckiae*
EB8	*Paenibacillus xylanisolvens*
EB9	*Bacillus licheniformis*
EB10	*Bacillus foraminis*
EB11	*Virgibacillus picturae*
EB12	*Oceanobacillus picturae*
EB13	*Bacillus subtilis*
EB14	*Bacillus aerius*
EB15	*Pseudomonas fluorescens*
EB16	*Bacillus subtilis*
EB17	*Bacillus aryabhattai /megaterium*
EB18	*Arthrobacter luteolus*
EB19	*Bacillus gibsonii*
EB20	*Paenibacillus sp.*

the other isolates were able to tolerate 10% NaCl concentration in media. Overall, the growth rate of endophytes decreased with increasing concentration of NaCl (Table 8) in the media.

DISCUSSION

In general Na^+ depresses K^+ uptake, but Hardikar et al. (2011) observed significant increase of K^+ in all tissue of seedlings with the increasing soil salinity in *Salvadora oleoides*. There was high selectivity of *S. oleoides* for K^+ over Na^+.

In practice, Na^+ is largely compartmentalised in vacuoles in halophytes (Flowers, 1977; Flowers et al., 1986). A range of metabolically inert organic compounds is also present and utilized to adjust the osmotic potential of the cytoplasm.

Maggigo et al. (2000) also observed increased growth in *S. persica* under saline conditions. Although the presence of NaCl is rarely an obligate requirement for growth of many halophyte (Flowers, 1977). The absence of salt in the nutrient solution strongly inhibited the growth of *S. persica* and other halophytes this was observed by (Maggigo et al., 2000).

In *Salvadora persica,* sodium content of leaves increased by 10% when imposed salinity was raised up to 30 dSm^{-1}, while potassium content in leaves reduced up to 18% at this salinity level. Maggio et al. (2000) also

Table 8. Salt tolerance of endophytic bacteria isolated from leaves of halophytes and salt tolerant plant species.

Isolate no.	Salt tolerance (NaCl %)			
	2.5%	5.0%	7.5%	10%
EB1	+	-	-	-
EB2	+	+	+	+
EB3	+	+	+	+
EB4	+	+	+	+
EB5	+	+	+	+
EB6	+	-	-	-
EB7	+	+	+	+
EB8	+	+	+	+
EB9	+	+	+	+
EB10	+	+	+	-
EB11	+	+	+	+
EB12	+	+	+	+
EB13	+	+	+	+
EB14	+	+	+	+
EB15	+	+	-	-
EB16	+	+	+	+
EB17	+	+	+	+
EB18	+	+	+	+
EB19	+	+	+	-
EB20	+	+	+	+

+= Growth; - = no growth

reported that in *S. persica* sodium content of plant grown under salinity showed 40 fold increase as compared to non-saline conditions. The increased Na^+ content plays an important role in osmotic adjustments. Contrary, in *S. oleoides,* K^+ and Na^+ content significantly increased in leaves in response to increasing soil salinity. There was a positive relationship between salt concentration applied and K^+ content in leaf. Similarly, a positive relationship was obtained between salt concentration and Na^+ content of leaves.

The high Na^+ content in leaf is due to compartmentation in leaf vacuoles in *Suaeda* (Maathuis et al., 1992). Whereas, K^+ content was reported to be very high in the leaves of *Salvadora oleoides*.

Endophytic bacteria are poorly investigated group of micro organism that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agriculture, and industrial areas (Strobel and Daisy, 2003). The mechanisms through which endophytes exist and respond to their surrounding must be better understood in order to be more predictive about which higher plants to seek study and spend time for isolating microfloral components. This may facilitate the product discovery process (Ravikumar et al., 2010). In the present study, from four different halophytes or salt tolerant plant leaves, 20 bacterial isolates were selected based on different morphological characters and salt tolerance. Other parts of the plant such as roots (Asraful et al., 2010; Zhang et al., 2010), stems, seeds (Zhang et al., 2010; Magani et al., 2010), petioles, tubers tissues, and flowers (Reiter and Sessitsch, 2000) can also be used in isolation of endophytes. Morphologically different 36 bacterial isolates has been isolated from leaves of mangrove and salt-marsh plants (Gayathri et al., 2010). Leaves of Gaynura procumbent plants have been used for isolation of cultivatable bacterial endophytes (Bhore et al., 2010).

It is well established that plant bacterial endophytes are to be found in most healthy plant tissues (Frommel et al., 1993; McInroy and Kloepper, 1995; Sturz, 1995). This particular host endophyte interaction has been variously defined as altruism, commensalisms, symbiosis or passivity to pathogenicity. Whatever the specific relationship involved, internal plant colonization by bacteria constitutes a vast and as yet little mapped ecological niche.

The diversity of a collection of twenty putative endophytic bacteria isolated from different tissues of the host was assessed using phenotypic characterization methods. Colony morphology gave an indication of the variation among the endophytes. The isolates studied were chosen for their dominance as well as uniqueness or differences with other in colony morphology. Interestingly, the proportion of Gram positive and Gram negative isolates in our study was almost similar. Earlier researchers have reported a predominance of Gram negative bacteria in the tissues of various plants (Stoltzfus et al., 1997; Elbeltagy, 2000). However, Zinniel et al. (2002) reported an equal presence of Gram negative and Gram positive bacteria.

Our observation revealed that, 85% of endophytic bacterial isolates were non-pigmented whereas only 15% were pigmented. Results of endophytes from mangrove leaves revealed 75% pigmented and 25% non-pigmented isolates (Gayathri et al., 2010).

Motility test results showed that majority of the isolates that is, 90% are highly motile. Studies on *Glycine max* and *Glycine soja* revealed that when grown on 2% agar, 78% of the endophytic isolates were found to be motile (Hung and Annapurna, 2004). Due to motility of these endophytes, there is an advantage for spreading of endophytes into the host plant.

Enzymes are the most important products. In the present study, while screening the endophytic bacterial isolates for two different enzymes, 10 isolates exhibited amylase and three isolates the urease activity. The assemblage of endophytes in young, mature and senescent leaves of *R. apiculata* and its possible role in mangrove litter degradation have been reported (Kumaresan and Suryanarayanan, 2001).

Endophytic bacteria residing within plant tissues have been reported to be promoting the plant growth directly or

indirectly through production of phytohormones, bio-control of host plant diseases and improvement of plant nutritional status (Pandey et al., 2008; Rosenblueth and Romero, 2006). They possess the capacity to solubilize phosphates as shown with the endophytic bacteria of soybean in phosphate assimilation (Hung and Annapurna, 2004). Phosphate solubilization by *Bacillus* sp. isolated from salt stressed environment had been observed by earlier researchers (Son et al., 2006). It is also evident in the present study that endophytic bacterial isolates showed four growth promoting activities, particularly ammonia and acetoin production by 6 and 5 isolates, respectively. Volatile substances, such as 2,3 butanediol and acetoin produced by bacteria are responsible for plant growth promotion, which is newly discovered mechanism (Ryu et al., 2003). Mixed acid fermenters produce complex mixture of acids like acetic, lactic, succinic and formic acids. Majority of the bacterial isolates were identified as *Bacillus* spp. Earlier studies also indicated the dominance of genera *Bacillus sp* and *Pseudomonas* spp. having PGP activity in salt stress (Tank and Saraf, 2010).

Occurrence of halophilic bacteria is well known in coastal marine biotopes including mangrove and salt marsh ecosystems. There are only two reports on halophilic endophytes in the coastal plants, one of them is the report of Kamalraj et al. (2008) who showed the effect of NaCl on endophytic fungal assemblage in the leaves of a mangrove *C. roxburghiana*. In the study, none of the endophyte showed growth above 300 mM NaCl concentration. Secondly, the endophytic bacteria isolated from five mangrove and two salt marsh leaves are reported to tolerate salt concentration upto 10% NaCl (Gayathri et al., 2010). However, in the present study, salt tolerance of endophytic bacteria was also observed upto 10% NaCl concentration and the growth rate of endophytes decreased with the increase in the salt concentration. Upadhyay et al. (2009) had reported that bacteria isolated from saline environment are more likely to withstand salt stress. Furthermore, if such bacterial strains also possess plant growth promoting properties they would be beneficial for use in mitigation of salt stress to enable agricultural crop production in saline soils (Egamberdiyeva and Islam, 2008).

Conclusion

This study demonstrated the occurrence and diversity of culturable endophytic bacteria from leaves of 4 different dominant halophytes and salt tolerant plant species dominant in coastal region of Gujarat. The successful colonization of these plants with such microbes suggests that they can be utilized in future applications, such as delivery of degradative enzyme for controlling certain plant diseases, plant growth promoting substances or other useful products. Also the halophilic endophytic bacteria having potential for plant growth promotion and phosphate solubilization can be possibly utilized for bio-remediation of salt affected soils for agricultural crop production. Therefore, further studies are needed for possible commercial utility of these potential bio-chemicals.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Bacon CW, White JF (2000). Microbial endophytes. Marcel Dekker Inc., New York, 341-388.

Bhore Subhash J, Nithya Ravichantar, Chye Ying Loh (2010). Screening of endophytic bacteria isolated from leaves of Sambung Nyawa [*Gynura procumbens* (Lour.) Merr.] for cytokinin-like compounds. Bioinformation 5(5):191-197.

Egamberdiyeva D, Islam KR (2008). Salt-tolerant rhizobacteria: plant growth promoting traits and physiological characterization within ecologically stressed environments. In: Ahmad I, Pichtel J, Hayat S (eds) Plant-bacteria interactions- strategies and techniques to promote plant growth. Wiley Press, Weilheim. 257-281.

El Shaer HM (2010). Halophytes and salt-tolerant plants as potential forage for ruminants in the Near East Region. Small Rumin. Res. 91:3-12.

Elbeltagy A, Nishioka K, Suzuki H, Sato T, Yu Sato, Morisaki H, Mitsui H, Minamisawa K (2000). Isolation and characterization of endophytic bacteria from wild and traditionally cultivated rice varieties. Soil Sci. Plant Nutr. 46:617-629.

Flowers TJ (1977). The mechanism of salt tolerance in halophytes. Annu. Rev. Plant Physiol. 28:89-121.

Flowers TJ, Colmer TD (2008). Salinity tolerance in halophytes. New Phytol. 179:945-950.

Flowers TJ, Hajibagheri MA, Clipson NJW (1986). Halophytes. Q. Rev. Biol. 61:313-337.

Frommel MI, Nawak J, Lazorovits G (1993). Treatment of potato tubers with a growth promoting *Pseudomonas* sp.: plant growth responses and bacterium distribution in the rhizosphere. Plant Soil 150:51-60.

Gayathri S, Saravanan D, Radhakrishnan M, Balagurunathan R, Kathiresan K (2010). Bioprospecting potential of fast growing endophytic bacteria from leaves of mangrove and salt-marsh plant species. Indian J. Biotechnol. 9:397-402.

Guo B, Dai J, Ng S, Huang Y, Leong C, Ong W, Carte BK (2000). Cytonic acids A and B: novel tridepside inhibitors of hCMV protease from the endophytic fungus *Cytonaema* species. J. Nat. Prod. 63: 602-604.

Hardikar SA, Panchal Nilesh S, Pandey AN (2011). Growth, water status and nutrient accumulation of seedlings of Salvadora oleoides (Decne.) in response to soil salinity. Trop. Ecol. 52(3): 253-264.

Harper JK, Ford EJ, Strobel GA, Arif, A., Grant, DM, Porco J, Tomer DP, Oneill K (2003). Pestacin: a 1,3 -dihydro isobenzofuran from *Pestalotiopsis microspora* possessing antioxidant and antimycotic activities. Tetrahedron 59:2471-2476.

Hung PQ, Annapurna K (2004). Isolation and characterization of endophytic bacteria in soybean (*Glycine* sp.). Omonrice 12: 92-101.

Johri BN (2006). Endophytes to the rescue of plants. Curr. Sci. 90: 1315-1316.

Kamalraj S, Sridevi S, Gangodevi V, Venkatesan A, Muthumary J (2008). Effect of NaCl on biochemical changes and endophytic assemblages in the leaves of a mangrove, *Ceriops roxburghiana* Arn. Indian J. Sci. Technol. 1: 1-7.

Kharwar RN, Verma VC, Strobel GA, Ezra D (2008). The endophytic fungal complex of *Catharanthus roseus* (L.) G. Don. Curr. Sci. 95: 228-232.

Kumaresan V, Suryanarayanan T (2001). Occurrence and distribution of endophytic fungi in a mangrove community. Mycol. Res. 105:1388-1391.

Long HH, Furuya N, Kurose D, Takeshita M, Takanami Y (2003). Isolation of endophytic bacteria from *Solanum* sp. and their antibacterial activity against plant pathogenic bacteria. J. Fac. Agric. 48(1-2):21-28.

Maathuis FJM, Flowers TJ, Yeo AR (1992). Chloride compartmentation in leaf vacuoles of the halophyte *Suaeda maritima* (L.) dum. and its relation to tonoplast permeability. J. Exp. Bot. 43(9):1219-1223.

Maggigo A, Reddy MP Jolly (2000). Leaf gas exchange and solute accumulation in the halophyte *Salvadora persica* grown under moderate salinity. Environ. Exp. Bot. 44:31-38.

Manousaki E, Kalogerakis N (2011). Halophytes present new opportunities in phytoremediation of heavy metals and saline soils. Ind. Eng. Chem. Res. 50:656-660.

McInroy JA, Kloepper JW (1995). Survey of indigenous bacterial endophytes from cotton and sweet corn. Plant Soil 173:337-342.

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

Petrini O (1991). Fungal endophytes of tree leaves. In Microbial ecology of leaves (eds. Andrews JH and Hirano SS), Springer-Verlag, New York, pp. 179-197.

Ravikumar S, Inbaneson SJ, Sengottuvel R, Ramu A (2010). Assessment of endophytic bacterial diversity among mangrove plants and their antibacterial activity against bacterial pathogens. Ann. Biol. Res. 1: 240-247.

Rosenblueth M, Romero EM (2006). Bacterial endophytes and their interactions with their hosts. Mol. Plant-Microbe Interact. 19:827-837.

Ryu C, Farag MA, Hu C, Reddy MS, Wei H (2003) Bacterial volatile promotes growth in Arabidopsis. Proc. Natl. Acad. Sci. USA 100:4927-4932.

Sahu MK, Sivakumar K, Kannan L (2005). Degradation of organic matters by the extracellular enzymes of actinomycetes isolated from the sediments and molluscs of the Vellar estuary. J. Aquat. Biol. 20:142-144.

Saikkonen K, Faeth SH, Helander M, Sullivan TJ (1998). Fungal endophytes: a continuum of interactions with host plants. Annu. Rev. Ecol. Syst. 29:319-343.

Sessitsch A, Reiter B, Berg G (2004). Endophytic bacterial communities of field-grown potato plants and their plant-growth promoting and antagonistic abilities. Can. J. Microbiol. 50:239-249.

Singh D, Chhonkar PK, Pandey RN (1999). Soil-plant-water analysis: a methods manual. Indian Agricultural Research Institute, New Delhi, pp. 57-71.

Son HJ, Park GT, Cha MS, Heo MS (2006). Solubilization of insoluble inorganic phosphates by a novel salt- and pH tolerant *Pantoea agglomerans R-42* isolated from soybean rhizosphere. Biores. Technol. 97:204-210.

Stierle A, Strobel GA, Stierle D (1993). Taxol and taxane production by *Taxomyces andreanae*. Science 260: 214-216.

Stoltzfus JR, So R, Malarvithi PP, Ladha JK, de Bruijn FJ (1997). Isolation of endophytic bacteria from rice and assessment of their potential for supplying rice with biologically fixed nitrogen. Plant Soil 194:25-36.

Strobel GA, Daisy B (2003). Bioprospecting for microbial endophytes and their natural products. Microbiol. Mol. Biol. Rev. 67(4):491-502.

Strobel GA, Ford E, Worapong J, Harper JK, Arif AM, Grant DM, Fung PCW, Chan K (2002). Isopestacin, an isobenzofuranone from *Pestalotiopsis microspora*, possessing antifungal and antioxidant activities. Phytochemistry 60:179-183.

Strobel GA, Long SM (1998) Endophytic Microbes Embody Pharmaceutical Potential. ASM News 64:263-268.

Strobel GA, Stierle A, Stierle D, Hess WM (1993). Taxomyces andreanae a proposed new taxon for a bulbilliferous hyphomycete associated with Pacific yew. Mycotaxon 47:71-78.

Sturz AV (1995). The role of endophytic bacteria during seed piece decay and potato tuberization. Plant Soil 175:257-263.

Sun L, Lu Z, Bie X, Lu F, Yang S (2006). Isolation and characterization of a co-producer of fengycins and surfactins, endophytic *Bacillus amyloliquefaciens* ES-2 from *Scuttellaria bacilensis* Georgi. World J. Microbiol. Biotechnol. 22:1259-1266.

Tan RX, Zou WX (2001). Endophytes: a rich source of functional metabolites. Nat. Prod. Rep. 18:448-459.

Tank N, Saraf M (2010). Salinity resistant plant growth promoting rhizobacteria ameliorates sodium chloride stress on tomato plants. J. Plant Interact. 5(1):51-58.

Upadhyay SK, Singh DP, Saikia R (2009). Genetic diversity of plant growth promoting rhizobacteria isolated from rhizospheric soil of wheat under saline condition. Curr. Microbiol. 59:489 496.

Verma VC, Gond SK, Kumara A, Kharwar RN, Strobel GA (2007). Endophytic mycoflora from leaf, bark, and stem of *Azadirachta indica* A. Juss. from Varanasi, India. Microb. Ecol. 54:119-125.

Waller F, Aehatz B, Baltruschat HJ, Becker K, Fischer M, Heier T, Huckelhoven R, Neumann C, Wettstein DV, Franken P, Kogel K (2005). The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc. Nat. Acad. Sci. 102(38):13386-13391.

Wiyakrutta S, Sriubolmas N, Panphut W, Thongon N, Danwisetkanjana K, Ruangrungsi N, Meevootisom V (2004). Endophytic fungi with anti-microbial, anti-cancer and anti-malarial activities isolated from Thai medicinal plants. World J. Microbiol. Biotechnol. 20:265-272.

Zhang B, Salituro G, Szalkowski D, Li Z, Zhang Y, Royo I, Vilella D, Dez M, Pelaez F, Ruby C, Kendall RL, Mao X, Griffin P, Calaycay J, Zierath JR, Heck JV, Smith RG, Moller DE (1999). Discovery of small molecule insulin mimetic with antidiabetic activity in mice. Science 284:974-981.

Zhang L, Duan ZY, Geng X, Li KG, Wei Z, Hu SP (2010). Isolation and identification of endophytic fungi of *Huperzia Serrata*. J. Jishou. Univ. 31:79-84.

Zinniel DK, Lambrecht P, Harris NB, Feng Z, Kuczmarski D, Higley P, Ishimaru CA, Arunakumari A, Barletta RG, Vidaver AK (2002). Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Appl. Environ. Microbiol. 68:2198-2208.

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Full Length Research Paper

Recognition of leptospirosis in dengue-suspected cases during dengue outbreak in Ceará State, Brazil

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Dengue fever frequently presents with non specific symptoms, making it difficult to be distinguished from other acute febrile illnesses, including leptospirosis. Given this, serum samples from 82 patients with clinical features of dengue-like illness were evaluated for dengue infection by IgM-enzyme-linked immunosorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR). Negative samples for dengue were tested for leptospirosis by IgM-ELISA and PCR. Dengue infection was detected in 35 patients. Six patients were positive to leptospirosis. This result shows that leptospirosis is underestimated in Ceará State, especially during epidemics of dengue with many reports, leading to an apparent low incidence of leptospirosis.

Key words: Dengue, leptospirosis, differential diagnosis, underrecognition, misdiagnosis.

INTRODUCTION

Dengue fever (DF) is an infectious disease caused by the dengue virus (DENV), of the family *Flaviviridae* and genus *Flavivirus*. Four serotypes can cause DF: DENV-1, DENV-2, DENV-3 and DENV-4 (Henchal and Putnak, 1990). DF is the most important arboviral disease in the world, and it causes more human illness and death than any of the known arthropod-borne viral infections. More than 2.5 billion people, in at least 100 countries, are at risk of dengue infections accounting for nearly 100 million cases/year globally (World Health Organization, 2012). Large epidemics have occurred frequently in Brazil, particularly in the northeastern region, including Ceará State, where DF has been reported since 1986. Major outbreaks have occurred in 1994, 2008, 2011 and 2012

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Table 1. Clinical and laboratorial features of patients tested for dengue and leptospirosis.

Disease	≤ 5 days from the onset of symptoms	≥ 5 days from the onset of symptoms	Dengue +			*Leptospira* +
			IgM ELISA	VI	Multiplex RT-PCR	IgM ELISA
Dengue	16	19	35	0	4	0
Leptospirosis	4	2	0	0	0	6

Dengue +: patients tested positive for dengue; *Leptospira* +: patients tested positive for leptospirosis; ELISA: enzyme-linked immunosorbent assay; RT-PCR: reverse transcriptase-polymerase chain reaction; IgM: immununoglobulin M; VI: virus isolation.

(Ceará, 2014).

In endemic areas, clinical manifestations of DF are often nonspecific and may be indistinguishable from other febrile syndromes such as influenza, oropouche fever, hantavirus infection and leptospirosis (Flannery et al., 2001; Levett et al., 2000; Manock et al., 2009).

Leptospirosis is a bacterial disease caused by *Leptospira*, and its infection may produce a wide clinical spectrum ranging from asymptomatic infections or undifferentiated febrile syndrome (UFS) to multiple organ failure or death (Libraty et al., 2007). Most leptospiral infections are asymptomatic or result in a mild disease, which can lead to a misdiagnosis of DF instead of leptospirosis in regions where both diseases are endemic (Daher et al., 2010).

DF and leptospirosis share many clinical similarities as well as geographic distribution and potentially fatal complications but need different treatments (Conroy et al., 2014). Up to now, there is no specific treatment for DF, but appropriate intensive supportive therapy may reduce mortality to less than 1% in some severe cases. Therefore, accurate and timely diagnosis is very important for patient management in dengue infection (Mehta et al., 2013). The treatment of leptospirosis requires specific antibiotic therapy, such that the earlier the diagnosis is made, the better the potential outcome of treatment, which can be seriously damaged by a misdiagnosis (Ellis et al., 2008).

This study aimed to evaluate the occurrence of leptospirosis in dengue-suspected cases during the 2008 dengue outbreak in Ceará State, Brazil. The study was approved by the Ethics Committee of the Hospital São José de Doenças Infecciosas (no. 031/2009).

MATERIALS AND METHODS

Serum samples collected from 82 patients with a clinical history of acute fever consistent with dengue infection were provided by Laboratory of Public Health of Ceará State and analyzed retrospectively. As previously published by Lima et al. (2011), the samples were assayed for DENV by enzyme-linked immunosorbent assay (ELISA) (Diagnostics PanBio®, Brisbane, Australia), multiplex reverse-transcription polymerase chain reaction (RT-PCR) (Lanciotti et al., 1992), and virus isolation (VI) using C6/36 cell monolayer cultures with the detection of infection by indirect immunofluorescence assay. In this work, dengue-negative samples were tested for qualitative detection of IgM antibodies to *Leptospira* in the serum using a *Leptospira* IgM ELISA commercial kit (Diagnostics PanBio®, Brisbane, Australia) and by PCR using LP1 and LP2 primers (Kee et al., 1994) and Ludwig commercial kit (Ludwig Biotechnology Ltd., Rio Grande do Sul, Brazil). All the PCR and RT-PCR fragments obtained were separated in 2% agarose gel, stained with Gel RedTM (Biotium Inc., Hayward, USA), and observed under ultraviolet light.

RESULTS AND DISCUSSION

Of the 82 patients, 35 (42.6%) were seropositive for dengue, of which all were ELISA-reactive and VI-negative (Table 1). Four patients (4.8%) were positive by RT-PCR and ELISA, of which 2 (2.4%) were infected with DENV-2 and 2 (2.4%) with DENV-3.

Serological tests for leptospirosis were performed in only 42 of 47 dengue-negative patients due to insufficient sample volume. Among the 42 patients analyzed, 6 (14.3%) were positive for leptospirosis by ELISA and PCR showing negative results. Thirty-six (85.7%) tested negative for both dengue and leptospirosis (Table 1).

The presence of leptospirosis in dengue-suspected cases in this study, and the rate of leptospirosis-infected patients (14.3%), corroborates several previous studies, which have demonstrated the occurrence of misdiagnoses between these diseases due to the similarity of the initial clinical features and the increase in the number of cases during rainy seasons, and supports further documented ranges of between 14 to 24% as reported previously in studies from different locations (Sanders et al., 1999; Libraty et al., 2007; Souza et al., 2007). The cases of leptospirosis in this study were detected in two male patients (33.3%) and in four female patients (66.7%). The most common symptoms identified were fever, headache, arthralgia, myalgia, retro-orbital pain, and asthenia; nausea/vomiting were observed less frequently. In some cases, diarrhea and hemorrhagic manifestations, which are not specific characteristic of leptospirosis presentation, were present. One patient died possibly due to improper treatment or undetermined diagnosis.

An early and accurate diagnosis is essential for clinical management, since proper treatment of patients differs

substantially for dengue and leptospirosis. Early diagnosis also allows the establishment of surveillance and control measures. Effective treatment for leptospirosis must be performed early, because delays or failure in the establishment of proper therapeutic can result in patient death (Daher et al., 2010).

One study limitation was the fact that paired samples were not used and thus, the microagglutination test, considered the gold standard for the diagnosis of leptospirosis, was not performed. Therefore, it is impossible to state categorically that a positive IgM for *Leptospira* necessarily implies an active infection, because anti-*Leptospira* IgM antibodies may remain detectable for several months after initial exposure. These detectable antibodies prevent differentiation between recent infections or a false-positive result (Cumberland et al., 2001). Besides, all samples were negative in PCR, probably due to freeze-thaw cycles of the samples, which may have affected the leptospiral genome detection. On the other hand, the adequate sensitivity of the IgM-ELISA used in this study to detect leptospirosis (PanBio®) decreases the likelihood of false-negative results (Libraty et al., 2007).

Forty patients (48.7%) had an undetermined diagnosis. Other UFS-causing pathogens, not studied here, may have affected these patients. In fact, it is known that patients with DF or leptospirosis can be misdiagnosed with other diseases, such as hantavirus infection, influenza and rubella; all of these could be responsible for the cases of nonspecific diagnosis of UFS (Libraty et al., 2007; Suharti et al., 2009).

This report is a strong indication that misdiagnosis is possible during dengue epidemics due to the lack of laboratory resources and similar non-specific symptoms, causing a false increase in the number of cases of DF and, consequently, an underreporting of other potentially fatal etiologies, such as leptospirosis.

The present study has shown that some cases of leptospirosis were not recognized in the 2008 dengue outbreak in Ceará. Therefore, it is reasonable to assert that leptospirosis can easily be confused with dengue, especially during outbreaks. Thus, differentiation and prompt diagnosis of leptospirosis during an outbreak is essential to enable the establishment of an effective antibiotic therapy and to avoid potentially fatal disease exacerbations due to improper treatment. Furthermore, it reinforces the need for implementation and expansion of surveillance investigations of febrile syndromes to improve the knowledge of the prevalence of leptospirosis, dengue, and other diseases, as they influence the population of Ceará State, Brazil.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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REFERENCES

Ceará. State Government of Ceará (2014). Fortaleza (CE): Center for Epidemiological Surveillance, [cited 2014 april 06]. http://www.saude.ce.gov.br/index.php/boletins

Cumberland P, Everard CO, Wheeler JG, Levett PN (2001). Persistence of antileptospiral IgM, IgG and agglutinating antibodies in patients presenting with acute febrile illness in Barbados 1979-1989. Eur. J. Epidemiol. 17:601-608.

Conroy AL, Gélvez M, Hawkes M, Rajwans N, Liles WC, Villar-Centeno LA, Kain KC (2014). Host biomarkers distinguish dengue from leptospirosis in Colombia: a case-control study. BMC Infect. Dis. 14: 35.

Daher EF, Lima RS, Silva-Júnior GB, Silva EC, Karbage NN, Kataoka RS, Carvalho Júnior PC, Magalhães MM, Mota RM, Libório AB (2010). Clinical presentation of leptospirosis: a retrospective study of 201 patients in a metropolitan city of Brazil. Braz. J. Infect. Dis. 14(1):3-10.

Ellis T, Imrie A, Katz AR, Effler PV (2008). Underrecognition of leptospirosis during a dengue fever outbreak in Hawaii, 2001-2002. Vector Borne Zoonotic Dis. 8:541-547.

Flannery B, Pereira MM, Velloso LF, Carvalho CC, Codes LG, Orrico GS, Dourado CM, Riley LW, Reis MG, Ko AI (2001). Referral pattern of leptospirosis cases during a large urban epidemic of dengue. Am. J. Trop. Med. Hyg. 65:657-663.

Henchal EA, Putnak R (1990). The dengue viruses. Clin. Microbiol. Rev. 3:376-396.

Kee SH, Kim IS, Choi MS, Chang WH (1994). Detection of leptospiral DNA by PCR. J. Clin. Microbiol. 32(4): 1035-1039.

Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV (1992). Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase polymerase chain reaction. J Clin. Microbiol. 30(3):545-551.

Levett PN, Branch SL, Edwards CN (2000). Detection of dengue infection in patients investigated for leptospirosis in Barbados. Am. J. Trop. Med. Hyg. 62:112-114.

Libraty DH, Myint KS, Murray CK, Gibbons RV, Mammen MP, Endy TP, Li W, Vaughn DW, Nisalak A, Kalayanarooj S, Hospenthal DR, Green S, Rothman AL, Ennis FA (2007). A comparative study of leptospirosis and dengue in Thai children. PLoS Negl. Trop. Dis. 1(3): e111.

Lima DM, Sabino-Santos JG, Oliveira AC, Fontes RM, Colares JK, Araújo FM, Cavalcanti LP, da Fonseca BA, Figueiredo LT, Pompeu MM (2011). Hantavirus infection in suspected dengue cases from State of Ceará, Brazil. Rev. Soc. Bras. Med. Trop. 44(6):795-796.

Mehta RC, Goswami Hm, Katara RK, Patel PS, Parikh UV, Vegad MM and Jain PY (2013). Importance of complete blood count and peripheral smear examination in early diagnosis of dengue patients. J. Infect. Dis. 2(1):22-24.

Manock SR, Jacobsen KH, de Bravo NB, Russell KL, Negrete M, Olson JG, Sanchez JL, Blair PJ, Smalligan RD, Quist BK, Espín JF, Espinoza WR, MacCormick F, Fleming LC, Kochel T (2009). Etiology of acute undifferentiated febrile illness in the Amazon basin of Ecuador. Am. J. Trop. Med. Hyg. 81(1):146-51.

Sanders EJ, Rigau-Pérez JG, Smits HL, Deseda CC, Vorndam VA, Aye T, Spiegel RA, Weyant RS, Bragg SL (1999). Increase of leptospirosis in dengue-negative after a hurricane in Puerto Rico in 1966. Am. J. Trop. Med. Hyg. 61:399-404.

Souza AI, Nogueira JM, Pereira MM (2007). Anti-Leptospira antibodies in patients in the State of Mato Grosso do Sul with clinical suspicion of dengue or viral hepatitis. Rev. Soc. Bras. Med. Trop. 40:431-435.

Suharti C, Van Gorp EC, Dolmans WM, Groen J, Hadisaputro S, Djokomoeljanto RJ, Osterhaus DME, Van der Meer JWM (2009). Hanta Virus infection during dengue virus infection outbreak in Indonesia. Acta Med. Indones. 41:75-80.

World Health Organization (2012). Dengue and severe dengue [Internet]; Geneva: World Health Organization; [cited 2012 Dec 22]. Available from: http://www.who.int/mediacentre/factsheets/fs117/en/index.html

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Full Length Research Paper

Antibiotic and heavy-metal resistance in motile *Aeromonas* strains isolated from fish

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***Aeromonas* spp. have been recognized as important pathogens causing massive economic losses in the aquaculture industry. This study examined the resistance of fish *Aeromonas* isolates to 15 antibiotics and 3 heavy metals. Based on the results, it is suggested that selective antibiotheraphy should be applied according to the *Aeromonas* species and the cultured-fish species. In addition, cadmium-resistant strains were associated with resistance to amoxicillin/clavulanic acid, suggesting that cadmium is a global factor related to co-selection of antibiotic resistance in *Aeromonas* spp.**

Key words: *Aeromonas* spp., antibiotic resistance, heavy-metal resistance, aquaculture, multi-antibiotics resistance.

INTRODUCTION

Motile *Aeromonas* spp. is widely distributed in aquatic environments and is a member of the bacterial flora in aquatic animals (Roberts, 2001; Janda and Abbott, 2010). In aquaculture, the bacterium is an emergent pathogen for motile *Aeromonas* septicemia, which causes massive economic losses when cultured fish are in stressful environments (Roberts, 2001). Although many studies have investigated vaccine development for preventing this disease, there is no vaccine available for aquaculture use (Sommerset et al., 2005), which has led to antibiotheraphy being selected as the best way for controlling infection due to *Aeromonas* spp. in the industry (Roberts, 2001). However, veterinarians encounter certain problems related to this approach, including a high diversity of *Aeromonas* spp. and the presence of multi-antibiotics resistance (MAR) strains.

Recent phylogenetic analysis has revealed high taxonomical complexities in the genus *Aeromonas*, with resulting ramifications in *Aeromonas* spp. (Janda and Abbott, 2010; Martinez-Murcia et al., 2011). *Aeromonas hydrophila*, *Aeromonas sobria* and *Aeromonas veronii* have previously been recognized as major pathogens in human and aquatic medicine, and some new species, especially *Aeromonas aquariorum*, are now frequently isolated from human clinical samples (Figueras et al., 2009; Aravena-Roman et al., 2011; Puthucheary et al., 2012). Our previous study found that *A. aquariorum* and *A. caviae* were major pathogens for aeromoniasis of eel (*Anguilla japonica*) (Yi et al., 2013). However, there is less information available on antibiotic resistance

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at the species level of the pathogens in aquatic medicine than in human medicine under critical phylogenetic identification procedures. Recently, many studies have shown emergency of MAR strains is *Aeromonas* spp. from clinical and aquatic environmental samples (Schmidt et al., 2000; Verner-Jeffreys et al., 2009). However, little information has been known about resistance to antibiotics and heavy metals resistances among clinical *Aeromonas* strains in Korea.The antibiotic resistance could result from antibiotic selection pressure in bacteria. On the other hand, heavy metals have been suggested as contributing to co-selection of antibiotic resistance by molecular mechanism such as cross-resistance, co-resistance and co-regulatory resistance (Baker-Austin et al., 2006; Seiler and Berendonk, 2012; Tacão et al., 2013). In addition, heavy metal resistant strains reportedly have a propensity for the MAR phenotype (Akinbowale et al., 2007; Matyar et al., 2010). In fact, there has been a little information on co-selection of antibiotic and heavy metal resistance in *Aeromonas* spp. The aim of this study was to determine the antibiotic resistance patterns at the species level in a collection of *Aeromonas* strains from eel, koi carp and pet fish in order to establish an antibiotheraphy regime for use in aquatic medicine under emergency situations related to infection. In addition, we explored the relationships between heavy metal tolerance and antibiotic resistance by comparing antibiotic resistance patterns between heavy-metal-resistant and heavy-metal-susceptible strains.

MATERIALS AND METHODS

The *Aeromonas* spp. used in this study comprised 117 strains isolated from diseased fish: 70, 11 and 36 strains isolated from eel, koi carp and imported pet fish, respectively. These isolates were well characterized by our previous phylogenetic analysis using *gyrB* and *rpoD* gene sequences (Kim et al., 2013; Yi et al., 2013). The 117 strains could be categorized as follows: 49 *Aeromonas veronii* strains (31 from pet fish, 13 from eel, and 5 from koi carp), 23 *A. aquariorum* strains (22 from eel and 1 from pet fish), 19 *A. hydrophila* strains (12 from eel, 6 from koi carp, and 1 from pet fish), 16 *A. caviae* strains (eel), 5 *Aeromonas jandaei* strains (4 from eel and 1 from pet fish), 2 *Aeromonas allosaccharophila* strains (pet fish), 2 *Aeromonas media* strains (eel), and 1 *Aeromonas trota* strains (eel). All strains were stored at -70°C using Cryocare Bacteria Preservers (Key Scientific Products).

The antibiotic susceptibility test (AST) was implemented using the Vitek2 system with a veterinary susceptibility test card for Gram-negative bacteria (AST-GN38), according to the manufacturer's instructions. Extended-spectrum β-lactamases (ESBL) producing test wells were loaded with cefepime (1 µg/ml), cefotaxime (0.5 µg/ml), or ceftazidime (0.5 µg/ml), or a combination thereof with clavulanic acid (4 µg/ml). Of 20 antibiotics, five different tests could not be evaluated for *Aeromonas* spp. because rifampicin, cefpirome, cephalexin, polymyxin B, and ESBL tests were not available for the species from Clinical and Laboratory Standards Institute (CLSI) criteria for resistance determination using the Vitek-2 AST system.

According to Matyar et al. (2008), the heavy-metal resistance of each strain was determined as the minimum inhibitory concentration (MIC) resulting from the agar dilution test using Mueller-Hinton medium containing Cd^{2+}, Cu^{2+} and Cr^{6+} at concen-trations ranging from 0.32 to 3200 µg/ml generated from $CdCl_2$, $CuSO_4$ and CrO_3, respectively. The *Escherichia coli* K-12 strain was used as the control; a tested strain was considered resistant if its MIC value was higher than that of the *E. coli* K-12 strain.

Levels of MAR to all isolates were quantified using the MAR index, defined as a/b, where 'a' represents the number of antibiotics to which the strain was resistant and 'b' represents the total number of antibiotics against which the individual isolate was tested (Krumperman et al., 1983). Overall MAR indexes were quantified as mean ± SD values for all strains grouped according to species and heavy-metal resistance. The obtained data were analyzed by SPSS (version 11.0) for Microsoft Windows. ANOVA and the t-test were used to identify significant differences in MAR values between groups, while Fisher's exact test was used to assess the significance of such differences. A probability value of $p<0.05$ was considered to be significant in all statistical analyses.

RESULTS

All of the *Aeromonas* strains were found to have the following overall percentage resistances for the indicated antibiotic agents: 99.2% to ampicillin (AM), 84.9% to piperacillin (PIP), 83.2% to tetracycline (TE), 80.7% to enrofloxacin (Eno), 59.7% to amoxicillin/clavulanic acid (AmC), 54.6% to sulfamethoxazole/trimethoprime (Sxt), 54.6% to ceftiofur (Tio), 44.5% to imipenem (Imi), 43.7% to marbofloxacin (Mar), 40.3% to nitrofurantoin (Nit), 34.5% to chloramphenicol (Chl), 26.9% to cefpodoxime (Pod), 24.4% to tobramycin (Tob), 15.1% to gentamicin (Gen) and 4.2% to amikacin (Ami). Table 1 lists the prevalence of resistant strains to 15 different antibiotic agents among major fish groups and *Aeromonas* spp. In the fish groups, AmC- and Chl-resistant strains were more frequently detected in fish strains cultured in Korea than in the imported pet fish strains. Eel strains were significantly more resistant to Pod and Tio, whereas resistance to Gen and Tob was more frequent among pet fish strains. The MAR index values did not differ significantly among fish groups.

Table 1 also showed antibiotic resistance and MAR indexes of major *Aeromonas* spp. Statistically significant differences in the prevalence of strains with resistance to AmC, Pod, Tio, Gen, Tob, Te, Chl and Sxt were observed among the *Aeromonas* spp. AmC-, Pod-, Tio- and Te-resistant strains were frequently detected in *A. caviae* and *A. aquariorum* strains. All *A. caviae* strains were susceptible to Imi. The levels of Gen and Tob resistance were higher in *A. veronii* strains than in the other *Aeromonas* spp. Chl and Sxt did not inhibit the growth of most *A. caviae* strains. The MAR index was highest in *A. caviae*, followed by *A. aquariorum*, *A. veronii* and *A. hydrophila*. This index differed significantly between *A. veronii* and both *A. caviae* and *A. aquariorum*, but not between *A. caviae* and *A. aquariorum* or between *A. hydrophila* and *A. veronii*.

The *E. coli* K-12 control strain exhibited MICs of 200, 1600 and 400 µg/ml to $CdCl_2$, $CuSO_4$ and CrO_3, respectively. Resistance to Cu was detected in only five

Table 1. Comparisons of the prevalence of resistance against 15 different antibiotic agents among different fish groups and major *Aeromonas* species.

Anibiotic	Conc. (μg/ml)	Eel (n=70)	Pet fish (n=36)	Koi (n=11)	p-value	*A. veronii* (n=49)	*A. caviae* (n=16)	*A. aquariorum* (n=23)	*A. hydrophila* (n=19)	p-value
AM	4—32	100	100	90.9	N/A	98.0	100.0	100.0	100.0	1
AmC	4/2—32/16	75.7	30.6	63.6	**<0.001**	32.7	93.8	95.7	78.9	**<0.001**
PIP	4—64	90	75	81.8	0.119	77.6	87.5	95.7	78.9	0.236
Pod	0.5—4	44.3	5.6	0	**N/A**	4.1	56.3	78.3	10.5	**<0.001**
Tio	1—2	70	27.8	27.3	**<0.001**	24.5	93.8	91.3	47.4	**<0.001**
Imi	2—8	47.1	44.4	36.4	0.852	36.7	0.0	91.3	57.9	**<0.001**
Ami	8—64	0	5.6	18.2	N/A	4.1	0.0	0.0	10.5	0.33
Gen	4—32	1.4	36.1	18.2	**<0.001**	26.5	0.0	0.0	10.5	**0.003**
Tob	8—64	10	47.2	27.3	**<0.001**	36.7	18.8	0.0	21.1	**0.002**
Eno	0.25—4	82.9	72.2	90.9	0.337	75.5	100.0	82.6	89.5	0.112
Mar	1—2	47.1	44.4	36.4	0.852	44.9	75.0	34.8	36.8	0.067
Te	2—8	82.9	91.7	72.7	0.222	79.6	100.0	100.0	73.7	**0.007**
Nit	16—64	35.7	47.2	36.4	0.507	36.7	62.5	34.8	42.1	0.304
Chl	4—32	42.9	19.4	36.4	**0.047**	18.4	87.5	17.4	57.9	**<0.001**
Sxt	1/19—16/304	60	52.8	27.3	0.122	49.0	93.8	43.5	47.4	**0.004**
MAR index		0.53±0.17	0.47±0.19	0.44±0.26		0.43±0.20	0.65±0.10 [a]	0.58±0.11 [a]	0.51±0.19	

[a]: Significantly different (p<0.05) from *A. veronii* strains; *AM, ampicillin; Amc, amoxicillin/clavulanic acid; PIP, piperacillin; Pod, cefpodoxime; Tio, ceftiofur; Imi, imipenem; Ami, amikacin; Gen, gentamicin; Tob, tobramycin; Eno, enrofloxacin; Mar, Marbofloxacin; Te, tetracycline; Nit, nitrofurantoin; Chl, chloramphenicol; Sxt, sulfamethoxazole/trimethoprime.

strains: four eel strains for *A. aquariorum* and one pet fish strain for *A. allosaccarophila*. Resistance to Cr was detected in only one *A. veronii* strain from pet fish. In contrast to the low prevalence of resistance to Cr and Cu, Cd-resistant strains were common among the present strains (74.4%). Resistance was present in 61.2, 82.6, 85.7 and 75.0% of *A. veronii*, *A. aquariorum*, *A. hydrophila* and *A. caviae* strains, respectively, with its prevalence not differing significantly among *Aeromonas* spp. However, the prevalence of Cd resistance was significantly higher in strains from the fish cultivated in Korea than in those from the imported pet fish (82.7 *vs*. 40.8%). The prevalence of resistance to AmC, Pod, Tio, Imi and Chl tends to be higher for Cd-resistant than for Cd-susceptible strains. However, a significant difference was observed for AmC between Cd-resistant and Cd-susceptible strains (Table 2). The MAR index values did not differ significantly between the presence and absence of Cd resistance.

Table 2. Comparisons of the prevalence of resistant strains against 15 different antibiotic agents between Cd-resistant and -susceptible strains

Antibiotic	Cd-R (n=87)	Cd-S (n=30)	p
AM	98.9	100.0	1.000
AmC	67.8	43.3	**0.029**
PIP	82.8	90.0	0.557
Pod	32.2	16.7	0.107
Tio	58.6	30.0	0.322
Imi	48.3	40.0	0.526
Ami	2.3	6.7	0.271
Gen	11.5	20.0	0.354
Tob	20.7	30.0	0.321
Eno	79.3	86.7	0.431
Mar	44.8	46.7	1.000
Te	81.6	93.3	0.152
Nit	37.9	46.7	0.518
Chl	36.8	30.0	0.658
Sxt	52.9	60.0	0.531
MAR index	0.50±0.19	0.50±0.17	

DISCUSSION

Comparing our data with those obtained in other studies (Vila et al., 2002; Akinbowale et al., 2007; Aravena-Roman et al., 2012) is difficult due to differences in the antibiotics used and sources isolated. Moreover, there is limited information available on the prevalence of antibiotic – resistant strains in *Aeromonas* spp. isolated from aquatic farming, as accurately identified using housekeeping genes. As we expected, there were frequent emergences among the present strains to quinolones,

Te, and Sxt regardless of the fish group. These antibiotics have been broadly used in both bath and oral therapies applied to cultured fish (including eels) in Korea. Although it is rare, there are also reports of the occurrence of resistance to these antibiotics in *Aeromonas* spp. isolated from clinical specimens for human, aquatic animals and environmental sources (Vila et al., 2002; Akinbowale et al., 2007; Aravena-Roman et al., 2012). However, we did not expect to find that the prevalence of strains with resistance to Gen, Tob, Te, Chl, and Sxt differed among *Aeromonas* spp. A survey of antibiotic use from the allied field of ornamental fish farming found aminoglycosides to be the fourth most frequently used drug type. In addition, Verner-Jeffreys et al. (2009) reported that 5.3, 31 and 61% of strains from ornamental fish were tolerant to Ami, Gen and Tob, respectively. In fact, 31 (63.3%) of the *A. veronii* strains included in the present study originated from imported ornamental fish, and 19 of the strains were resistant to one or more aminoglycosides. This could explain why aminoglycoside-resistant strains were detected frequently in *A. veronii* in the present study. In contrast, higher prevalences of Chl, Sxt and Te resistances were responsible for the characteristics of *A. caviae* and/or *A. aquariorum* strains isolated from eel and koicarp strains farmed in Korea (Kim et al., 2013; Yi et al., 2013). This discrepancy in the prevalence of antibiotics-resistant strains might be due to the resistance mechanism of *Aeromonas* spp. under antibiotic selection pressure induced by antibiotic use differing with the aquaculture environment. It could be that various global factors are relevant to the antibiotic resistance of bacteria.

The investigated *Aeromonas* spp. was susceptible to all the tested antibiotics except aminopenicillins. *Aeromonas* spp. has been considered to be a naturally occurring phenotype for resistance to ampicillin, and hence we expected the prevalence of ampicillin-resistant strains to be significantly higher among the present strains. However, some unexpected higher prevalence rates were observed for the resistance to AmC, Pod, Tio and Imi among eel strains, of which major species were *A. aquariorum* and/or *A. caviae*. Since Pod, Tio, and Imi are used as off-label drugs in aquaculture worldwide (including Korea), these antibiotics have rarely been used in aquatic animal farming. Some *Aeromonas* strains with resistance to these antibiotics have been found in rainbow-trout and shrimp farming. The authors of these previous reports have speculated that the resistances result from an inflow of the antibiotic from domestic animal farming to aquatic farming (Akinbowale et al., 2007; Matyar et al., 2010). However, those studies found very low prevalences of antibiotics when compared with our data. The differences among fish groups might be due to variousnon-antibiotic factors (e.g. cadmium) driving the co-selection of antibiotic resistance according to different methods of fish management.

A. aquariorum was the predominant species among Imi-resistant strains, followed by *A. hydrophila* and *A. veronii*. However, all *A. caviae* strains were susceptible to Imi. These observations are supported by previous studies (Rossolini et al., 1995; Wu et al., 2012) showing species-related distributions of *cphA* and related carbapenemase-encoding genes; for example, the genes were found in *A. aquariorum*, *A. hydrophila* and *A. veronii* but not in most *A. caviae* strains. In addition, the simultaneous resistance to all β-lactams was observed in 18 and 3 strains of *A. aquariorum* and *A. hydrophila*, respectively, which might be due to three chromosomally coordinately controlled β-lactamase genes for penicillinnase, cephalosporinase and carbapenemase, as previously reported in *A. sobria* (Walsh et al., 1995). On the other hand, the β-lactams resistance of *A. caviae* might be mediated by Class-C AmpC-type cephalosporinases, such as CAV-1 and MOX-4, that were previously identified in *A. caviae* strains (Fosse et al., 2003; Ye et al., 2010). The cephalosporinase could hydrolyze broad and extended-spectrum cephalosporin but not carbapenem; in addition, it is not inhibited by clavulanic acid. Therefore, our data might reflect that the different antibiotic resistances among *Aeromonas* spp. are due to the differences in their genetic backgrounds.

The method of antibiotic susceptibility testing applied in this study resulted in the frequent detection of MAR strains in *A. caviae* and *A. aquariorum*. This contrasts with previous studies showing frequent occurrences in *A. hydrophila* strains isolated from aquatic farming (Saavedra, 2004; Akinbowale et al., 2007). This discrepancy might be due to inaccuracies in the species-level identification, and differences in etiological species for aeromoniasis in the aquatic animals and antibiotics investigated. MAR *A. caviae* and *A. aquariorum* strains could be major pathogens for opportunistic infections in antibiotic treatments for controlling primary bacterial diseases in Korea aquaculture.

Heavy metals have been known to be selective agents for antibiotic resistance due to them sharing common structural and functional characteristics of efflux pumps associated with antibiotic and heavy-metal resistance (Nies, 2003; Baker-Austin et al., 2006; Hacioglu et al., 2013). To our knowledge, the present study is the first to show co-selection of Cd and AmC resistance among a collection of *Aeromonas* strains according to fish groups. In addition, the prevalence of Tio and Pod resistances tended to be higher among Cd-resistant strains than among Cd-susceptible strains ($p>0.05$). These results suggest that cadmium could be a suitable inducer for the production of inducible β-lactamases and/or co-selective efflux pumps related to resistance to β-lactams and β-lactamase inhibitors, at least in *Aeromonas* spp. In disagreement with previous studies showing a relationship between MAR and heavy-metal resistance (Akinbowale et al., 2007; Matyar et al., 2010), we found that Cd had no effect on the occurrence of MAR strains in *Aeromonas* strains.

Put together, these observations indicate that veterinarians need to carefully consider the possibility of secondary infection of these MAR strains during antibiotic treatments. In addition, the various antibiotic resistance patterns in the present study indicate the need for antibiotic susceptibility testing before applying antibiotics during an *Aeromonas* outbreak. In addition, Cd could be a global factor for co-selection for resistance to β-lactams, especially AmC.

Conflict of interest

There are no potential conflicts of interest.

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REFERENCES

Akinbowale OL, Peng H, Grant P, Barton MD (2007). Antibiotic and heavy metal resistance in motile aeromonads and pseudomonads from rainbow trout (*Oncorhynchus mykiss*) farms in Australia. Int. J. Antimicrob. Agents. 30:177-182.

Aravena-Roman M, Harnett GB, Riley TV, Inglis TJ, Chang BJ (2011). *Aeromonas aquariorum* is widely distributed in clinical and environmental specimens and can be misidentified as *Aeromonas hydrophila*. J. Clin. Microbiol. 49:3006-3008.

Aravena-Roman M, Inglis TJ, Henderson B, Riley TV, Chang BJ (2012). Antimicrobial susceptibilities of *Aeromonas* strains isolated from clinical and environmental sources to 26 antimicrobial agents. Antimicrob. Agents.Chemother. 56:1110-1112.

Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV (2006). Co-selection of antibiotic and metal resistance. Trends. Microbiol. 14:176-182.

Figueras MJ, Alperi A, Saavedra MJ, Ko WC, Gonzalo N, Navarro M, Martinez-Murcia AJ (2009). Clinical relevance of the recently described species *Aeromonas aquariorum*. J. Clin. Microbiol. 47:3742-3746.

Fosse T, Giraud-Morin C, Madinier I, Labia R (2003). Sequence analysis and biochemical characterisation of chromosomal CAV-1 (Aeromonas caviae), the parental cephalosporinase of plasmid-mediated AmpC 'FOX' cluster. FEMS. Microbiol. Lett. 222:93-98.

Hacioglu N, Tosunoglu M (2014). Determination of antimicrobial and heavy metal resistance profiles of some bacteria isolated from aquatic amphibian and reptile species. Environ. Monit. Assess. 186:407-413.

Janda JM, Abbott SL (2010). The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clin. Microbiol. Rev. 23:35-73.

Kim DC, Yi SW, Cho HS, You MJ, Shin GW (2013). Phylogenetic Identification and Distribution of Enterotoxin Genes in Aeromonas Strains Isolated from Pet Fish. J. Anim. Vet. Adv. 12: 557-564.

Krumperman PH (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Appl. Environ. Microbiol. 46:165-170.

Martinez-Murcia AJ, Monera A, Saavedra MJ, Oncina R, Lopez-Alvarez M, Lara E, Figueras MJ (2011). Multilocus phylogenetic analysis of the genus *Aeromonas*. Sys. Appl. Microbiol. 34:189-199.

Matyar F, Kaya A, Dincer S (2008). Antibacterial agents and heavy metal resistance in Gram-negative bacteria isolated from seawater, shrimp and sediment in Iskenderun Bay, Turkey. Sci. Total. Environ. 407:279-285.

Nies DH (2003). Efflux-mediated heavy metal resistance in prokaryotes. FEMS. Microbiol. Rev. 27:313-339.

Puthucheary SD, Puah SM, Chua KH (2012). Molecular characterization of clinical isolates of Aeromonas species from Malaysia. PloS one 7:e30205.

Roberts RJ (2001). The bacteriology of teleosts. In: Roberts RJ (eds) Fish pathology, W.B. Saunders, New York, NY. pp. 297-331.

Rossolini GM, Zanchi A, Chiesurin A, Amicosante G, Satta G, Guglielmetti P (1995). Distribution of cphA or related carbapenemase-encoding genes and production of carbapenemase activity in members of the genus *Aeromonas*. Antimicrob. Agents. Chemother. 39:346-349.

Saavedra MJ (2004). Resistance to β-lactam antibiotics in *Aeromonas hydrophila* isolated from rainbow trout (*Onchorhynchus mykiss*). Int. Microbiol. 7:207-211.

Seiler C, Berendonk TU (2012). Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front. Microbiol. 3:399.

Schmidt AS, Bruun MS, Dalsgaard I, Pedersen K, Larsen JL (2000). Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Appl. Environ. Microbiol. 66:4908-4915.

Sommerset I, Krossoy B, Biering E, Frost P (2005). Vaccines for fish in aquaculture. Expert. Rev. Vaccines. 4:89-101.

Tacão M, Moura A, Correia A, Henriques I (2014). Co-resistance to different classes of antibiotics among ESBL-producers from aquatic systems. Water. Res. 48:100-107.

Verner-Jeffreys DW, Welch TJ, Schwarz T, Pond MJ, Woodward MJ, Haig SJ, Rimmer GS, Roberts E, Morrison V, Baker-Austin C (2009). High prevalence of multidrug-tolerant bacteria and associated antimicrobial resistance genes isolated from ornamental fish and their carriage water. PloS one 4:e8388.

Vila J, Marco F, Soler L, Chacon M, Figueras MJ (2002). *In vitro* antimicrobial susceptibility of clinical isolates of *Aeromonas caviae*, *Aeromonas hydrophila* and *Aeromonas veronii* biotype *sobria*. J. Antimicrob. Chemother. 49:701-702.

Walsh TR, Payne DJ, MacGowan AP, Bennett PM (1995). A clinical isolate of *Aeromonas sobria* with three chromosomally mediated inducible beta-lactamases: a cephalosporinase, a penicillinase and a third enzyme, displaying carbapenemase activity. J. Antimicrob. Chemother. 35:271-279.

Wu CJ, Chen PL, Wu JJ, Yan JJ, Lee CC, Lee HC, Lee NY, Chang CM, Lin YT, Chiu YC, Ko WC (2012). Distribution and phenotypic and genotypic detection of a metallo-beta-lactamase, *CphA*, among bacteraemic *Aeromonas* isolates. J. Med. Microbiol. 61:712-719.

Yi SW, You MJ, Cho HS, Lee CS, Kwon JK, Shin GW (2013). Molecular characterization of *Aeromonas* species isolated from farmed eels (Anguilla japonica). Vet. Microbiol. 164:195-200.

Ye Y, Xu XH, Li JB (2010). Emergence of CTX-M-3, TEM-1 and a new plasmid-mediated MOX-4 AmpC in a multiresistant *Aeromonas caviae* isolate from a patient with pneumonia. J. Med. Microbiol. 59:843-847.

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Full Length Research Paper

Role of lignocellulolytic thermophilic fungus *Thermoascus aurantiacus* MTCC 375 in paddy straw digestibility and its implication in biogas production

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Paddy straw was pretreated with *Thermoascus aurantiacus* MTCC 375, a ligno-cellulolytic thermophilic fungus, to enhance its biodegradability. The potential of microbial pretreatment under aerobic condition on paddy straw digestibility was investigated at regular intervals of 1, 2, 3, 4 and 5 days by determining the change in proximate (total solids (%) and volatile solids (%)) and chemical composition (cellulose, hemicellulose, lignin and silica content). The pretreatment of 5 days significantly (P ≤ 0.05) reduced the concentrations of cellulose, hemicelluloses, lignin and silica content in the paddy straw by 34.25, 39.19, 34.12 and 10.59%, respectively. A maximum of 30% increase in biogas production was observed from one day pretreated paddy straw as compared to untreated paddy straw. However, biogas production from paddy straw supplemented with enzyme containing digested biogas slurry without giving aerobic treatment was found to be more than the samples given aerobic treatment. In both cases, biogas production was greater than the control by 63.2 and 30.7%, respectively.

Key words: Paddy straw, pretreatment, lignin, *Thermoascus aurantiacus*, biodegradability, enzyme containing digested biogas slurry, biogas production.

INTRODUCTION

Rising energy consumption, depletion of fossil fuels and increased environmental concerns has shifted the focus of energy generation towards biofuel use. Due to increasing fuel prices and environmental concerns, it has become necessary to develop alternative energy sources like biogas. Among potential alternative bioenergy resources, agricultural wastes such as paddy straw, sugarcane bagasse, blends of cassava, potato peels, etc. have been identified as the prime source of biofuels and other value added products.

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Rice being a major cereal crop in India, leads to the production of much larger quantity of paddy straw. About 150 million tonnes of paddy straw were produced in India during 2011-12 (Anonymous, 2012). From such a large quantity of paddy straw, only a minor portion is used as animal feed and household fuel while the remaining paddy straw is disposed off by burning. One tonne paddy straw burning releases 3 kg particulate matter, 60 kg CO, 1460 kg CO_2, 199 kg ash and 2 kg SO_2 (Jenkins and Bhatnagar, 2003), which causes many respiratory diseases.

For effective utilization of paddy straw, intensive research and development studies are being carried out throughout the world. High concentration of organic matter in paddy straw makes it a suitable substrate for biogas production. However, high lignin (6-14%) content in the cell wall of paddy straw hinders the accessibility of cellulase to cellulose and hemicellulases to pentoses, thereby reducing the hydrolysis efficiency significantly. In addition to lignin, high concentration of silica (8-12%) in its epidermal surface acts as a physical barrier preventing microbial attachment (Wiidyastuti et al., 1987) for hydrolytic process.

Therefore, the paddy straw needs to be pretreated, to enable cellulose to be more accessible to the microbial / enzymatic attack. Many physical (mechanical and non mechanical), chemical (alkaline hydrolysis, acid hydrolysis, oxidative delignification and solvent extraction), physicochemical (ammonia fibre explosion, CO_2 and steam explosion) and biological pretreatments (lignocellulolytic microorganisms and the enzymes) have been proposed (Saratale et al., 2008; Hendricks and Zeeman, 2009). However, the physical and chemical pretreatments require high energy and corrosion resistant, high pressure reactors, which increase the case of pretreatment. Furthermore, the chemical pretreatments can be detrimental to the methanogens apart from generating acidic or alkaline water, which needs pre-disposal treatment to ensure environment safety (Keller et al., 2003).

Some of these problems can be solved by biological pretreatment of paddy straw which involves the use of either whole micro-organisms or enzymes produced by microbes to enhance its digestibility. Both fungi and bacteria are being used for biotreatment of paddy straw. Fungal pretreatment of paddy straw has been well employed for improving its digestibility (Sahni, 2010; Sinegani et al., 2005). Advantages of biological pretreatment include inexpensive, low energy requirement and mild environmental conditions (Saratale et al., 2008). Fungi degrade lignin by secreting enzymes collectively termed as lignolytic enzymes (laccase, lignin peroxidase and manganese peroxidase). Most of the research concerning biodegradation of lignin has been focused on *Phanerochaete chrysosporium*, *Streptomyces viridosporus, Pleurotous eryngii, Trametes trogii*, *Fusarium proliferatum*, etc. (Regalado et al., 1997).

No doubt, reports are available on biological pretreatment of paddy straw; however, the effect of pretreatment on biogas production is less explored. Therefore, the present study was undertaken to optimize the conditions for biological pretreatment of paddy straw by thermophilic *Thermoascus aurantiacus* MTCC 375, a lignocellulolytic fungus and to study the implications of enhanced paddy straw digestibility on biogas production.

MATERIALS AND METHODS

Procurement of the materials

Paddy straw was procured from the research field of Punjab Agricultural University, Ludhiana after harvesting the crop. The paddy straw was chopped to 3-4 cm with a chopping machine and was stored in polythene bags at room temperature. Microbial culture of *T. aurantiacus* MTCC 375 was procured from Institute of Microbial Technology, Chandigarh India and was maintained on potato dextrose agar slants at 45±2°C by monthly transfers. Digested biogas slurry was procured from a working biogas plant of School of Energy Studies for Agriculture, PAU, Ludhiana, India.

Pretreatment of paddy straw

For pretreatment of paddy straw, lignolytic enzymes were produced from *T. aurantiacus* MTCC 375 using digested biogas slurry as medium and paddy straw as a substrate. Sterilized digested biogas slurry medium (250 g) was inoculated with 10^7 spores/ml and incubated at 45±2°C for 4 days as per the method described by Dar and Phutela (2013). The lignin degrading enzymes like laccase, manganese peroxidase and lignin peroxidase were measured by the method of Shandilya and Munjal (1983), Paszczynski et al. (1988) and Tien and Kirk (1988) respectively. Two hundred and fifty grams of paddy straw was soaked and mixed in polybags with enzyme containing digested biogas slurry (250 g). The mixture was dispensed in polybags and incubated at 45°C for aerobic pretreatment studies. Five sets of such bags in triplicate were prepared. A control was also used where uninoculated digested biogas slurry was mixed with paddy straw. Samples from one set of bag was taken from incubator at an interval of 24 h, dried at 100°C overnight and was used for proximate and chemical analysis of pretreated straw.

Biogas production from pretreated paddy straw

Biogas production experiments were carried out in two litre capacity digesters following monophasic method and biogas produced was measured by water displacement method. Pretreated paddy straw (250 g) containing digested biogas slurry was mixed with 100 ml cattle dung and was fed to biogas digester. The digested biogas slurry acts as inoculum for biogas production whereas cattle dung acts as inducer for enhancing biogas production from paddy straw. The digester was properly sealed with rubber cork and araldite. The biogas production data was taken for a period of 35 days.

Biogas production from paddy straw supplemented with enzyme containing digested biogas slurry

Lignolytic enzyme containing digested biogas slurry (250 ml) was mixed with soaked paddy straw (250 g) and 100 g of cattle dung. The mixture was put in the digester without any pretreatment and

Table 1. Change in chemical and proximate composition of paddy straw by enzyme containing media.

Treatment (Day)	Composition of paddy straw (%)							
	T S	V S	TOC	Ash	Cellulose	Hemicellulose	Lignin	Silica
Control#	98.5±0.38	86.39±0.35	47.98±0.43	16.15±0.58	36.2±0.41	27.3±0.69	8.5±0.29	11.8±0.32
1	95.55±0.57	82.82±0.55	46.01±0.56	17.38±0.49	34.6±0.78	24.2±0.51	7.4±0.38	11.35±0.43
2	93.56±0.96	81.07±0.90	45.04±0.37	17.85±0.37	34±0.35	23.8±0.51	7.1±0.27	11.2±0.33
3	92.13±0.88	80.05±0.63	44.47±0.61	18.05±0.50	31.8±0.61	23.2±0.41	6.8±0.32	10.8±0.55
4	91.04±0.89	78.15±0.36	43.42±0.42	18.93±0.64	28.6±0.74	20.6±0.43	6.2±0.54	10.65±0.43
5	90.12±0.69	77.5±0.28	43.06±0.33	19.95±0.39	23.8±0.43	16.6±0.68	5.6±0.32	10.55±0.39
Percentage change from control	8.5 (↓)	10.30 (↓)	10.25 (↓)	23.53 (↑)	34.25(↓)	39.19 (↓)	34.12 (↓)	10.59 (↓)
CD (5%)	2.33	1.72	1.61	1.55	1.74	1.69	1.13	NS

Control: control contains soaked paddy staw, uninoculated digested biogas slurry and cow dung; TS: total solids; VS: volatile solids; TOC: total organic carbon; CD: critical difference at 5% level; ± values indicate % standard error for triplicate data; (↓): decrease ; (↑): increase.

biogas produced was measured for a period of 35 days.

Chemical analysis

Standard methods of AOAC (2000) were followed for the determination of proximate and chemical composition of paddy straw, that is, total solids, volatile solids, cellulose, hemicellulose, lignin and silica.

Statistical analysis

The standard error (SE at 5% level) and critical difference (5% level) were calculated for triplicate data.

RESULTS AND DISCUSSION

Effect of enzymetic pretreatment conditions on paddy straw digestibility

Chopped and soaked paddy straw was pretreated with enzyme containing digested biogas slurry (DBS) and its effect on paddy straw digestibility was determined. The change in chemical (TS, VS, Ash and TOC contents) and proximate (cellulose, hemicellulose, lignin and silica contents) composition of paddy straw with enhanced biogas production was taken as criteria for paddy straw digestibility. The results are presented in Table 1.

Results from Table 1 indicate that there was a smooth decrease in total solids and volatile solids with increase in the incubation period. The total solids decreased from 95.25 (in control) to 90.12% in 5 days treatment. Volatile solids also decreased from 82.62 (in control) to 77.5%. However, ash content was found to be increasing with a maximum increase of 10.30% in 5 days treated sample. The cellulose content decreased from 36.2 (control) to 23.8% and hemicellulose decreased from 27.3 (control) to 16.6% in 5 days. The decrease in cellulose and hemicellulose content might be the result of breakdown or hydrolysis of cellulose and hemicellulose into fermentable sugars (Jalc et al., 1998). This observation clearly indicates that the fungus has active cellulases and hemicellulases. Lignin content also decreased from control (8.5%), showing maximum reduction of 34.12% in 5 days treated sample (5.6%). There was decrease in silica content but the decrease is not significant as critical difference came out to be non significant. This demonstrates that the *T. aurantiacus* MTCC 375 is lignocellulolytic in nature.

Similar results were found by Huang et al. (2007) who used two lignolytic micro-organisms *viz. P. chrysosporium* (white-rot fungi) and *Streptomyces badius* (actinomycetes) for bio-delignification of rice straw and found that lignin was degraded by 41 and 31% by *P. chrysosporium* and *S. badius*, respectively. Zafar et al. (1980) also observed that cellulose content of rice straw treated with *Pleurotus sajor caju* decreased from 45.0 to 17.8%. Shi et al. (2009) pretreated cotton stalks with *P. chrysosporium* and found a significant decrease in lignin, that is, 19.38 and 35.33% for submerged and solid state cultivation, respectively.

Biogas production from enzymatically pretreated paddy straw under aerobic condition

Results from Table 2 show that the biogas production was enhanced in pretreated straw as compared to untreated straw. However, with the increase of pretreatment period, there was decrease in biogas production. Highest biogas production (168.6 l/Kg PS) was found in 1 day pretreated paddy straw which showed an increase of 30.7% over control. This increase in biogas production might be due to the increase in paddy straw digestibility by enzyme pretreatment. The presence of anaerobic fungi and methanogens in cow dung and digested biogas slurry (Davies et al., 1993) might be other contributing

Table 2. Biogas production from enzymatically pretreated paddy straw.

Parameter	Control#	Pretreatment period (Day)					CD (5%)
		1	2	3	4	5	
Biogas (l/250 g PS)	32.3±3.85	42.2±2.40	40.0±1.10	39.3±2.35	35.2±2.65	34.4±2.60	2.13
Biogas (l/kg PS)	129.3±2.35	168.6±2.10	160.4±2.30	157.0±2.00	140.8±2.40	137.7±2.65	7.99
Biogas (l/Kg TS)	339.1±3.95	451.1±2.45	427.5±1.90	407.0±2.50	365.3±2.60	349.2±2.20	9.28
Biogas (l/Kg VS)	390.9±3.55	514.9±2.55	499.7±1.96	478.1±2.02	428.5±3.25	424.3±3.60	10.05
%age change from control	0.0	30.7(↑)	23.8(↑)	21.7(↑)	9.0(↑)	6.5(↑)	2.63

Control: soaked paddy staw, uninoculated digested biogas slurry and cow dung; T S: total solids; VS: volatile solids; PS: paddy straw; CD: critical difference at 5% level; ± values indicate % standard error for triplicate data; (↓): decrease ; (↑): increase.

Table 3. Biogas production and chemical composition of paddy straw supplemented with enzyme containing digested biogas slurry.

Parameter	Control	Paddy straw + enzyme containing digested biogas slurry
TS (%)	98.5±0.38	96.35±0.55
VS (%)	86.39±0.35	83.44±0.61
Ash (%)	16.15±0.58	17.2±0.40
Cellulose (%)	36.2±0.41	35±0.44
Hemicellulose (%)	27.3±0.69	26.6±0.61
Lignin (%)	8.5±0.29	8±0.35
Silica (%)	11.8±0.32	11.6±0.46
Biogas (l/250g PS)	32.3±3.85	52.7±2.54
Biogas (l/kg PS)	129.3±2.35	210.8±2.72
Biogas (l/kg TS)	339.1±3.95	563.1±3.31
Biogas (l/kg VS)	390.9±3.55	643.1±2.44
%age change from control	0.0	63.2(↑)

TS: Total solids; VS: volatile solids; PS: paddy straw; ± values indicate % standard error for triplicate data; (↓): decrease ; (↑): increase.

factors for increase in biogas production.

In the present study, there may have been more transfer of H_2 between methanogenic bacteria and anaerobic fungi in one day pretreated paddy straw, thus resulting in more carbon flow than others through hydrogenosomes which ultimately shift the end product formation towards acetate, H_2 and formate (though the latter two are utilized by the methanogens as precursors of methane production). While in the case of the samples given the 2, 3, 4 and 5 days aerobic pretreatment, with the increase in pretreatment period, more death of methanogens and anaerobic fungi may have occurred, thus resulting in less flow of carbon through the hydrogenosomes and ultimately less production of acetate, H_2 and formate and ultimately less biogas production.

Biogas production from paddy straw supplemented with enzyme containing digested biogas slurry

Results from Table 3 showed that there was 63.2% enhancement in biogas production from the paddy straw which was directly put in the digester after mixing with enzyme containing digested biogas slurry (DBS) than that of the control. It produced 210.8 l biogas/kg of paddy

straw which was quite higher than the control (129.3 l biogas/kg of paddy straw) and other samples pretreated aerobically by enzyme activated digested biogas slurry (Table 2). High biogas production might be due to utilization of volatile fatty acids by the microorganisms (anaerobic fungi and methanogens), thus not inhibiting the methanogenesis as was found in the case of aerobically treated samples (Davies et al., 1993).

Conclusion

The thermophilic fungus *T. aurantiacus* MTCC 375 is both cellulolytic as well as lignolytic in nature as it degrades both cellulose and lignin. There was decrease in silica content but the decrease was not significant as critical difference was not significant. Biogas production from paddy straw supplemented with enzyme containing digested biogas slurry without giving aerobic treatment was found to be more than the samples given aerobic treatment, although biogas production was enhanced as compared to the control in both cases.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Anonymous (2012). Ministry of Agriculture, Government of India. (http://www.indiastat.com/agriculture/2/foodgrains/17180/rice/17194/stats.aspx).

AOAC (2000). Association of Official Analytical Chemists, Official Methods of Analysis, 17th Edition, Maryland, USA.

Dar RA, Phutela UG (2013). Production of lignolytic enzymes from *Thermoascus aurantiacus* MTCC 375 for enhancing biogas production from paddy straw. M.Sc. Thesis. Punjab Agricultural University, Ludhiana.

Davies DR, Theodorou MK, Lawrence MIG, Trinci APJ (1993). Distribution of anaerobic fungi in the digestive tract of cattle and their survival in faeces. J. Gen. Microbiol. 139:1395-1400.

Hendricks ATWM, Zeeman G (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. 100:10-18.

Huang HL, Zeng GM, Tang L, Yu HY, Xi XM, Chen ZM, Huang GH (2007). Effect of biodelignification of rice straw on humification and humus quality by *Phanerochaete chrysosporium* and *Streptomyces badius*. Int. Biodeterior. Biodegrad. 61:331-36.

Jalc D, Nerud F, Siroka P (1998). The effectiveness of biological treatment of wheat straw by white rot fungus. Folia Microbiol. 43:687-89.

Jenkins BM, Bhatnagar AP (2003). Electric potential from paddy straw in Punjab and optimal size of power generation station. Bioresour. Technol. 37:35-41.

Keller FA, Hamilton JE, Nguyen QA (2003). Microbial pretreatment of biomass potential for reducing severity of thermo-chemical biomass pretreatment. Appl. Biochem. Biotechnol. 105:27-41.

Paszczynski A, Huynhv B, Crawford R (1988). Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. 29:37-41

Regalado V, Rodriguez F, Carnicero A, Fuente G, Falcon MA (1997). Lignin degradation and modification by soil inhabiting fungus *Fusarium proliferatum.* Appl. Environ. Microbiol. 63:3716-3718.

Sahni N (2010). Pretreatment of paddy straw by lignocelluloytic fungi for enhancing biogas production. M.Sc. Thesis. Punjab Agricultural University, Ludhiana.

Saratale GD, Chen SD, Lo YC, Saratale RG, Chang JS (2008). Outlook of biohydrogen production from lignocellulosic feedstock using dark fermentation-a review. J. Sci. Ind. Res. 67:962-79.

Shi J, Shivappa RRS, Chinn M, Howell N (2009). Effect of microbial pretreatment on enzymatic hydrolysis and fermentation of cotton stalks for ethanol production. Biomass Bioenergy 33:88-96.

Shandilya JR, Munjal RL (1983). Some experiments with casing layer. Tai-wan-Yang-ku: Taiw Mush 7(1):32-38.

Sinegani AAS, Emtiazi G, Hajrasuliha S, Shariatmadari H (2005). Biodegradation of some agricultural residues by fungi in agitated submerged cultures. Afr. J. Biotechnol. 10:1058-1061.

Tien M, Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium.* Methods Enzymol. 161:238-49.

Wiidyastuti Y, Terada F, Kajikawa H, Abe A (1987). Digestion of rice straw cell wall constituents in various rumen conditions. Jpn. Agric. Res. Quart. 21:59-64.

Zafar SI, Kausar TK, Shah FH (1980). Biodegradation of cellulose component of rice straw by *Pleurotus sajor caju*. Folia Microbiol. 26:394-97.

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Antifungal activity of chitosan-silver nanoparticle composite against *Colletotrichum gloeosporioides* associated with mango anthracnose

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Chitosan loaded with various metal ions such as Ag^{+}, Cu^{2+}, Zn^{2+}, Mn^{2+} and Fe^{2+} has been reported to exert strong antimicrobial activity. In this study, the silver-nanoparticles (AgNPs) were synthesized at 95°C using chitosan as the reducing agent and stabilizer. The UV-Vis spectrum displayed peak in a range between 415-420 nm, the characteristic surface plasmon resonance band of silver nanoparticles. The size, shape and aggregation properties of the resultant nanoparticles were examined using field emission scanning electron microscopy coupled with energy dispersive X-ray spectroscopy. The measurement results indicated that the chitosan-silver nanoparticle (chitosan-AgNP) composite having the mean hydrodynamic diameter range of 495-616 nm were apparently smooth and the silver nanoparticles with the size distribution was from 10 to 15 nm. Chitosan-AgNP composites had a zeta potential of +50.08 mV to +87.75 mV. *In vitro* conidial germination assay indicated that chitosan-AgNP composite exhibited significantly higher antifungal activity against *Colletotrichum gloeosporioides* than its components at their respective concentrations. *In vivo* assay using detached mango fruit cv. Alphonso showed that anthracnose was significantly inhibited by chitosan-AgNP composite. Therefore, this study suggests that postharvest decay in mango can be minimized by chitosan-AgNP composites and its application on a commercial scale needs to be exploited.

Key words: Chitosan, silver nanoparticle, antifungal activity, mango fruits, *Colletotrichum gloeosporioides*.

INTRODUCTION

Chitosan, a deacetylated derivative of chitin, is the second most abundant natural hydrophilic linear polysaccharide found in the nature after cellulose. It is made up of N-acetyl-2 amino-2-deoxy-D-glucose (glucosamine) and 2-amino-2-deoxy-D-glucose (N-acetyl-glucosamine) residues (Aranaz et al., 2010). Due to its unique biological properties, such as non-toxicity, biodegradability, biofunctionality and biocompatibility, many applications have been reported either alone or blended with other natural polymers (starch, gelatine and

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alginates) in the food, pharmaceutical, textile, agriculture, water treatment and cosmetics industries (Harish Prashanth and Tharanathan, 2007). This biodegradable material can also improve the quality of agricultural products and extend shelf life by minimizing microbial growth in the product because of its positively charged (poly cationic) nature (Zhong et al., 2009; El Hadrami et al., 2010). Antimicrobial properties of chitosan have been shown against many bacteria, filamentous fungi and yeasts (Kong et al., 2010). It has got significant importance in plant protection because of its dual function; fungistatic or fungicidal effects and induction of defence responses (Kendra and Hadwiger, 1984; Sudarshan et al., 1992; Tsai and Su, 1999; Bautista-Banos et al., 2006). The antimicrobial activity of chitosan depends on its molecular weight, deacetylation degree, pH of chitosan solution and the target organism (Helander et al., 2001; Jeon et al., 2001; Zhong et al., 2009). The antifungal activity of chitosan against *Colletrotichum gloeosporioides* has been reported (Ali et al., 2010). Antifungal activity of chitosan has also been reported on *Alternaria alternata, Botrytis cinerea*, *Rhizopus stolonifer* and *Phytophthora capsici* (El Ghaouth et al., 1992; Xu et al., 2007). Chitosan possesses high chelating capacity with various metal ions such as Ag^{+}, Cu^{2+}, Zn^{2+}, Mn^{2+} and Fe^{2+} in acidic conditions and these chitosan metal complexes exert strong antimicrobial activity (Kong et al., 2010).

The antimicrobial property of elemental silver (Ag^{+}) has been studied extensively and has found many applications in medicine than any other inorganic metal ion, while at the same time having shown no harm to human cells (Russell et al., 1994). Silver can be used for management of plant pathogens in a relatively safer way as compared to synthetic fungicides (Park et al., 2006) as it displays multiple modes of inhibitory action (Clement and Jarrett, 1994). Previous studies provided evidence of the applicability of silver for controlling plant pathogenic fungi such as *Bipolaris sorokiniana*, *Magnaporthe grisea* (Jo et al., 2009), *Golovinomyces cichoracearum* or *Sphaerotheca fusca* (Lamsal et al., 2011) and *Raffaelea* sp. (Kim et al., 2009). In recent years, considerable research has been done for incorporation of silver-nanoparticles into ultrathin fibers for many important applications, such as using wound dressing materials in the medical field (Zhuang et al., 2010). Chitosan has been used as both a reducing agent and stabilizer to form AgNPs (Murugadoss and Chattopadhyay, 2008). The binding interaction between chitosan and the silver-nanoparticles results in stabilization of the chitosan-AgNP composites and hence, the nanoparticles attached to the polymer chains will disperse in the solution when the composite dissolved.

Mango (*Mangifera indica* L.) is commercially important tropical fruit crop in India, accounting for > 54% of the total mango produced worldwide. India exports fresh mangoes to more than 50 countries (Tharanathan et al., 2006). Anthracnose, caused by the *C. gloeosporioides* is the most important post-harvest disease of mango (Arauz, 2000) and cause decay during storage and transport. Chemical fungicides have been the most effective way to control postharvest decay of mango, but their use has caused the development of fungicide resistance, and increasing public conflict (Lin et al., 2011). Benzimidazole fungicides have been used for management of *Colletotrichum* diseases during the last 38 years and numerous cases of resistance have been reported due to it's very specific mode of action (Hewitt, 1998; Peres et al., 2004). In view of wide-spread benzimidazole fungicide resistance across *Colletotrichum* species, alternative safe chemical control strategies need to be developed to manage pre and postharvest anthracnose diseases. Previous studies showed that chitosan significantly inhibited growth of *Colletotrichum* sp. even at lower concentrations (Bautista-Banos et al., 2003; Munoz et al., 2009). Sanpui et al. (2008) has demonstrated that chitosan-AgNP composites were highly effective in inhibiting certain bacteria than chitosan alone. As no work has been carried out on the antifungal activities of chitosan-AgNP composites, we hypothesized that chitosan can be useful in plant protection due to its dual function of defense response in plants and antifungal effect, and its activity can be enhanced in combination with metal ion in the form of nanoparticles. The objective of this study was to evaluate the chitosan-AgNP composite for its antifungal activity against *C. gloeosporioides in vitro* and its efficacy in reduction of anthracnose disease in mango fruit.

MATERIALS AND METHODS

Chemicals

Chitosan low molecular weight (85% deacetylated), and Whatman No. 1 filter paper were purchased from Sigma-Aldrich, USA. Silver nitrate ($AgNO_3$, 99.9%) was supplied by Merck, India. Acetic acid (glacial, 99–100%) was from SD fine chemicals, India. Tween-80 and sodium hydroxide were procured from HiMedia Laboratories, India. All the chemicals were used as received without further purification. We used double distilled and deionized water throughout the study (MilliQ/Millipore System, Billerica, MA, USA).

Synthesis of the chitosan-AgNP composite

Nanoparticles were prepared by adopting the one-pot synthesis method of Sanpui et al. (2008) with slight modifications. Briefly, a 100 ml aqueous solution containing 0.2 g of chitosan was kept on a magnetic stirrer with hot plate at a temperature of 95±1°C with constant stirring. This was followed by addition of freshly prepared 2.0 ml solution of different concentrations (10, 15, 20, 25 and 30 mM) of silver nitrate and 300 µl of 0.3 M NaOH, respectively. The pH of the solution was measured to be 10.0. Formation of AgNPs occurs spontaneously in about a minute, by turning the resultant solution to yellow indicating the formation of AgNPs in the medium.

The reaction was allowed to continue for additional 30 min and cooled to room temperature. Powdered yellow colored solid was then collected by filtration through Whatman No. 1 filter paper. The powder was washed with deionized water four times, then air dried and used for further studies.

Analytical measurements and characterization

Ultraviolet-visible spectroscopy (UV–vis) spectra of all the synthesized nanoparticle samples were recorded at room temperature using a TECAN infinite 200 (Seestrasse, Mannedorf, Switzerland) in the range 250–550 nm. The dynamic light scattering (DLS) and Zeta potential measurements of the liquid samples were performed by using Zeta pals (Brookhaven Instrument Corporation, Holtsville, NY, USA) at 25°C. Size, shape and aggregation properties of the nanoparticles were examined by field emission scanning electron microscopy (FESEM). For FESEM studies, a drop of the aqueous suspension of nanoparticle was placed on aluminium stub with double sided carbon tape and kept for drying at 70°C for three hours. Then, samples were kept in desiccator containing silica gel for 72 h, and gold particles were sputtered over the coated material to prevent the charging effect and kept under vacuum pressure for 30 min before analysis. The samples were examined under FESEM using a Zeiss-Ultra 55 model microscope (Carl Zeiss Promenade, Jena, Germany) equipped with an energy dispersive X-ray spectroscopy (EDS) capability. The quantity of silver present in the nanocomposite was estimated using atomic absorption spectrophotometer. A stock solution of silver at a concentration of 1000 µg mL^{-1} was prepared by dissolving 1.574 g of $AgNO_3$ (equivalent to 1g of metallic silver) in nitric acid : water (1: 1) and diluted to 1000 ml with Milli Q water and stored in the dark. A Thermo M series atomic absorption spectro-photometer (Thermo Electron Corporation: Chromatography and Mass Spectrometry, River Oaks Parkway San Jose, CA, USA) was used with a silver hollow-cathode-lamp, at an operating current of 2 mA, and a wavelength and spectral band pass of 328.1 and 1 nm, respectively.

Germination of treated-conidia of *C. gloeosporioides* with chitosan-AgNP composite

C. gloeosporioides isolate (Cm 50 NCBI accession number EF025937) recovered from mango was grown on potato dextrose agar (PDA) for 7 days under cool white fluorescent light (67.5 mmol m^{-2} s^{-1}) at 25 ± 1°C to promote sporulation. Conidia were washed from the PDA plate with 5 ml of sterile distilled water and adjusted to 1.5 x 10^6 conidia/ml using a haemocytometer (Than et al., 2008). A solution of 50 µl containing different concentrations of chitosan-AgNPs composite (loaded with 30 mM $AgNO_3$) and chitosan (0.1, 1, 10, 100 and 1000 µg/ml) dissolved in 0.1% (v/v) acetic acid was added to the well of the cavity slide containing 50 µl spore suspension. An equal volume (50 µl) of sterile distilled water with 0.1% (v/v) acetic acid was added to the control well. All cavity slides were placed in a moisture chamber containing ~95% humidity and incubated for 12 h at 25± 1°C, germination of conidia in 20 randomly chosen fields were determined at 400x magnification under Zeiss bright field microscope (Axio Scope.A1, Gottingen, Germany). Conidia were considered germinated when the length of the germ tube equalled or exceeded the length of the conidia. Percent inhibition of spore germination over control was calculated using the following formula. I = (C-T/C) x100, Where, I = percentage inhibition of conidial germination in test pathogen, C = number of germinated conidia in control and T = number of conidia germinated in treatment. Each treatment had three replicates, and each replicate contained nine slides. The experiment was repeated three times.

Coating preparation

The coating solutions were prepared by dissolving chitosan-AgNPs composite loaded with 30 mM $AgNO_3$ and chitosan (0.5 and 1.0% w/v) respectively at 40°C in a 0.5% (v/v) acetic acid solution, since chitosan is only soluble in an acidic medium. Then, Tween 80 at 0.1% (v/v) was added for improving wettability. The resulting mixture was agitated vigorously with heating using a magnetic stirrer during 2 h until chitosan was dissolved (Garcia et al., 2010).

Coating and antifungal activity of chitosan-AgNP composites on anthracnose development on mango fruits

The fruits of mango cv. 'Alphonso' collected from 15 year old tree grown in the experimental farm of Indian Institute of Horticultural Research, Bangalore, India were used in the experiments. The healthy fruits were selected at a semi-ripe stage with similar size and weight (250 g). The fruits were washed in running water and surface sterilized with 0.1% sodium hypochlorite solution for 2 min and then washed with sterile distilled water thrice and air dried. The fruits were dipped for 15 min in sterile water (control), 0.5% acetic acid, chitosan (0.5% and 1%) and chitosan-AgNPs composite loaded with 30 mM $AgNO_3$ (0.5% and 1%) with and without addition of 0.1% Tween-80 (v/v). Chemical check of carbendazim at 0.001, 0.01%, 0.001 and 0.0001% were also done for coating mangoes and then coated fruits were air dried for 30 min at 25°C. Mango fruits were then wounded on the same side (distal and proximal) to a depth of 2 mm by puncturing them with a sterilized pin. Each wound site was then inoculated with 20 µl of spore suspension (1.5 x 10^6 spore/ml) of *C. gloeosporioides*. Treated and control fruits were then placed onto wire mesh in plastic boxes (45 cm height x 40 cm length x 15 cm width) containing water, maintaining ~95% relative humidity and incubated at 24±1°C for 7 days. Lesion area (Cm^2) was scored separately by measuring length and breadth of each infection site. The experiment was carried out with three replicates, each replicate contain 12 fruits, thereby, making a total of 36 fruits per treatment. The experiment was repeated three times.

Statistical analysis

All data were statistically analysed using one-way analysis of variance (ANOVA) to identify the origin of significance and followed by a Fishers test to separate means and treatments using Graph pad Prism V.500 for windows (Graph pad software, San Diego, California, USA). Means were compared between treat-ments by least significant difference (LSD) at the 1% level ($p < 0.01$). Percentage data were arcsin-transformed before analysis according to y = arcsin [sqr (_/100)].

RESULTS

Synthesis and characterization of chitosan-AgNP composites

As substantiated by the formation of yellow colored powder, reduction of Ag+ to Ag NPs was observed in the presence of NaOH and at an elevated temperature. This indicated chitosan produced Ag-NPs under alkaline

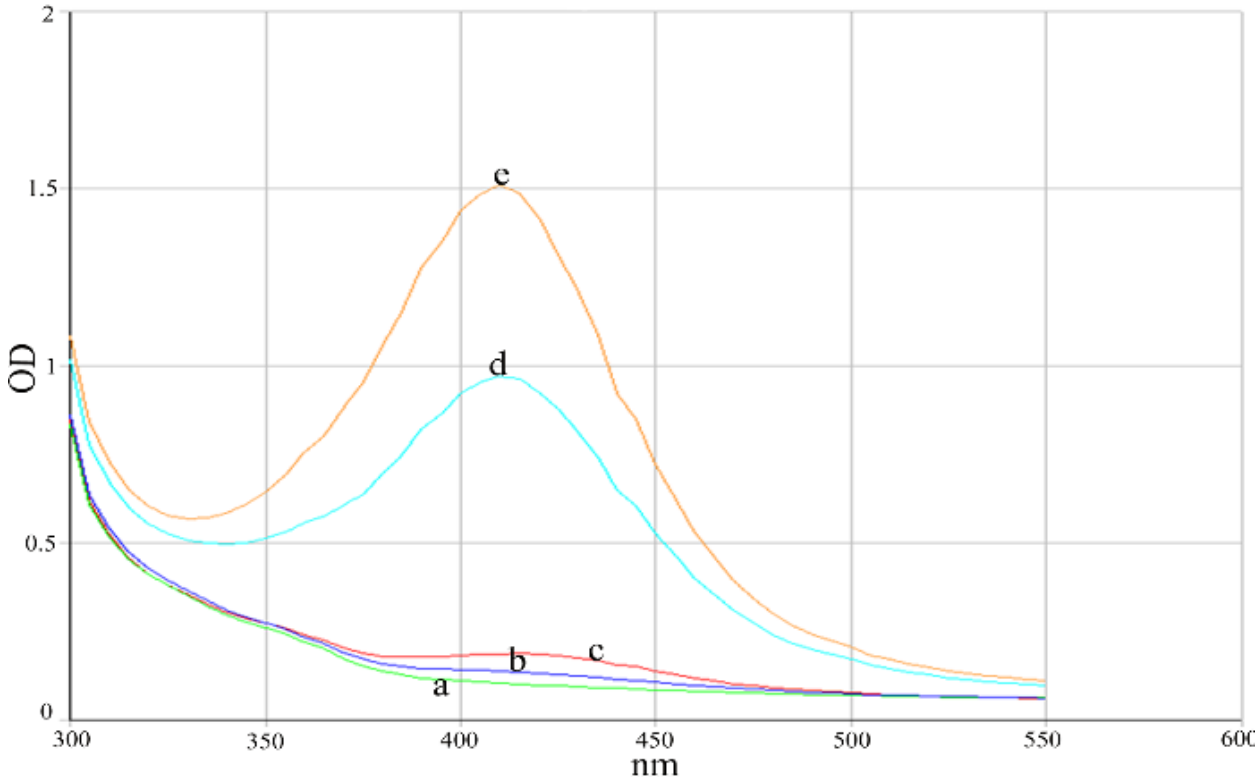

Figure 1. UV-Vis absorbance spectra of chitosan-AgNP composite dissolved in 0.1% (v/v) acetic acid in water. The concentration of silver ions in the original solution used for the preparation of NPs were a) 10 mM, b) 15 mM, c) 20 mM, d) 25 mM, and e) 30 mM $AgNO_3$ respectively. The amount of chitosan used for synthesis was 0.2g in 100 ml.

Table 1. Result of dynamic light scattering and zeta potential.

Concentration of $AgNO_3$	Particle size (nm)	Zeta potential (mV)
10 mM	494.5	50.08
15 mM	589.7	62.11
20 mM	616.1	76.52
25 mM	592.2	85.37
30 mM	594.8	87.75

conditions. The chitosan worked as both reducing agent and also stabilizer for the production of NPs. The yellow powder was found to be insoluble in water. The yellow powder of composite was dissolved in acetic acid and the UV-Vis spectrum was measured. The spectra showed that with the increase in concentration of $AgNO_3$, there was a gradual increase in the intensity of peak in a range between 415-420 nm (Figure 1), the characteristic surface plasmon resonance (SPR) band of silver nanoparticles, indicating the formation of silver nanoparticle. The result showed that with the increase in concentration of silver ion, the concentration of silver nanoparticle formation has increased. Table 1 showed the size distribution profiles of the chitosan-AgNP composites loaded with different concentrations of $AgNO_3$. The mean hydrodynamic diameters of nanoparticles loaded with 10, 15, 20, 25 and 30 mM of $AgNO_3$ was 495, 590, 616, 592 and 595 nm, respectively when analysed with DLS. The zeta potentials were enhanced significantly with the increase in concentration of $AgNO_3$ (Table 1). Chitosan-AgNP composites had a zeta potential varying from +50.08 mV to +87.75 mV. When the same samples used for DLS studies were analysed through SEM, chitosan-AgNP particles showed spherical shape with solid dense structure having particle sizes in the range between 10-15 nm (Figure 2). When the elemental composition of chitosan-AgNP was determined by EDS, silver signal was detected, indicating the presence of significant amounts of silver in nano composite (Figure 3). In addition, carbon signal originated from the carbon tape used for coating the material and gold signal because of the gold sputtering on the surface of the film to prevent charging effect and to improve conductivity (Table 2). The nano-composite prepared from 30 mM $AgNO_3$ had 30.281 mg of silver per gram of chitosan-Ag nanoparticle composite when analysed through atomic absorption spectrophotometer.

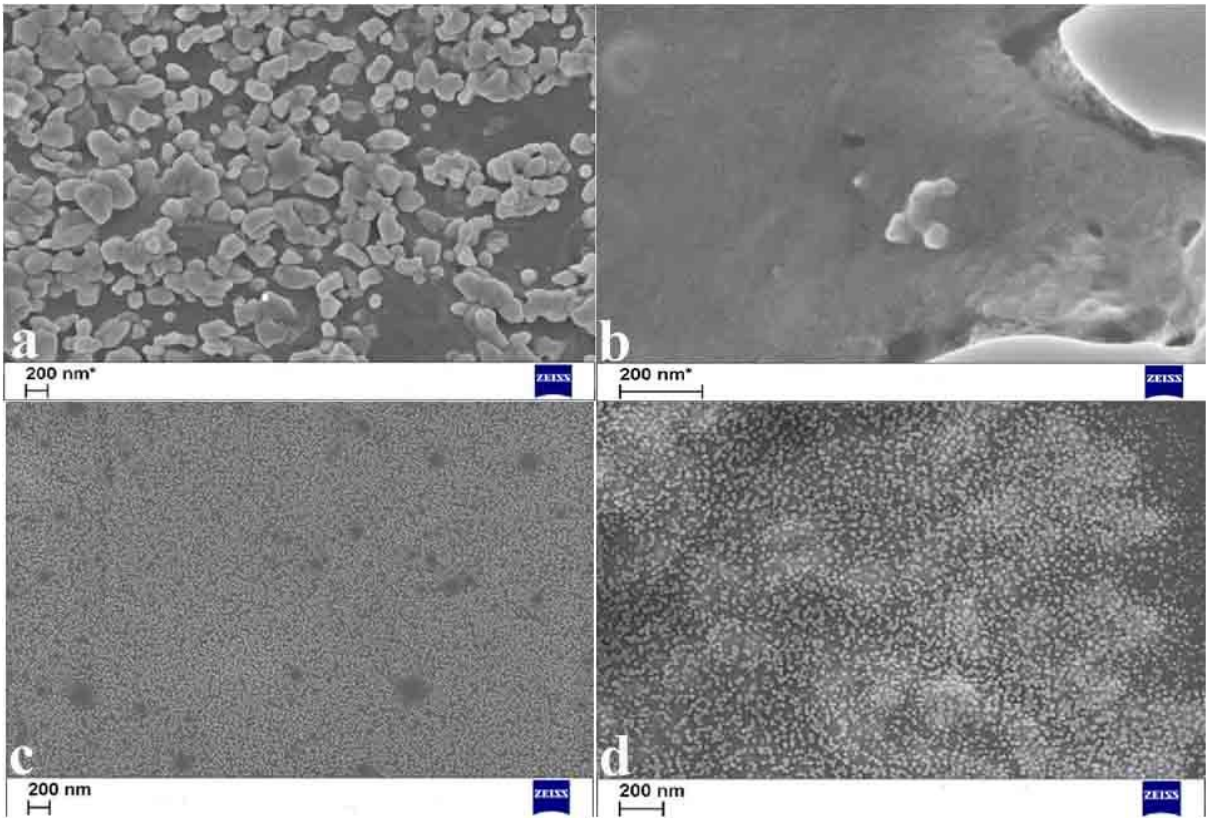

Figure 2. SEM micrograph; a) Chitosan aggregates, b) $AgNO_3$, c) Chitosan-AgNP composite (30 mM $AgNO_3$) at 50 K X, and d) 300 K X magnification.

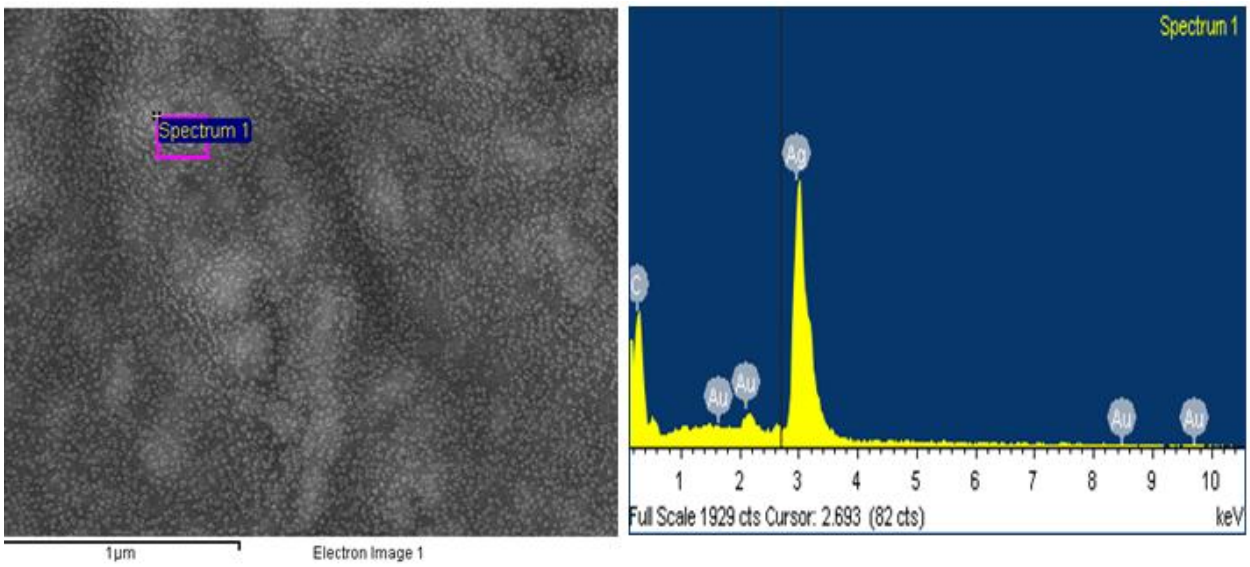

Figure 3. SEM image and corresponding EDS spectra for chitosan-AgNP composite.

Antifungal activity of chitosan-AgNP composite

In this study, the effect of chitosan and chitosan-AgNP composite (Figure 4) loaded with 30 mM $AgNO_3$ (zeta potential of 87. 75 mV) on conidial germination of *C. gloeosporioides* were determined and shown in Figure 6.

Table 2. Chemical composition of elements present in the chitosan-AgNP composite determined by EDS.

Element	Weight (%)
Carbon*	19.64
Silver	75.60
Gold**	4.76

*Carbon signal originated from carbon tape; **Gold signal from gold sputtering on the surface of the film.

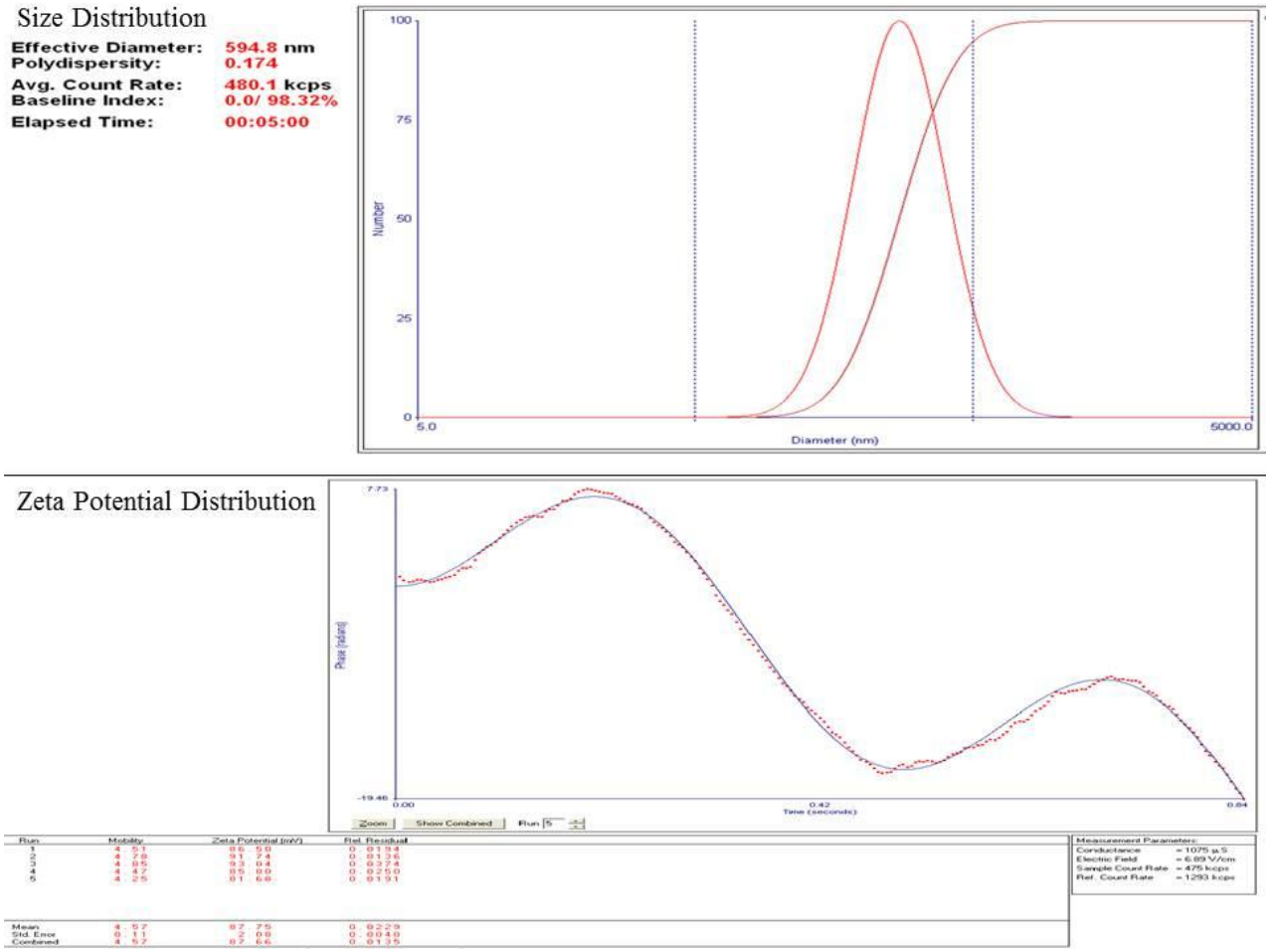

Figure 4. Size distribution and zeta potential distribution of 30 mM $AgNO_3$ treated chitosan-AgNPs composite.

Chitosan-AgNP composite (treated with 30 mM $AgNO_3$) markedly reduced conidial germination of *C. gloeosporioides* than chitosan. Chitosan-AgNP composite with 0.1 (0.00001%), 1.0 (0.0001%) and 10.0 µg/ml (0.001%) concentration inhibited spore germination by 44, 70 and 78%, respectively. Spore germinations were completely inhibited with concentrations of 100.0 µg/ml (0.01%) (Figure 5). Normal conidial germination of *C. gloeosporioides* was found in sterilized distilled water with 0.1% (v/v) acetic acid after 12 h of incubation on the glass slide. Complete spore germination was observed at 0.1% acetic acid, so dispersion of composites with low concentration of acetic acid did not cause any adverse effect. The results showed that chitosan-AgNP composite treatment suppressed germination and was found to be more effective than its counterpart.

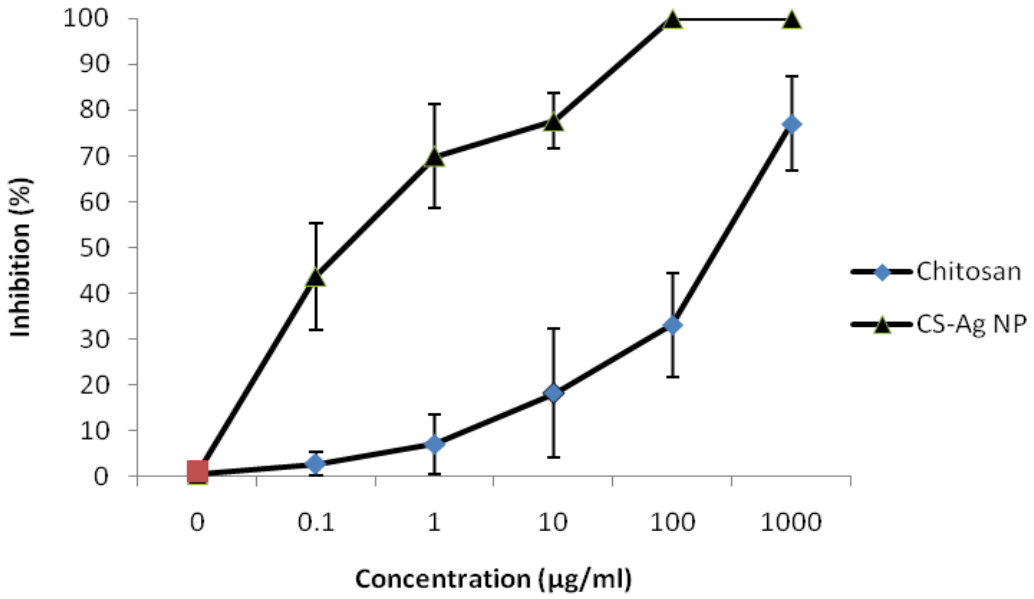

Figure 5. Effect of chitosan, and chitosan-AgNP composite on conidial germination of *C. gloeosporioides* after 12 h of incubation.

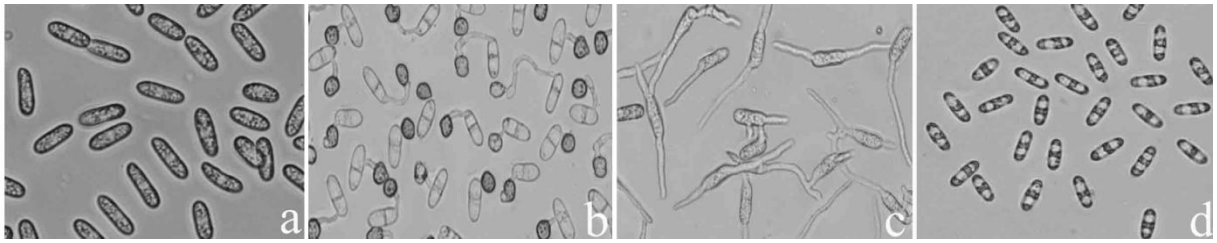

Figure 6. Effect of chitosan-AgNP composite on conidial germination of *C. gloeosporioides* a) Normal conidia, b) Water control with 0.1% (v/v) acetic acid (appressoria formation), c) Conidial germination in chitosan (100 µg/ml) with 0.1% (v/v) acetic acid, d) Complete inhibition of conidial germination by chitosan-AgNP composite.

Effect of chitosan-AgNP composite on anthracnose of mango

The chitosan-AgNP composite, at 0.5 and 1% concentration, showed a reduction of anthracnose by 45.7 and 71.3% respectively. Chitosan at 0.5 and 1% concentration showed 35.5 and 41.8% reduction of disease (Table 3). On the other hand, combination of chitosan-AgNP composite with 0.1% Tween-80 reduced the disease by 75.8% at 0.5% and 84.6% at 1% concentration. Chitosan and Tween-80 combinations at 0.5 and 1% concentrations showed 51.9 and 65.7% reduction of disease incidence, respectively. While carbendazim at 0.0001 and 0.001% showed 49.3 and 63.0% inhibition when compared with untreated fruits (Table 3). The anthracnose incidence was significantly ($P < 0.01$) lower in all chitosan- AgNP particle treatments as compared to other treatments (Table 3 and Figure 7).

DISCUSSION

Chitosan structure differs in molecular weight and degree of deacetylation. The primary amine and hydroxyl group in chitosan shows high affinity towards metal ions by chelation. Chitosan a biodegradable and nontoxic polymer in the presence of NaOH and at an elevated temperature reduces and stabilizes $AgNO_3$ to silver nanoparticles. Different concentrations of silver nitrate were reduced to corresponding silver nanoparticles at particular temperature. The nanoparticle formation was characterised by UV-Vis spectrum. The UV-Vis absorption spectrum showed sharp peak in a range between 415-420 nm, the characteristic surface plasmon resonance (SPR) band of silver nanoparticles (Wei et al., 2009), which supports the formation of silver nanoparticles on chitosan matrix. For nano suspensions, size distribution and zeta potential are important characteristic parameters

Table 3. Efficacy of chitosan-AgNP composite and treatments on anthracnose of mango caused by *C. gloeosporioides.*

Treatment[B]	Lesion size[A] (Cm^2)
Control	2.38± 0.08a
Acetic acid	2.23± 0.07 (14.84)b
Chitosan 0.5%	1.57± 0.08 (35.53)c
Chitosan 1%	1.32± 0.05 (41.78)d
Chitosan 0.5% + Tween 80	0.91± 0.04 (51.87)g
Chitosan 1% + Tween 80	0.40± 0.03 (65.74)i
Chitosan-AgNP 0.5%	1.12± 0.07 (45.67)e
Chitosan-AgNP 1%	0.25±0.01(71.28)j
Chitosan-AgNP 0.5%+Tween 80	0.14± 0.01 (75.79)k
Chitosan-AgNP 1%+Tween 80	0.03± 0.04 (84.55)l
Carbendazim 0.0001%	1.01± 0.04 (49.26)f
Carbendazim 0.001%	0.49± 0.03 (63.04)h
CD1%	0.26

[A]Values in parentheses indicate percent inhibition over control. Percentage of inhibition was calculated based on data collected after seven days of inoculation. Percentage of inhibition was calculated using formula [C-T/C] (100)], where C is the lesion size on control fruit and T is the lesion size on treated fruit (cm^2). Percentage data were arcsin-transformed before analysis according to y = arcsin [sqr. (_/100)]. Data are the means and standard deviation of three independent experiments. Each experiment contained three replicates. Each replicate contained 12 fruits and two inoculation points. Each row values followed by a different lower case letter are significantly different at $p < 0.01$, according to Fishers LSD test. [B]Healthy mangoes cv. Alphonso, treated with different concentrations of chitosan-AgNP composite in comparison with other test chemicals and control, placed in plastic boxes (45 cm height x 40 cm length x 15 cm width) containing water to maintain humidity and used for bioassay against *C. gloeosporioides* Cm 50.

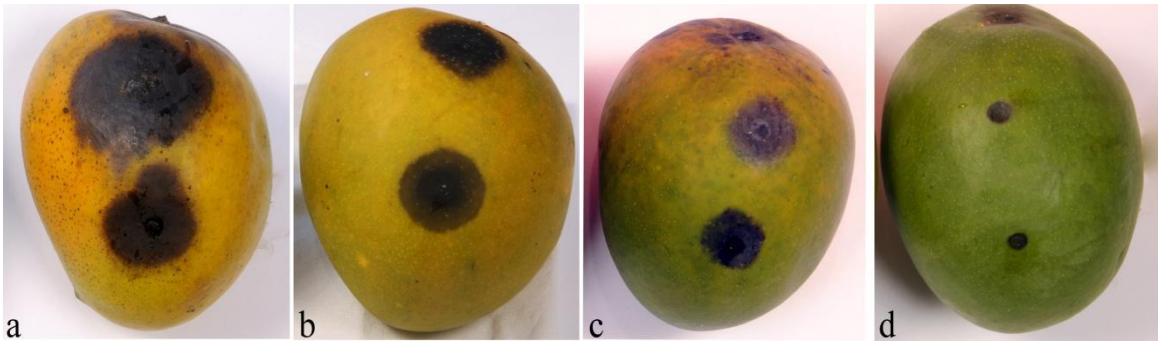

Figure 7. Effect of nanocomposite on mango against anthracnose a) Control, b) carbendazim (0.001%), c) 1% chitosan, and d) 1% Chitosan-AgNP composite with Tween-80.

parameters (Muller et al., 2001). When the Chitosan-AgNP composites loaded with different concentrations of $AgNO_3$ were analysed with DLS, samples showed the size distribution in range between 495 - 616 nm. The zeta potential of ± 30 mV is required as a minimum for electrostatic repulsion of the physically stable nanosuspension, which indicates the degree of repulsion between adjacent, similarly charged particles and also stability of nanoparticles in a solution (Muller et al., 2001; Du et al., 2009). The zeta potentials were enhanced significantly with the increase in loading of silver into nanocomposite ranging from +50.08 to +87.75 mV. These data indicated that nanocomposites prepared with different concentrations of $AgNO_3$ were highly stable. To conform the presence of silver nanoparticles in the composite, SEM measurements were carried out, chitosan-AgNP composites showed spherical particles with solid dense structure having particle sizes in the range between 10-15 nm. The significant decrease in size of nanocomposite can be justified by two major explanations, first is association of

large number of water molecules with nanocomposite when we investigate via DLS, while in the case of SEM imaging water was removed during sample preparation. The second significant difference is because of large chitosan particles present in bulk solution during DLS analysis, while in SEM no bigger particles has been considered for diameter measurement. The EDS taken during SEM imaging showed prominent silver signal, indicating the presence of silver in nanocomposite.

The major postharvest losses of mango are due to fungal infection, physiological disorders, and physical injuries, chitosan coating has the potential to prolong the storage life and control decay of mangoes (Kittur et al., 2001). Researchers have used some plant essential oils as post-harvest botanical fungicides in the management of anthracnose disease of mango fruits (Abd-AllA and Haggag, 2013). Mohamed et al. (2013) showed antifungal activity of chitosan film on inhibition of *C. gloeosporioides* associated with mango. In the present study, we have evaluated the applicability of the chitosan-AgNP composite as a fruit coating material to inhibit the fungal growth of *C. gloeosporioides*. Nanocomposites showed a greater effect in reducing the percentage of rotting fruit tissue. Tween alone has no significant effect on inhibition of spores, but the addition of non-ionic surfactant Tween 80 to nanocomposite enhanced the wettability and adhesion property of coating solution, which did not allow the normal development of spores and exhibited higher disease reduction as compared to other treatments.

Previously, chitosan nanoparticles with the enhanced zeta potential have shown excellent inhibitory effects on microorganisms (Qi et al., 2004). In the present study, chitosan-AgNP composite markedly reduced conidial germination of *C. gloeosporioides* as compared to control and other counterparts. In mango, the post-harvest phase of anthracnose caused by *C. gloeosporioides* is the most devastating and economically significant phase, and this phase is directly linked to the field phase where the infection takes place on developing fruit and infections remain quiescent in the form of appresoria and subcuticular hyphae until the onset of ripening (Arauz, 2000). At present, the elimination of quiescent infection is achieved commercially by thermal and chemical treatments or a combination of both (McMillan, 1987). Temperature and time control is critical, because fruit can be easily damaged by over exposure to heat, and the process is time consuming and labour intensive.

In the present study, we demonstrated that chitosan-AgNP composites were more effective than chitosan in inhibiting spore germination of *C. gloeosporioides* and inhibition of anthracnose on mango. In their nanosized form, silver appear to be more toxic than their bulk sized counterparts. Thus, these nanocomposites can be utilized as coating material in preventing quiescent infections of *C. gloeosporioides* on mango to prevent post-harvest losses.

Conclusion

Silver nanoparticles with size of 10-15 nm were prepared using low molecular weight chitosan as a reducing and stabilizing agent at 95°C. The results of UV-Vis spectrum, EDS and FESEM confirmed the presence of silver nanoparticles and also structure of chitosan-AgNP composite. The resultant composite successfully inhibited conidial germination of *C. gloeosporioides* and also reduced the anthracnose incidence on mango. This could find applications in preventing quiescent infections of *Colletotrichum* on mango to prevent enormous crop losses and promote exports.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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REFERENCES

Abd-AllA M, Haggag WM (2013). Use of some plant essential oils as post-harvest botanical fungicides in the management of anthracnose disease of mango fruits (*Mangifera indica* L.) caused by *Colletotrichum Gloeosporioides* (Penz). Int. J. Agric. For. 3: 1-6.

Ali A, Muhammad MTM, Sijam K, Siddiqui Y (2010). Potential of chitosan coating in delaying the postharvest anthracnose (*Colletotrichum gloeosporioides* Penz.) of Eksotika II papaya. Int. J. Food Sci. Technol. 45: 2134-2140.

Aranaz I, Harris R, Heras A (2010). Chitosan amphiphilic derivatives. Chemistry and applications. Curr. Org. Chem. 14: 308-330.

Arauz LF (2000). Mango anthracnose: economic impact and current options for integrated management. Plant Dis. 84: 600-611.

Bautista-Banos S, Hernandez-Lauzardo AN, Velazquez-del Valle MG, Hernandez-Lopez M, Ait Barka E, Bosquez-Molina E, Wilson CL (2006). Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. Crop Prot. 25: 108-118.

Bautista-Banos S, Hernandez-Lopez M, Bosquez-Molina E, Wilson CL (2003). Effects of chitosan and plant extracts on growth of *Colletotrichum gloeosporioides*, anthracnose levels and quality of papaya fruit. Crop Prot. 22: 1087-1092.

Clement JL, Jarret PS (1994). Antimicrobial silver. Met-Based Drugs. 1: 467-482.

Du WL, Niu SS, Xu YL, Xu ZR, Fan CL (2009). Antibacterial activity of chitosan tripolyphosphate nanoparticles loaded with various metal ions. Carbohydr. Polym. 75: 385-389.

El Ghaouth A, Arul J, Asselin A, Benhamou N (1992). Anti-fungal activity of chitosan on post-harvest pathogens: Induction of morphological and cytological alterations in *Rhizopus stolonifer*. Mycol. Res. 96: 769-779.

El Hadrami A, Adam LR, El Hadrami I, Daayf F (2010). Chitosan in Plant Protection. Mar. Drugs 8**:** 968-987.

Garcia M, Diaz R, Martinez Y, Casariego A (2010). Effects of chitosan coating on mass transfer during osmotic dehydration of papaya. Food Res. Int. 43: 1656–1660

Harish Prashanth KV, Tharanathan RN (2007). Chitin/chitosan: modifications and their unlimited application potential—an overview. Trends Food Sci. Technol. 18: 117-131.

Helander IM, Nurmiaho-Lassila EL, Ahvenainen R, Rhoades J, Roller S (2001). Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. Int. J. Food Microbiol. 71: 235–244.

Hewitt HG (1998). Fungicide resistance. In: Hewitt, H.G. (Ed.). Fungicides in Crop Protection Centre for Agriculture and Biosciences International, Walling Ford, UK, pp. 155-181.

Jeon YJ, Park PJ, Kim SK (2001). Antimicrobial effect of chito oligosaccharides produced by bioreactor. Carbohydr. Polym. 44: 71-76.

Jo YK, Kim BH, Jung G (2009). Antifungal activity of silver ions and nanoparticles on phytopathogenic fungi. Plant Dis. 93: 1037-1043.

Kendra DF, Hadwiger LA (1984). Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin from *Pisum sativum*. Exp. Mycol. 8: 276-281.

Kim SW, Kim KS, Lamsal K, Kim YJ, Kim SB, Jung M, Sim SJ, Kim HS, Chang SJ, Kim JK (2009). An *in vitro* study of the antifungal effect of silver nanoparticles on oak wilt pathogen *Raffaelea* sp. J. Microbiol. Biotechnol. 19**:** 760-764.

Kittur FS, Saroja N, Habibunnisa, Tharanathan RN (2001). Polysaccharide-based composite coating formulations for shelf-life extension of fresh banana and mango. Eur. Food Res. Technol. 213: 306–311.

Kong M, Chen XG, Xing K, Park HJ (2010). Antimicrobial properties of chitosan and mode of action: A state of the art review. Int. J. Food Microbiol. 144: 51-63.

Lamsal K, Kim SW, Jung JH, KimYS, Kim KS, Lee YS (2011). Inhibition effects of silver nanoparticles against powdery mildews on cucumber and pumpkin. Mycobiology 39: 26-32.

Lin J, Gong D, Zhu S, Zhang L, Zhang L (2011). Expression of PPO and POD genes and contents of polyphenolic compounds in harvested mango fruits in relation to Benzothiadiazole-induced defense against anthracnose. Sci. Hortic. 130**:** 85-89.

McMillan RT Jr (1987). Effectiveness of various postharvest treatments for mango decay control. Proc. Fla. State Hortic. Soc. 100: 7-9.

Mohamed C, Clementine KA, Didier M, Gerard L, Marie Noelle DC (2013). Antimicrobial and physical properties of edible chitosan films enhanced by lactoperoxidase system. Food Hydrocolloids 30: 576-580.

Muller RH, Jacobs C, Kayser O (2001). Nanosuspensions as particulate drug formulations in therapy: rationale for development and what we can expect for the future. Adv. Drug Deliv. Rev. 47**:** 3-19.

Munoz Z, Moret A, Garces S (2009). Assessment of chitosan for inhibition of *Colletotrichum* sp. on tomatoes and grapes. Crop Prot. 28: 36-40.

Murugadoss A, Chattopadhyay A (2008). A'green'chitosan–silver nanoparticle composite as a heterogeneous as well as micro-heterogeneous catalyst. Nanotechnology 19: 1-9.

Park HJ, Kim SH, Kim HJ, Choi SH (2006). A new composition of nanosized silica-silver for control of various plant diseases. Plant Pathol. J. 22: 295-302.

Peres N, Souza N, Peever T, Timmer L (2004). Benomyl sensitivity of isolates of *Colletotrichum acutatum* and *C. gloeosporioides* from citrus. Plant Dis. 88: 125-130.

Qi L, Xu Z, Jiang X, Hu C, Zou X (2004). Preparation and antibacterial activity of chitosan nanoparticles. Carbohydr. Res. 339: 2693-2700.

Russell A, Path F, Sl FP, Hugo W (1994). Antimicrobial activity and action of silver. Prog. Med. Chem. 31: 351-370.

Sanpui P, Murugadoss A, Prasad PVD, Ghosh SS, Chattopadhyay A (2008). The antibacterial properties of a novel chitosan–Ag-nanoparticle composite. Int. J. Food Microbiol. 124: 142-146.

Sudarshan NR, Hoover DG, Knorr D (1992). Antibacterial action of chitosan. Food Biotechnol. 6: 257-272.

Than PP, Jeewon R, Hyde KD, Pongsupasamit S, Mongkolporn O, Taylor PWJ (2008). Characterization and pathogenicity of *Colletotrichum* species associated with anthracnose disease on chilli (*Capsicum* spp.) in Thailand. Plant Pathol. 57: 562-572.

Tharanathan RN, Yashoda HM, Prabha TN (2006): Mango (*Mangifera indica* L.), "The King of Fruits"—An Overview. Food Rev. Int. 22(2): 95-123.

Tsai GJ, Su WH (1999). Antibacterial activity of shrimp chitosan against *Escherichia coli*. J. Food Prot. 62: 239-243.

Wei D, Sun W, Qian W, Ye Y, Ma X (2009). The synthesis of chitosan-based silver nanoparticles and their antibacterial activity. Carbohydr. Res. 344: 2375-2382.

Xu J, Zhao X, Han X, Du Y (2007). Antifungal activity of oligochitosan against *Phytophthora capsici* and other plant pathogenic fungi *in vitro*. Pest Biochem. Physiol. 87: 220–228.

Zhong Z, Li P, Xing R, Chen X, Liu S (2009). Preparation, characterization and antifungal properties of 2-(α-arylaminophosphonate)-chitosan. Int. J. Biol. Macromol. 45: 255-259.

Zhuang X, Cheng B, Kang W, Xu X (2010). Electrospun chitosan/gelatin nanofibers containing silver nanoparticles. Carbohydr. Polym. 82: 524–527.

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Biocontrol efficiency of native mycorrhizal isolates in ashwagandha (*Withania somnifera* L.)

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Ashwagandha (*Withania somnifera* L.) is an angiospermic medicinal herb, well recognized for the immense therapeutic potential of its roots containing several withanolides. *W. Somnifera*, however is susceptible for many plant pathogens like *Ralstonia*, *Phytophthora*, *Fusarium* and many others. The present investigation on ashwagandha was undertaken to screen the efficacy of arbuscular mycorrhizal fungi against different diseases under mesh house conditions. The results indicated that the plants affected with *Ralstonia* and *Phytophthora* showed root rots and wilting symptoms respectively, at different incubation intervals. Among all the isolates AM-G-4, AM-G-5b, AM-G-9a, AM-B-1 and AM-B-10a showed inhibitory action against both *Ralstonia* and *Phytophthora* diseases.

Key words: *Withania somnifera* L., angiospermic, *Ralstonia, Phytophthora,* inhibitory action.

INTRODUCTION

Withania somnifera (L.) commonly known as 'ashwagandha' or '*asgandh*' has a high repute in traditional Indian medicine, and it is one of the most extensively used plant in Ayurveda and Unani medicines. It is wide spread in Africa, Mediterranean region and the Middle East. The plant roots are a major source of alkaloids including tropine, pseudotropine and somniferine (Shilpi *et al*. 2013). Use of vesicular-arbuscular mycorrhizal (VAM) fungi in biocontrol of soil-borne plant pathogens has increased its significance in recent years.

VAM fungi are known to reduce the inoculums multiplication and infection of nematodes, viruses and bacteria as well. In addition to certain exceptions, VAM fungi increase the growth and yield of certain crop plants. VA mycorrhizal fungi retard the development of pathogens in root systems and increase disease severity in non-mycorrhizal roots. This systemic influence can be attributed to better nutrition, which enhanced the plant growth and physiological stimulation in mycorrhizal plants. The roots colonized by a VAM fungus exhibit high chitinolytic activity. These enzymes can be effective against the other fungal pathogens. Under direct influence of mycorrhizal fungi, roots tissues become more resistant to pathogenic attack. This induced resistance is strictly limited to the site of host-endophyte interaction and will only affect soil-borne pathogen. Thus, application of selected VA mycorrhizal fungi offers the possibility of increasing resistance against soil-borne pathogens.

Among all the major constraints for growing this crop

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Table 1. Details of ashwagandha rhizosphere soil used for isolation of AM fungi at different locations of India.

S/N	Sampling place	Samples code	Isolate	Soil type	Soil pH
1	Bangalore (5 samples)	BNG-1	AM-BNG-1	Red soil	7.85
2		BNG-2	AM-BNG-2	Red soil	8.36
3		BNG-3	AM-BNG-3	Red soil	7.63
4		BNG-4	AM-BNG-4	Red soil	8.36
5		BNG-5	AM-BNG-5, AM-BNG-5a	Red soil	7.93
6	Gadag (20 samples)	G-1	AM-G-1	Black soil	7.60
7		G-2	AM-G-2, AM-G-2a	Black soil	8.09
8		G-3	AM-G-3	Black soil	7.69
9		G-4	AM-G-4	Black soil	8.32
10		G-5	AM-G-5, AM-G-5a, AM-G-5b	Black soil	7.64
11		G-6	AM-G-6	Black soil	7.39
12		G-7	AM-G-7, AM-G-7a	Black soil	7.01
13		G-8	AM-G-8	Black soil	8.39
14		G-9	AM-G-9, AM-G-9a	Black soil	8.00
15		G-10	AM-G-10	Black soil	8.67
16		G-11	AM-G-11	Black soil	8.02
17		G-12	AM-G-12	Black soil	8.53
18		G-13	AM-G-13, AM-G-13a	Black soil	8.26
19		G-14	AM-G-14	Black soil	7.59
20		G-15	AM-G-15	Black soil	7.69
21		G-16	AM-G-16, AM-G-16a	Black soil	8.23
22		G-17	AM-G-17	Black soil	7.96
23		G-18	AM-G-18, AM-G-18a, AM-G-18b	Black soil	8.65
24		G-19	AM-G-19	Black soil	7.85
25		G-20	AM-G-20, AM-G-20a	Black soil	7.36
26	Bellary (5 samples)	B-1	AM-B-1, AM-B-1a	Medium black	7.69
27		B-2	AM-B-2, AM-B-2a, AM-B-2b	Medium black	8.01
28		B-3	AM-B-3	Black soil	7.45
29		B-4	AM-B-4, AM-B-4a	Medium black	7.68
30		B-5	AM-B-5	Black soil	7.98
31	Bellary (5 samples)	B-6	AM-B-6	Medium black	8.63
32		B-7	AM-B-7, AM-B-7a	Medium black	6.90
33		B-8	AM-B-8, AM-B-8a	Black soil	8.31
34		B-9	AM-B-9	Medium black	7.62
35		B-10	AM-B-10, AM-B-10a	Medium black	6.85
36	Koppal (10 samples)	K-1	AM-K-1	Black soil	8.90
37		K-2	AM-K-2	Black soil	7.25
38		K-3	AM-K-3	Black soil	6.35
39		K-4	AM-K-4, AM-K-4a	Black soil	8.36
40		K-5	AM-K-5	Red soil	7.30
41		K-6	AM-K-6	Black soil	5.86
42		K-7	AM-K-7, AM-K-7a	Black soil	5.50
43		K-8	AM-K-8	Black soil	7.52
44		K-9	AM-K-9	Black soil	8.63
45		K-10	AM-K-10, AM-K-10a	Red soil	8.32
46		A-1	AM-A-1	Black soil	7.95

Table 1. Contd.

47	Arabhavi (5 samples)	A-2	AM-A-2, AM-A-2a	Red soil	6.90
48		A-3	AM-A-3, AM-A-3a	Black soil	7.48
49		A-4	AM-A-4	Black soil	8.65
50		A-5	AM-A-5, AM-A-5a, AM-A-5b	Red soil	7.80

Table 2. Disease severity recorded based on symptoms appearance and days taken for expression of symptoms.

Days taken for expression of symptoms	Disease severity
≤25	++++
26-50	+++
51-75	++
76	+
>75 days (upto harvest)	-

are diseases like wilt and root rot caused by soil borne pathogens belonging to species of *Ralstonia* and *Phytophthora* (Kamalakannan et al., 2005). Many effective pesticides have been tried against soil borne pathogens but not considered as long term solution because of concerns about exposure risks, health and environmental hazards, high cost, residue persistence, development of resistance to pesticides and elimination of natural enemies. Keeping these points in view, the present investigation on ashwagandha was undertaken to screen the efficacy of AM fungi against diseases under mesh house conditions.

MATERIALS AND METHODS

Collection of soil and root samples

A total of 50 rhizosphere soil and ashwagandha root samples were collected from the major ashwagandha growing fields spread across five districts in the Indian state of Karnataka for isolation of AM fungi. The details on the soil type and locations from where the samples were collected have been given in Table 1.

Identification and mass production of arbuscular mycorrhizal fungi (AMF)

Identification and mass production of AMF spore population density and species richness of AMF from each soil sample was estimated by the method of wet sieving and decanting (Gerdemann and Nicolson, 1963)). Assessment and the percentage of AMF colonization in the roots of ragi were determined by using the trypan blue in lactophenol method (Phillips and Hayman, 1970). For the identification of AMF, intact spores were picked up from the filter paper and mounted on micro slides in lactophenol, and were observed under binocular research microscope. The morphology of spores and sporocarps of AMF were observed, and then their characters were used for identification by referring the Manual for identification of AM fungi (Schenck and Perez, 1990). Mass inoculums production of AMF was carried out in the roots of ragi under mesh house condition, by the method described by Selvaraj et al. (2009).

Screening methodology

The experiment was conducted under Mesh house condition (12 to 15°C minimum and 26 to 30°C maximum temperature), at Department of Agricultural Microbiology, College of Agriculture, UAS Dharwad, India during 2012 to 2013. Seventy five native AM fungal isolates and five standard cultures of AM fungi were evaluated for the biological control of naturally occurring diseases in ashwagandha under pot culture condition (Srivastava et al., 1991). Sterilized sand, decomposed animal dung, and sterilized sandy clay loam soil (1:1:2) (10 kg per pot) was used as potting mixture. AMF inoculums (30 g/pot) was added to each pot, and thoroughly mixed with surface soil. Ten ashwagandha seeds were sown in each pot. The experiment was laid out in Completely Randomized Design (CRD), with five replications and 81 treatments consisting of various AM fungi isolates maintained in pot culture. Uninoculated AM fungi were used as control check.

Record of observations

The observation on disease expression was recorded from the day symptoms which appeared and continued until fresh infections ceased to appear and the disease severity was scored based on symptoms appeared and days taken for expression of symptoms as given in Table 2.

Root rot index was calculated by the scale suggested by Chidananda Prabhu (1987) given as following:

Root rot index (0-5 scale)
0 – Healthy (No symptoms at all)
1 – Slight discolouration at the collar region, roots are healthy
2 – Discolouration 1 to 1.5 inch from ground level, taproots discoloured and secondary roots still healthy.
3 – Complete discolouration of tap roots including 2 to 2.5 inch of the stem a root discoloured.

Table 3. Screening for biocontrol ability of AM fungi isolates in ashwagandha against different pathogens

S/N	Isolate	Disease severity		Root rot index
		Ralstonia	*Phytophthora*	
1	AM-BNG-1	++	+++	2.00
2	AM-BNG-2	+++	++++	5.00
3	AM-BNG-3	++	+++	3.00
4	AM-BNG-4	++++	+++	4.50
5	AM-BNG-5	++	++++	4.50
6	AM-BNG-5a	+++	+++	3.50
7	AM-G-1	+	+++	1.50
8	AM-G-2	++	+++	2.00
9	AM-G-2a	++++	+++	4.00
10	AM-G-3	+++	++	3.50
11	AM-G-4	-	-	0.00
12	AM-G-5	+++	++++	3.00
13	AM-G-5a	+++	++	3.00
14	AM-G-5b	-	-	0.00
15	AM-G-6	+++	+++	2.50
16	AM-G-7	++	++++	2.00
17	AM-G-7a	++++	+	4.50
18	AM-G-8	+++	++	3.00
19	AM-G-9	+++	+++	2.50
20	AM-G-9a	-	-	0.00
21	AM-G-10	+++	+	3.00
22	AM-G-11	+++	++++	2.50
23	AM-G-12	+++	++	3.00
24	AM-G-13	+	++++	1.50
25	AM-G-13a	+++	++	2.00
26	AM-G-14	+++	+++	3.00
27	AM-G-15	+++	++	3.00
28	AM-G-16	+	+++	1.00
29	AM-G-16a	++	++	3.00
30	AM-G-17	++	+++	2.50
31	AM-G-18	++++	++	4.50
32	AM-G-18a	++	+++	3.50
33	AM-G-18b	+++	++	4.00
34	AM-G-19	+++	+++	3.50
35	AM-G-20	++++	++	4.50
36	AM-G-20a	+++	+++	3.50
37	AM-B-1	-	-	0.00
38	AM-B-1a	+++	+	3.00
39	AM-B-2	+++	+++	3.50
40	AM-B-2a	++	+	2.50
41	AM-B-2b	+++	+++	3.00
42	AM-B-3	++++	++	4.50
43	AM-B-4	+	++++	1.50
44	AM-B-4a	+++	+	2.50
45	AM-B-5	+++	+++	3.50
46	AM-B-6	++	+++	2.00
47	AM-B-7	+++	++	3.50
48	AM-B-7a	+++	+++	3.00
49	AM-B-8	+++	++	3.50
50	AM-B-8a	+	+++	1.50

Table 3 . Contd.

51	AM-B-9	+++	+++	3.00
52	AM-B-10	+++	++	2.50
53	AM-B-10a	-	-	0.00
54	AM-K-1	+++	++++	3.00
55	AM-K-2	+	++	1.00
56	AM-K-3	++	+++	2.00
57	AM-K-4	++	+++	2.50
58	AM-K-4a	+++	+	3.00
59	AM-K-5	+++	++	3.50
60	AM-K-6	++	+++	2.00
61	AM-K-7	+++	++	3.00
62	AM-K-7a	++	++++	2.00
63	AM-K-8	++	+++	2.50
64	AM-K-9	+++	++	3.50
65	AM-K-10	++	+++	2.50
66	AM-K-10a	+++	+++	3.50
67	AM-A-1	+++	+++	3.50
68	AM-A-2	++	+	2.00
69	AM-A-2a	+++	+++	4.00
70	AM-A-3	++	+	2.50
71	AM-A-3a	+	++++	1.00
72	AM-A-4	++	+++	2.00
73	AM-A-5	++	++	2.50
74	AM-A-5a	+++	++	4.00
75	AM-A-5b	++	++	3.00
76	*G. caladonicum*	+	+++	1.50
77	*G. mosseae*	++	++	2.50
78	*G. bagyaraji*	+	+++	1.00
79	*G. etinucatum*	++	+	2.00
80	*G. macrocarpum*	+	++	1.50
81	Control	++++	+++++	5.00

****** Values are means of three replications.

4 – Majority of secondary roots discoloured and root tips infected.
5 – All roots rotting and complete discolouration

RESULTS AND DISCUSSION

The rhizosphere soil samples used for the isolation of AM fungi were analysed for pH and soil types were listed (Table 1). The pH of the soils ranged from 5.50 to 8.90. The soil type was found to be black in all the locations except a few locations, wherein red soils were noticed. All fifty samples showed the presence of AM fungi. Supriya and Kaushik (2011) also worked on periodical survey of various places in India to collect and identify different VAM species associated with medicinal plants.

Based on morphological and structural parameters, almost all AM fungi isolates belonged to the probable genera *Glomus* except for five isolates which belonged to the genera *Gigaspora.* Presence of several species of different genera has already been reported by Supriya and Kaushik (2011). The VAM species identified were: *Glomus aggregatum; Glomus fasciculatum; Glomus geosporum; Glomus monosporum Glomus mosseae; Glomus claroideum; Glomus etunicatum; Glomus coronatum; Glomus intraradices; Glomus macrocarpum; Gigaspora margarita; Gigaspora rosea; Gigaspora gigantea; Sclerocystis sinuosa; Acaulospora scrobiculata* and *Acaulospora laevis.* In the present investigation, AM fungi were found to have a significant effect on controlling different diseases of ashwagandha.

The results indicated that the plants affected with *Ralstonia* showed wilting and root rot symptoms at different interval that is 20 to 76 day after sowing (DAS; Table 3). Among all the isolates AM-G-4, AM-G-5b, AM-G-9a, AM-B-1 and AM-B-10a showed inhibitory action against *Ralstonia* disease, ten isolates showed disease symptoms at 76 DAS, twenty-three isolates showed disea-

disease symptoms between 51 to 76 DAS, 36 isolates between 36 to 50 DAS and seven isolates showed early symptoms within 25 DAS. Similar results were also reported by Mallesh et al. (2009) the plants inoculated with *Ralstonia solanacearum* showed early symptoms of disease within 20. These results are in accordance with the findings of Vijayakumari (2004), Ramaprasad (2005) that conducted pathogenicity studies with *R. solanacearum* and *Fusarium chlamydosporum* on *Coleus* and proved that they caused early symptom and maximum disease incidence.

The results indicated that the plants affected with *Phytophthora* exhibited tip burning and drying symptoms at different interval that is 20 to 76 DAS. Among all the isolates AM-G-4, AM-G-5b, AM-G-9a, AM-B-1 and AM-B-10a showed inhibitory action against *Phytophtora* disease and eight isolates showed disease symptoms at 76 DAS, twenty-three isolates showed disease symptoms between 51 to 76 DAS, 34 isolates between 36 to 50 DAS and eleven isolates showed early symptoms within 25 DAS. Similar disease symptoms were found by Nelson (2008) in tomato crop infected with *Phytophthora.* Boby and Bagyaraj (2003) also observed the reduction of root rot incidence in *Coleus forskohlii* by microbial inoculants.

REFERENCES

Boby VU, Bagyaraj DJ (2003). Biological control of root-knot of *Coleus forskohlii* Briq. using microbial inoculants. World J. Microbiol. Biotech. 19: 175-180.

Gerdemann JW, Nicolson TH (1963). Spores of mycorrhizal Endogone species extracted from soil by wet-sieving and decanting. Trans. Br. Mycol. Soc. 46: 235-244.

Kamalakannan A, Mohan L, Valluvaparidasan V, Mareeswari P, Karuppiah R (2005). First report of Macrophomina root rot (*Macrophomina phaseolina*) on medicinal coleus (*Coleus forskohlii*) in India. Pl. Pathol. 55 : 302.

Mallesh SB, Lingaraju S, Byadgi AS, Hegde YR, Mokashi AN, Krishnaraj PU (2009). Bioefficacy of rhizobacteria on root knot/wilt complex in coleus and ashwagandha. Karnataka J. Agric. Sci. 22 (5):116-1 120.

Nelson SC (2008). Late Blight of Tomato (*Phytophthora infestans*). Plant Disease. 15:45-47.

Phillips JF, Hayman DS (1970). Improved procedures for clearing roots and staining parasite and vesicular arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Bri. Mycol. Soc. 55: 158-161.

Ramaprasad S (2005) Studies on management of root rot complex of *Coleus forskohlii* (Wild.) Briq; caused by *Fusarium chlamydosporum* (Frag. and Cif.) Booth, *Rhazoctonia bataticola (T* aub.) Butler and Sclerotium *rolfsii* Kuhn. M. Sc. (Agri.), Thesis, Univ. Agric. Sci., Dharwad (India).

Schenck NC, Perez Y (1990). Manual for the Identification of Arbuscular Mycorrhizal Fungi. University of Florida, Gainesville, Florida, USA.

Selvaraj T, Nisha MC, Rajeshkumar S (2009). Effect of indigenous arbuscular mycorrhizal fungi on some growth parameters and phytochemical constituents of *Pogostemon patchouli* Pellet. Maejo. Int. J. Sci. Technol. 3: 222-234.

Shilpi KS, Sudeep T, Rakesh P (2013). Rhizospheric biological weapons for growth enhancement and *Meloidogyne incognita* management in *Withania somnifera* cv. Poshita. Biol. Control**.** 65(2): 225–234.

Srivastava KJ, Qadri SMH, Tiwari BK, Bhonde SR, Pandey UB (1991). Control of purple blotch disease of onion bulb crop in *kharif* season. Indian Phytopathol. 44: 251-253.

Supriya G, Kaushik P (2011). Analysis of vesicular arbuscular mycorrhiza associated with medicinal plants in Uttarakhand state of India. World Appl. Sci. J. 14 (4): 645-653.

Vijayakumari SG (2004). Studies on bacterial wilt of Coleus *forskohlii* caused by *Ralstonia solanacearum*. M. Sc. (Agri.) Thesis, Univ. Agric. Sci., Bangalore (India).

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Full Length Research Paper

Mosquito repellent from Thai essential oils against dengue fever mosquito (*Aedes aegypti* (L.)) and filarial mosquito vector (*Culex quinquefasciatus* (Say))

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Repellent activity of Thai essential oils derived from ylang-ylang (*Cananga odorata*), lemon grass (*Cymbopogon citratus*) and citronella grass (*Cymbopogon nardus*) were evaluated against female *Aedes aegypti* (L.) and *Culex quinquefasciatus* (Say) and compared a commercially available repellents (IR3535, ethyl butylacetylamino propionate 12.5% w/w; Johnson's Baby Clear Lotion Anti-Mosquito®). Each test repellent was applied at 0.17 and 0.33 µl/cm^2 on the forearm of volunteers. All essential oils at 0.17 µl/cm^2 showed lower protection time and percentage of protection against two mosquito species than at 0.33 µl/cm^2. All essential oils exhibited higher repellent activity than chemical repellent. The essential oil of *C. citratus* at 0.33µl/cm^2 exhibited excellent repellent activity with 98.67% protection from bites of *A. aegypti* for 116.67±55.75 min and 99.75% protection from bites of *C. quinquefasciatus* for 128.33±12.89 min. However, repellent activity in order of protection time and percentage of protection against two mosquito species in three essential oils was *C. citratus* oil >*C. odorata* oil >*C. nardus* oil. Our data showed that *C. citratus* oil is an effected green repellent for mosquitoes that is safe for humans and environmentally friendly.

Key words: Repellent, *Aedes aegypti*, *Culex quinquefasciatus*, Thai essential oil.

INTRODUCTION

Mosquito is a serious insect to public health, which transmits several dangerous diseases such as dengue, filariasis, malaria, yellow fever and Japanese encephalitis. Every year at least 500 million people in the world suffer from one or the other tropical diseases that include dengue, malaria and filariasis (Madhumathy et al., 2007; Kumar et al., 2012). However, dengue worldwide disease is transmitted by *Aedes aegypti*, approximately 2.5 billion people from 100 countries live in areas infested with these mosquito vectors (Borah et al., 2010). Reported cases of dengue and dengue hemorrhagic fever have shown an exponential increase over the last 30 years

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with the number of cases reported to the WHO between 2000 and 2007 doubling over those in the previous decade; Southeast Asian and Western Pacific countries bear the brunt of global disease burden due to dengue (WHO, 2009a). *Culex quinquefasciatus* is an important vector of filariasis in tropical and sub-tropical regions, about 90 million people worldwide suffer from these diseases.

Thus, mosquito control and personal protection from mosquito bites are currently the most important measures to control mosquito transmitted diseases. However, repellents base on chemical insecticides are considered to be useful in reducing and preventing the mosquito vectors. On the other hand, chemical repellents are not safe for human, especially children because they may cause skin irritation, hot sensation rashes or allergy (Das et al., 2003). In recent years, there was an increase in public concern on the safety of many chemical products that instigated a renewed interest on the use of natural products from plant origin for mosquito vector management. In addition, plant essential oils in general have been recognized as an important natural resource of insecticides and insect repellents, various essential oils have also been documented to exhibit acute toxic effects against insects, including mosquitoes (Pavela, 2008). The repellents base on plant essential oils are effective for mosquito control, environment-friendly, easily biodegradable, and readily available in many areas of the world, have no ill effect on non-target organisms (Govindarajan, 2011).

Many researchers have reported the repellent activity of plant essential oils against female mosquito vectors. Essential oils of *Cymbopogon citratus*, *Cinnamomum zeylanicum*, *Mentha piperita*, *Rosmarinus officinalis* and *Zingiber officinalis* showed repellent activity against *A. aegypti* and *C. quinquefasciatus* (Govindarajan, 2011; Khandagle et al., 2011; Kumar et al., 2011). Furthermore, the US Environmental Protection Agency (USEPA) has registered citronella, lemon and eucalyptus oil as insect repellent ingredients for application on the skin, these natural products are been frequently used due to their relative low toxicity, comparable efficacy, and customer approval (Katz et al., 2008).

Therefore, the objective of this study was to investigate the repellency of Thai essential oils derived from *Cymbopogon citratus*, *Cymbopogon nardus* and *Cananga odorata* against females of *A. aegypti* and *C. quinquefasciatus* and to compare them with chemical repellent, IR3535 (12.5% w/w Ethyl butylacetylamino propionate; Johnson's Baby Clear Lotion Anti-Mosquito®).

MATERIALS AND METHODS

Test mosquitoes

A. aegypti and *C. quinquefasciatus* eggs were obtained from the Armed Forces Research Institute of Medical Sciences (AFRIMS), Thailand. These eggs were brought to the laboratory of Entomology and Environment Program, Plant Production Technology Section, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok. The eggs were hatched in a glass cup with 250 ml of water. We then transferred batches of 200 larvae to white plastic trays (30 x 35 x 5 cm). Fish food (HIPRO®) was added (0.1 g for 1st and 2nd instar larvae, 0.3 g for 3rd instar larvae, and 0.5 g for 4th instar larvae at 0800 am and 0400 pm each day) to each tray for the successive two weeks until pupation of all larvae. The pupae were collected weekly and kept in a holding cage (size 30 x 30 x 30 cm) for emergence. Adult mosquitoes were reared at 25-30°C, and a relative humidity of 80±10% with a photoperiod of 12 h light followed by 12 h dark (12L:12D). We provided adults with soaked cotton balls containing a 5% glucose solution. Adult females, 5 to 7 days post eclosion, were used in these experiments. Prior to testing, the glucose-saturated cotton balls were removed from the holding containers for 12 h prior to blood feeding. Six hours prior to blood feeding, the water soaked cotton ball was removed.

Plant materials and herbal essential oils

Various parts of three species from Thai plants (flower of *Cananga odorata*, stem of *C. citratus* and stem of *C. nardus*) were collected from Nakhon Ratchasima province, Thailand. All plants were identified, by plant taxonomist of Plant Production Technology Section, Faculty of Agricultural Technology, KMITL. The various plant parts were extracted for essential oils by water distillation. One kilogram of dried and finely ground material from each plant was placed in an extraction column connected to a round-bottomed distillation flask containing distilled water. The flask was heated to approximately 100°C and allowed to boil until distillation was completed. The liquid formed, together with the distillate oils, were collected in a separating funnel. The mixture was then allowed to settle for 1 day, after which the water (lower) layer was slowly drawn off until only the oil remained. These essential oils were prepared at 0.17 and 0.33µl/cm^2 in ethyl alcohol. All formulations were kept at room temperature before testing.

Chemical repellent

IR3535 (12.5% w/w ethyl butylacetylaminopropinnate; Johnson's Baby Clear Lotion Anti-Mosquito®), a common chemical repellent for children in Thailand, was purchased from IDS Manufacturing Co. Ltd., Thailand.

Repellent bioassay

The three essential oils were tested against *A. aegypti* and *C. quinquefasciatus* females under laboratory conditions using the arm in cage method (Barnard, 2005) following WHO (2009b). Six human volunteers were recruited from the healthy students and lecturers of Entomology and Environment Laboratory, Plant Production Technology Section, Faculty of Agricultural Technology, KMITL. The volunteers for the repellency test had no history of dermatological disease or allergic reaction to mosquito bites or repellents. All volunteers signed an informed consent form after having received a full verbal/written explanation of the test objectives. The research proposal was approved by the research committee of Faculty of Agricultural Technology, KMITL. The timing of the tests depended on the mosquitoes, for *A. aegypti* was tested during the day time from 0800 am to 0400 pm, while *C. quinquefasciatus* was tested during night time from 0400 pm to 1200 pm. Before testing, the volunteer's arms were washed and cleaned thoroughly with distilled water and the left arm was used for treatment and the right arm for control. Both arms of volunteers were covered with rubber

Table 1. Repellency of herbal essential oils and chemical repellent against *A. aegypti* and *C. quinquefasciatus* at 0.17 µl/cm^2.

Test repellent	Mosquito sp.	Protection time (Mean±SD) (min)	Protection (%)
C. odorata oil	*A. aegypti*	53.20±5.79c[1]	98.80
	C. quinquefasciatus	90.0±0 a	98.67
C. citratus oil	*A. aegypti*	60.67±6.45b	98.80
	C. quinquefasciatus	65.0±2.35b	99.20
C. nardus oil	*A. aegypti*	58.33±7.64bc	96.92
	C. quinquefasciatus	65.0±2.35b	98.94
IR3535	*A. aegypti*	3.0±0d	77.54
	C. quinquefasciatus	3.0±0d	75.73

[1]Means of protection time in each column, followed by the same letter are not significantly different (one-way ANOVA and Duncan's multiple Range Test, P<0.05).

sleeve with a window area of 3 x 10 cm on the ventral part of forearm. One hundred microlitres of each repellent was applied to the treatment area of left forearm of each volunteer and allowed to dry on the skin for 1 min. After applying the test repellent, the volunteer was instructed not to rub, touch or wet the treated area. The right arm acting as a control was exposed for up to 30 s to mosquito cage (30 x 30 x 30 cm) containing 250 nulliparous female mosquitoes (4-5 days old). If at least two mosquitoes landed on or bit the control arm, the repellency test was then continued. The test continued until at least two bites occurred in a three-minute period. However, if no mosquitoes bit during a three-minute period, the arm was withdrawn from the mosquito cage. The protection time or repellency test period was carried out every 15 min until at least two mosquitoes bit during the three-minute period and then the repellency test was stopped. The time between applications of the repellents and the two first mosquitoes bite was recorded as the protection time. Percentage of protection was calculated for each repellent using the following formula (Manimaran et al., 2013):

$$\text{Percentage of protection} = 100 - \left(\frac{\text{No. of mosquitoes biting or landing} \times 100}{\text{No. of mosquitoes released}} \right)$$

Statistical analysis

The mean protection time was used as a standard measure of repellency of essential oil repellents and IR3535 (chemical repellent) against *A. aegypti* and *C. quinquefasciatus*. Differences in significance were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) comparisons by SPSS for windows (version 6.0).

RESULTS

Table 1 and Figure 1 shows the protection time in minute and percentage of protection of herbal essential oil repellents at 0.17 µl/cm^2 and IR3535 (chemical repellent) against *A. aegypti* and *C. quinquefasciatus* in the laboratory. There were differences in repellency among the herbal essential oil repellents and IR3535 by mosquito species. The essential oils of *C. citratus* and *C. odorata* gave the highest repellency for the longest lasting period and percentage protection against *A. aegypti* and *C. quinquefasciatus* for 60 to 90 min with 98.80 to 98.67% protection. All herbal essential oils provided 96.92 to 98.80% protection from *A. aegypti* for 53 to 60 min of protection time, and also provided 98.94 to 99.20% protection from *C. quinquefasciatus*. On the other hand, the chemical repellent showed 75.73 to 77.54% protection from both mosquito species for 3 min. The results of 0.33 µl/cm^2 of herbal essential oil repellents and IR3535 against two mosquito species are shown in Table 2 and Figure 2. *C. citratus* oil gave the highest repellency with 98.67% protection from bites of *A. aegypti* up to a mean time of 116 min and with 99.75% protection from bites of *C. quinquefasciatus* up to a mean time of 128 min. IR3535 gave protection for only 3 min and 78.80 to 77.30% protection from bites of two mosquito species. However, all herbal essential oil repellents provided higher protection time and percentage of protection against two mosquito species than IR3535. All herbal essential oil repellent provided lower repellency activity (97.07 to 98.67% protection for 78 to 116 min) against *A. aegypti* than *C. quinquefasciatus* (98.94 to 99.75% protection for 86 to 128 min).

DISCUSSION

Our study clearly revealed that all essential oils from Thai herbs at 0.33 µl/cm^2 offered protection against the mosquito species tested for more than 80 min. *C. citratus* oil exhibited the highest repellent activity with 98.0 to 99.0% protection from bites of two mosquitoes for more than 120 min. The result coincides with the earlier result of other researchers, which reported that essential oils from *C. citratus* showed repellent activity against *A. aegypti*, *C. quinquefasciatus*, *Culex tritaeniorhynchus*, *Anopheles subpictus* and *Anopheles dirus* (Govindaraja, 2011; Sritabutra and Soonwera, 2013). Besides, *C. citratus* oil also showed the insecticidal effect against housefly (*Musca domestica*), showing 100% knockdown at 30 min and KT_{50} values of 5.14 min (Sinthusiri and

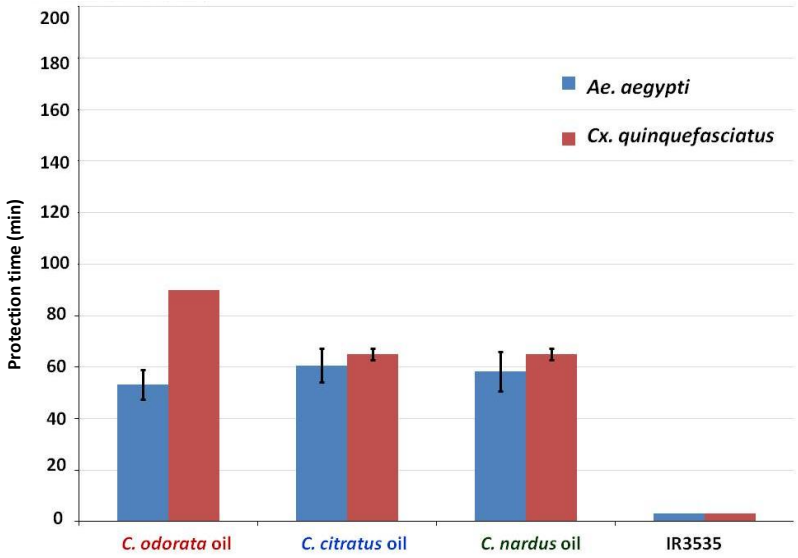

Figure 1. Comparison of protection times for repellent testing at 0.17 µl/cm^2 against mosquito species

Table 2. Repellency of herbal essential oils and chemical repellent against *A. aegypti* and *C. quinquefasciatus* at 0.33 µl/cm^2.

Test repellents	Mosquito sp.	Protection time (mean ± SD) (min)	Protection (%)
C. odorata oil	*A. aegypti*	86.67±10.40b[1]	98.94
	C. quinquefasciatus	126.0±15.77a	99.20
C. citratus oil	*A. aegypti*	116.67±55.75 a	98.67
	C. quinquefasciatus	128.33±12.89a	99.75
C. nardus oil	*A. aegypti*	80.33±16.07b	97.07
	C. quinquefasciatus	86.67±10.40b	98.94
IR3535	*A. aegypti*	3.0±0c	78.80
	C. quinquefasciatus	3.0±0c	77.30

[1]Means of protection time in each column, followed by the same letter are not significantly different (one-way ANOVA and Duncan's multiple Range Test, P<0.05).

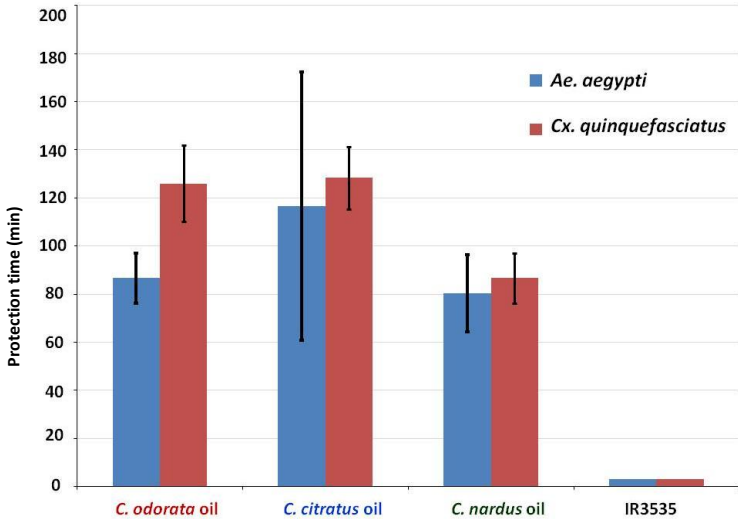

Figure 2. Comparison of protection times for repellent testing at 0.33 µl/cm^2 against mosquito species.

Soonwera, 2013). However, *C. odorata* oil also showed excellent repellent activity against the mosquito species tested with 98.94 to 99.20% protection for 86 to 126 min. Moreover, *C. odorata* oil also exhibited high potential for oviposition-deterrent and ovicidal action against *A. aegypti*, *C. quinquefasciatus* and *A. dirus* (Phasomkusolsil and Soonwera, 2012). The essential oil of *C. odorata* flowers has been shown to possess repellency against mosquito bites (*A. aegypti*) and two grain storage insects, *Sitophilus zeamias* and *Tribolium castaneum* (Trongtokit et al., 2005; Nerio et al., 2009; Caballero-Gallardo et al., 2011). However, these essential oils in this study exhibited protection time against *A. aegypti* bites of nearly 1 h and less than 2 h. Even though, essential oil repellents can be short-lived in their effectiveness, they can evaporate completely. Thus, many researchers have demonstrated improved repellency of plant-derived topical repellent products after formulating with a base or fixative materials, such as vanillin, salicylic acid and mustard and coconut oils (Stuart et al., 2000; Tawatsin et al., 2001; Das et al., 2003). There are many factors that affect the efficacy of repellent against mosquitoes, such as species and density of mosquito (Barnard et al., 1998), age of person, sex and biochemical attractiveness to biting mosquitoes (Golenda et al., 1999), ambient temperature, humidity and wind speed (Service, 1980).

In addition, *C. citratus* oil have been traditionally used in Thai medicine for analgesic, antifungal, anti inflammatory, antiseptic, antiviral, bactericidal digestive and tonic. While, *C. odorata* oil is used topically as a sedative, antiseptic, hypotensive and aphrodisiac. In addition, it is used in foods and beverages as a flavoring agent and in consumer product manufacturing as a fragrance for cosmetics and soaps (Burdock and Carabin, 2008). However, *C. nardus* oil also has been traditionally used in Thai medicine to repel mosquitoes in rural of Thailand and this oil produces the most used natural repellents in the world (Trongtokit et al., 2005). Citronella oil mosquito repellency has also been verified by Kim et al. (2005), including effectiveness in repelling *A. aegypti*, but requires reapplication after 30 to 60 min. Research also indicates that citronella oil is an effective repellent for body louse, head louse and stable flies (Baldacchino et al., 2013; Mumcuoglu et al., 1996, 2004).

Fortunately, all herbal essential oil repellents provide higher repellency activity than IR3535. Moreover, allergic reaction in this study clearly indicated that all herbal essential oil repellents did not show any allergic effects such as headache, itching, breathing difficulty, skin irritation and hot sensation rashes. While, IR3535 repellent maybe unsafe for children, the physiological mechanism of IR3535 action is a neuroexcitatory effect (Faulde et al., 2010). Therefore, essential oils from three species of Thai herbs in this study can be considered for their use in green repellent or friendly repellents in mosquito control instead of chemical repellent that could reduce mosquito resistance, safe repellents for humans and friendly to the environment.

Mosquito repellent is one of the success methods in controlling mosquito transmitted diseases. While, chemical repellents are not safe for human and unfriendly to the environment. All essential oils in this study exhibited high repellent activity against two mosquito vectors (*A. aegypti* and *C. quiquefasciatus*) and safe for human skin, friendly to the environment. Thus, three essential oils from Thai herbs showed high potential for use as environmental friendly repellent against mosquito vectors.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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REFERENCES

Baldacchino F, Tramut C, Salem A, Lienard E, Deletre E, Franc M, Martin T, Duvallet G, Jay-Robert P (2013). The repellency of lemongrass oil against stable flies, tested using video tracking. Parasite 20: 21.

Barnard DR (2005). Biological assay methods for mosquito repellents. J. Am. Mosq. Control Assoc. 21: 12-16.

Barnard DR, Posey KH, Smith D, Schreck CE (1998). Mosquito density, biting rate and cage size effects on repellent tests. Med. Vet. Entomol. 12: 39-45.

Borah R, Kalita MC, Kar A, Talukdar AK (2010). Larvicidal efficacy of *Toddalia asiatica* (Linn.) Lam against two mosquito vectors *Aedes aegypti* and *Culex quinquefasciatus*. Afr. J. Biotechnol. 9: 2527-2530.

Burdock GA, Carabin IG (2008). Safety assessment of Ylang-Ylang (*Cananga* spp.) as a food ingredient. Food Chem. Toxicol. 46: 433-445.

Caballero-Gallardo K, Olivero-Verbel J, Stashenko EE (2011). Repellent activity of essential oils and some of their individual constituents against *Tribolium castaneum*. Herbst. J. Agric. Food Chem. 59: 1690-1696.

Das NG, Baruah I, Talukdar PK, Das SC (2003). Evaluation of botanicals as repellents against mosquitoes. J. Vector Borne. Dis. 40: 49-53.

Faulde MK, Abiez G, Nehring O (2010). Insecticidal, acaricidal and repellent effect of DEET and IR3535-impregnated bed nets using a novel long-lasting polymer-coating technique. Parasitol. Res. 106: 957-965.

Golenda CF, Solberg VB, Burge R, Gambel JM, Wirtz RA (1999). Gender-related efficacy difference to an extended duration formulation of topical N, N-diethyl-m-toluamide (DEET). Am. J. Trop. Med. Hyg. 60: 654-657.

Govindarajan M (2011). Larvicidal and repellent properties of some essential oils against *Culex tritaeniorhynchus* Giles and *Anopheles subpictus* Grassi (Diptera: Culicidae). Asian Pac. J. Trop. Med. 4: 106-111.

Katz T, Miller J, Hebert A (2008). Insect repellent: historical perspectives and new developments. J. Am. Acad. Dermatol. 58: 865-871.

Khandagle AJ, Tare VS, Raut KD, Morey RA (2011). Bioactivity of essential oils of *Zingiber officinalis* and *Achyranthes aspera* against mosquitoes. Parasitol. Res. 109: 339-343.

Kim J-K, Kang C-S, Lee J-K, Kim Y-R, Han H-Y, Yun HK (2005). Evaluation of repellency effect of two natural aroma mosquito repellent compounds, citronella and citronellal. Entomol. Res. 35:117-112.

Kumar S, Wahab N, Warikoo R (2011). Bioefficacy of *Mentha piperita* essential oil against dengue fever mosquito *Aedes aegypti* L. Asian Pac. J. Trop. Med. 1: 85-88.

Kumar SV, Mani P, John Bastin TMM, Ravikumar G (2012). Mosquito larvicidal, oviposition deterrent and repellent properties of *Acalypha indica* L. extracts against *Aedes aegypti*, *Anopheles stephensis* and *Culex quinquefasciatus*. Int. J. Med. Biosci. 1: 33-41.

Madhumathy AP, Aivazi AA, Vijayan VA (2007). Larvicidal efficacy of *Capsicum annum* against *Anopheles stephensi* and *Culex quinquefasciatus*. J. Vector Borne. Dis. 44: 223-226.

Manimaran A, Cruz MMJJ, Muthu C, Vincent S, Ignacimuthu S (2013). Repellent activity of plant essential oils formulation against three diseases causing mosquito vectors. J. Agric. Technol. 9(4): 845-854.

Mumcuoglu K, Galun R, Bach U, Miller J, Magdassi S (1996). Repellency of essential oils and their components to the human body louse, *Pediculus humanus humanus*. Entomol. Exp. Appl. 78: 309-314.

Mumcuoglu KY, Magdassi S, Miller J, Ben-Ishai F, Zentner G, Helbin V, Friger M, Kahana F, Ingber A (2004). Repellency of citronella for head lice: double-blind randomized trial of efficacy and safety. Isr. Med. Assoc. J. 6: 756-759.

Nerio LS, Olivero-Verbel J, Stashenko EE (2009). Repellent activity of essential oils from seven aromatic plants grown in Colombia against *Sitophilus zeamais* Motschulsky (Coleoptera). J. Stored Prod. Res. 45: 212-214.

Pavela R (2008). Insecticidal properties of several essential oils on the house fly (*Musca domestica* L.). Phytother. Res. 22: 274-278.

Phasomkusolsil S, Soonwera M (2012). The effects of herbal essential oils on the oviposition deterrent and ovicidal activities of *Aedes aegypti* (Linn.), *Anopheles dirus* (Peyton and Harrison) and *Culex quinquefasciatus* (Say). Trop. Biomed. 29: 138-150.

Service MW (1980). Effects of wind on the behaviour and distribution of mosquitoes and blackflies. Int. J. Biometeorol. 24: 359-360.

Sinthusiri J, Soonwera M (2013). Efficacy of herbal essential oils as insecticides against the housefly, *Musca domestica* L. Southeast Asian J. Trop. Med. Public Health 44: 188-196.

Sritabutra D, Soonwera M (2013). Repellent activity of herbal essential oils against *Aedes aegypti* (Linn.) and *Culex quinquefasciatus* (Say). Asian Pac. J. Trop. Dis. 3: 271-276.

Stuart AE, Brooks CJ, Prescott RJ, Blackwell A (2000). Repellent and antifeedant activity of salicylic acid and related compounds against the biting midge, *Culicoides impunctatus* (Diptera: Ceratopogonidae). J. Med. Entomol. 37: 222-227.

Tawatsin A, Wratten SD, Scott RR, Thavara U, Techadamrongsin Y. (2001). Repellency of volatile oils from plants against three mosquito vectors. J. Vector Ecol. 26: 76-82.

Trongtokit Y, Rongsriyan Y, Komalamisra N, Apiwathnasorn C (2005). Comparative repellency of 38 essential oils against mosquito bites. Phytother. Res. 19: 303-309.

WHO (2009a). Dengue: guidelines for diagnosis, treatment, prevention, and control. Geneva.

WHO (2009b). Guidelines for efficacy testing of mosquito repellents for human skins. WHO/HTM/NTD/WHOPES/2009.4.

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Full Length Research Paper

Multiple antibiotic-resistance of *Enterococcus* isolated from coastal water near an outfall in Brazil

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Marine outfalls contribute to the environmental protection of coastal zones. However, these structures may serve as vehicles for microbiological contamination. This study aimed to investigate the occurrence of antimicrobial-resistant bacteria in water samples collected from 67 stations located in nearby areas of the ocean outfall in Fortaleza, Brazil. 81 *Enterococcus* strains were isolated, identified and distributed in the following groups of species: *Enterococcus faecalis* (n = 37; 45.7%), *Enterococcus faecium* (n = 30; 37%), *Enterococcus mundtii* (n = 9; 11.1%), *Enterococcus raffinosus* (n = 2; 2.5%), *Enterococcus dispar* (n = 2; 2.5%) and *Enterococcus durans* (n = 1; 1.2%). Antimicrobial resistance was observed in 47 (58%) of the strains, and the most predominant profile was the concurrent resistance to ampicillin, clindamycin, penicillin and vancomycin. In 31 strains were detected phenotypically, plasmid resistance factors. The data reported in this study should serve as an alert to public health authorities, since they suggest that the area near the submarine outfall in Fortaleza may contribute to antimicrobial-resistant enterococci spread.

Key words: Enterococci, seawater, multidrug-resistant bacteria, public health.

INTRODUCTION

The coexistence of different interests in coastal areas, that is, the presence of highly populated cities, recreational areas and extensive shellfish farming (Scroccaro et al., 2010) justifies the construction of structures which contribute to mitigate the impact caused by adding residual water into the aquatic environment. Thus, marine outfalls must be considered part of an integrated environmental protection system for coastal zones (Mendonça et al., 2013). However, these structures need to be constantly monitored, once they serve as vehicle to pollution due to organic enrichment and microbiological contamination (Gubitoso et al., 2008).

Among the bacteria constantly associated to domestic sewage, those which belong to the *Enterococcus* genus

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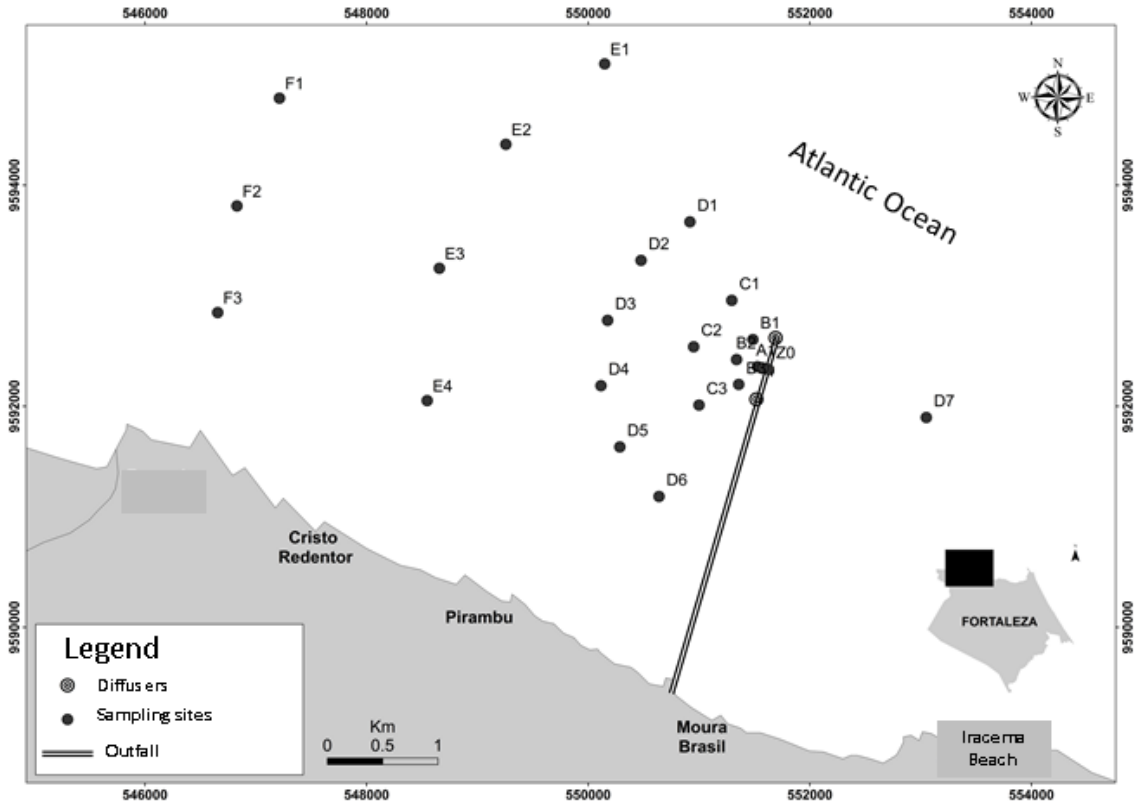

Figure 1. Water samples from multiple collection points around the marine outfall in Fortaleza, Ceara, Brazil.

are certainly noteworthy. These micro-organisms are ranked as excellent indicators for saline waters, since they have a higher survival time levels and greater resistance as compared to the *Escherichia coli* species and other thermal tolerant coliforms (Dufour, 1994). In addition, their ability to survive in extreme ranges of temperature, pH and salinity must be highlighted, as well as their resistance to detergent action, which prevents their removal from the environment (Hayes et al., 2003).

The presence of enterococci in recreational waters, sediments and beach sand affects the quality of these habitats and poses a risk to bathers' health (Erdem-Kimiran et al., 2007). Besides, beaches that are domestic waste recipients contribute to the spread of microorganisms that carry antibiotic-resistant genes (Iversen et al., 2004).

The main objective of this article was to identify strains of *Enterococcus* spp. in water samples collected near the ocean outfall in Fortaleza, Ceara, Brazil, determine the antimicrobial susceptibility profile, and investigate if the antimicrobial resistance was plasmid coded.

MATERIALS AND METHODS

Sampling sites

Water samples from 67 stations divided into surface (AS, BS, CS, DS, ES, FS), middle (AM, BM, CM, DM, EM, FM), bottom (AB, BB, CB, DB, EB, FB) and raw sewage (RS) were analyzed. All stations are located in a nearby area around the marine outfall in Fortaleza, Ceara, Brazil (Figure 1). Samples were collected in Van Dorn bottles and stored until bacteriological analysis in sterile amber glasses with 1 L capacity.

Isolation and identification of *Enterococcus*

Seawater samples were dilluted (1:9) in 1% saline solution and inoculated using Azide Broth (Difco), incubated at 35°C/48 h. After the incubation period, aliquots from positive (turbid) tubes in the Azide Broth were taken and plated on m-Enterococcus Agar (Difco), incubated at 35°C/48 h. Colonies with similar characteristics with the genus *Enterococcus* were isolated on Brain Heart Infusion Agar (BHI-Difco) and subjected to characterization by Gram staining. All isolates identified as Gram-positive cocci (n=81) were identified to the species level in accordance with Carvalho et al. (2004). The strains were maintained in BHI Agar (pH 7.5) at 18-20°C until antibiogram procedures.

Antibiogram

Antimicrobial susceptibility was verified by disk diffusion method using Müeller-Hinton agar (Difco) (CLSI, 2010). The following antimicrobials were tested: Ampicillin (Amp 10 µg), Penicillin (Pen 10 U.I), Gentamicin (Gen 10 µg), Streptomycin (Str 10 µg), Tetracycline (Tcy 30 µg), Chloramphenicol (Chl 30 µg), Clindamycin (Cli 2 µg) and Vancomycin (Van 30 µg). For this procedure, the cell concentration for all strains was adjusted to a turbidity level similar

Table 1. Distribution of *Enterococcus* species in sampling sites from the marine outfall (Fortaleza, Brazil).

Specie	Number	Sampling site (number of isolates)
E. faecalis	37	RS (n=22), A1S (n=2), A1B (n=2), B2M (n=2), B2B (n=2), A1M (n=1), B1S (n=1), B3S (n=1), C3B (n=1), C4B (n=1), D2M, F3S (n=1)
E. faecium	30	RS (n=7), F3S (n=3), A1S (n=2), A1M (n=2), B1S (n=2), B2S (n=2), B2M (n=2), C2M (n=2), D2M (n=2), F3M (n=2), A1B (n=1), B2B (n=1), C1M (n=1), C2B (n=1)
E. mundtti	9	B1M (n=3), RS (n=1), A1B (n=1), B1S (n=1), B3S (n=1), F2B (n=1), F1S (n=1)
E. raffinosus	2	B1M (n=1), B2S (n=1)
E. dispar	2	A1M (n=1), D2M (n=1)
E. durans	1	C2M (n=1)

*RS: Raw sewage. S: surface. M: middle. B: bottom.

Table 2. Number of antibiotic-resistant *Enterococcus* strains isolated from water samples collected in the nearby area around the marine outfall (Fortaleza, Brazil).

Specie	Number	Resistance to							
		Cli	Van	Pen	Tcy	Str	Amp	Gen	Chl
E. faecalis	37	22	14	13	12	12	11	12	6
E. faecium	30	16	9	8	7	7	7	5	2
E. mundtti	9	5	2	1	3	3	1	-	-
E. raffinosus	2	2	2	1	-	-	1	-	1
E. dispar	2	-	-	-	-	-	-	-	-
E. durans	1	-	-	-	-	-	-	-	-
Total	81	45	27	23	22	22	20	17	9

* Cli: Clindamycin 2 µg; Van: Vancomycin 30 µg; Pen: Penicillin 10 U.I; Tcy: Tetracycline (Tcy 30 µg); Str: Streptomycin 10µg; Amp: Ampicillin 10 µg; Gen: Gentamicin 10 µg; Chl: Chloramphenicol 30 µg.

to MacFarland 0.5 scale. The inoculation procedure for plates containing Müeller-Hinton medium was made using swabs, followed by application of the antimicrobial discs. All plates were incubated at 35°C for 24 h. Zones of inhibition were measured using a digital caliper (Digimess) and each strain behavior was classified as sensitive, intermediate or resistant, according to CLSI (2010) recommendations. The *Enterococcus faecalis* ATCC 29212 strain was used as a control. Multiple antibiotics resistance (MAR) index was determined according to Krumperman (1983).

Plasmid curing

Multiple resistant strains were selected and submitted to plasmid curing according to Molina-Aja et al. (2002) with modifications. We use Luria-Bertani broth (LB), supplemented with 0.85% NaCl and acridine orange at 50 µg/mL. Strains grown under constant shaking in LB medium for 24 h at 30°C were once again subjected to antibiotic susceptibility testing (described above) against the antimicrobials to which they were resistant. Resistance was classified as plasmid dependent when affected by plasmid curing.

RESULTS

The identification of 81 isolates from 20 sampling sites revealed the presence of six species: *Enterococcus faecalis* (n = 37; 45.7%), *E. faecium* (n = 30; 37%), *Enterococcus mundtii* (n = 9; 11.1%) (Table 1).

The antimicrobial susceptibility profile test showed that 53 (65.4%) strains were resistant to at least one antimicrobial, and the number of clindamycin-resistant isolates was high and should be highlighted (n = 45, 55.5%) (Table 2).

Twenty four multi-resistant profiles were detected from a total of 47 (58%) strains, the most predominant being Amp+Cli+Pen+Van (n=8) (Table 3). The species with the highest number of isolates with multiple-resistant profiles was *E. faecalis* (n=26), followed by *E. faecium* (n=13), *Enterococcus mundtii* (n=6), *Enterococcus raffinosus* (n=1) and *Enterococcus dispar* (n=1). MAR levels ranged from 0.25 to 0.87 (Table 3)

In 31 strains were detected phenotypically, plasmid resistance factors. Resistance to at least two of the following antimicrobials was verified in 27 (57.4%) of the multiple-resistant strains: Cli, Van, Pen, Chl, Str, Tet, Amp and Gen (Table 4).

DISCUSSION

In Fortaleza, there is a large number of Enterococci in the vicinity of outfalls and nearby areas. In this study, the

Table 3. Multiple resistance profiles to antimicrobials by *Enterococcus* strains isolated from water samples collected in the nearby area around the marine outfall (Fortaleza, Brazil).

Profile	n	*E. faecalis* n=26	*E. faecium* n=13	*E. mundtti* n=6	*E. raffinosus* n=1	*E. dispar* n=1	MAR
Amp+Cli+Pen+Van	8	3	3	2			0.5
Amp+Cli+Str+Pen+Tcy+Van	3	1		1	1		0.75
Amp+Cli+Chl+Str+Pen+Tcy+Van	3	2	1				0.87
Amp+Cli+Chl+Str+Pen+Van	2	1	1				0.75
Cli+Str+Gen+Tcy	3	2	1				0.5
Cli+Str+Gen+Tcy	3	2	1				0.5
Cli+Str+Gen	3	2	1				0.37
Cli+Est+Tet	3	1	1	1			0.37
Cli+Van	3	1	1	1			0.25
Amp+Cli+Gen+Pen+Tcy+Van	2	2					0.75
Cli+Str	2	2					0.25
Cli+Gen	2	1	1				0.25
Cli+Tcy	2	1	1				0.25
Amp+Cli+Str+Gen+Pen+Tcy+Van	1	1					0.87
Amp+Cli+Str+Pen+Van	1	1					0.62
Cli+Gen+Pen+Tcy+Van	1	1					0.62
Cli+Chl+Gen+Tcy	1	1					0.5
Cli+Gen+Tcy	1	1					0.37
Cli+Chl+Van	1					1	0.37
Cli+Pen+Van	1		1				0.37
Str+Gen+Tcy	1		1				0.37
Chl+Tcy	1	1					0.5
Pen+Van	1	1					0.5
Chl+Gen	1			1			0.5

*n: number of strains. Amp: Ampicillin 10 µg; Cli: Clindamycin 2 µg; Pen: Penicillin 10 U.I; Van: Vancomycin 30 µg; Str: Streptomycin 10µg; Tcy: Tetracycline (Tcy 30 µg); Chl: Chloramphenicol 30 µg; Gen: Gentamicin 10 µg. MAR: Multiple Antibiotic Resistance index.

species *E. faecalis* and *E. faecium* were the most frequently isolated. This result is similar to those obtained by Graves and Weaver (2010), who reported a diversity of ten species of *Enterococcus* (*E. faecalis* - 30.6%; *Enterococcus pseudoavium* - 24%, *Enterococcus casseliflavus* - 12.8%; *E. faecium* - 11.2%, *E. mundtii* 7.9%, *Enterococcus gallinarum* - 6.2%; *E. dispar* - 3.7%; *Enterococcus hirae* - 2.1%, *Enterococcus durans* – 0.8% and *Enterococcus flavescens* - 0.8%) in water samples in a wetland close to College Station, Texas. According to the authors, the distribution of these species depends on environmental factors and specific points and sources of the sewer system. This statement was confirmed in this study, since 30 (37%) strains were isolated from the station corresponding to the raw sewage (RS) and only 8 (9.9%) were derived from more distant stations (F1S, FSB, F3S and F3M) from the RS.

The relationship between water quality and sewage discarding via submarine outfall has been discussed. In Tijuana-Mexico, Gersberg et al. (2008) makes statistical comparisons of the bacterial water quality - including enterococci densities of the ocean, both before and after discharge of sewage to the South Bay Ocean Outfall (SBOO). The authors observed that only four (of the 11 total) showed significant improvement, that is, decreased frequency of incidence of bacterial indicator thresholds, after SBOO discharge began.

Regarding antimicrobial resistance data, values similar to those presented in this study were reported by Oliveira and Piñata (2008) when they evaluated the antimicrobial drug resistance of 160 strains of *Enterococcus* from two recreational beaches in São Paulo, Brazil. The authors found a 51.9% rate of antimicrobial resistance, and showed the problem of the discharge of domestic sewage on beaches.

In the present study, the highest percentages of resistance were observed for Cli, Van, Pen, Tcy and Str (Table 2). Moore et al. (2010), researching antimicrobial resistance in bacteria in the waters of rivers and streams in Northern Ireland, observed Cli, Van, Pen and Tcy

Table 4. Multiple resistance profiles of *Enterococcus* strains isolated from water samples collected in the nearby area around the marine outfall (Fortaleza, Brazil) before and after plasmid curing.

Collection station	Antimicrobial resistance profiles			
	Before plasmids curing	**n**	**After plasmids curing**	**n**
AS	Cli+Van	1	-	1
AM	Cli+Chl+Van	1	Cli	1
AB	Cli+Str+Tcy	1	Cli+Str+Tcy	1
BS	Cli+Van	1	Cli	1
BM	Cli+Van	1	Cli	1
BM	Cli+Pen+Van	1	Cli	1
BB	Gen+Van	1	Gen	1
DM	Tcy+Chl	1	Tcy	1
FS	Tcy+Cli	1	Amp+Cli+Pen+Van	1
FS	Tcy+Cli	1	Amp+Cli+Pen+Van	1
FS	Amp+Cli+Pen+Van	1	Amp+Cli+Pen+Van	1
FB	Amp+Cli+Pen+Van	1	Tcy+Cli	1
FB	Amp+Cli+Pen+Van	1	Tcy+Cli	1
FB	Amp+Cli+Pen+Van	1	Amp+Cli+Pen+Van	1
FM	Amp+Cli+Pen+Van	1	Amp+Cli+Pen+Van	1
FM	Amp+Cli+Pen+Van	1	Amp+Cli+Pen+Van	1
	Cli+Gen	2	Cli	6
	Cli+Str	2	Van	1
	Pen+Van	1	Cli+Tcy	2
	Cli+Str+Gen	3	Cli+Str	1
	Str+Gen+Tcy	2	Cli+Str+Tcy	3
	Str+Tcy+Van	1	Cli+Pen+Van	2
	Cli+Str+Tcy	1	Str+Gen+Tcy	2
	Cli+Str+Gen+Tcy	3	Cli+Str+Gen	1
RS	Amp+Cli+Pen+Van	2	Amp+Cli+Pen+Van	8
	Cli+Chl+Gen+Tcy	1	Amp+Cli+Pen+Tcy+Van	2
	Amp+Cli+Str+Pen+Van	1		
	Cli+Gen+Pen+Tcy+Van	1		
	Amp+Cli+Str+Pen+Tcy+Van	3		
	Amp+Cli+Gen+Pen+Tcy+Van	2		
	Amp+Chl+Cli+Str+Pen+Van	1		
	Amp+Cli+Chl+Str+Pen+Van	1		
	Amp+Cli+Chl+Str+Pen+Tcy+Van	3		
	Amp+Cli+Str+Gen+Pen+Tcy+Van	1		

*S: Surface. M: Middle. B: Bottom. RS: Raw Sewage. n: number of strains. Cli: Clindamycin 2 µg; Van: Vancomycin 30 µg; Chl: Chloramphenicol 30 µg; Str: Streptomycin 10 µg; Tcy: Tetracycline (Tcy 30 µg); Pen: Penicillin 10 U.I; Gen: Gentamicin 10 µg; Amp: Ampicillin 10 µg.

resistant profiles. According to these authors, the resistance profile expressed by environmental strains allows the identification of the type of polluting source that the ecosystem has been receiving. Mudryk et al. (2010) showed a 27% resistance to Cli in beach and sediment isolates from the National Park at the South Coast of the Baltic Sea, a lower percentage than the one detected in this study. Cli belongs to the family of Lincosamides, and according to Moellering (1991) resistance to such antimicrobial is common in enterococci, which may be considered a case of intrinsic resistance, with interspecies associations. For Lüthje and Schwarz (2007), bacterial resistance to lincosamide is related to efflux mechanisms and mutations.

The susceptibility of enterococcal isolates to clindamycin was reported by Schmitz et al. (1999) - only 4.4 of all *E. faecalis* strains from 20 European university hospitals were clindamycin-susceptible. Thus, these authors call attention to the fact that lincosamides cannot be considered as a therapeutic option for enterococcal infec-

tions.

Vancomycin was the second antimicrobial agent to which the strains showed a higher resistance (34%). Iversen et al. (2002) observed that 60% of the residual water samples from four sewage treatment plants in Sweden, 36% of hospital sewage effluent and 19% of those of treated sewage had vancomycin-resistant strains. Similarly, Talebi et al. (2007) detected resistance to Van in 19% of *Enterococcus* strains isolated in three sewage treatment plants in Iran. For Whitman et al. (2003), the presence of resistant enterococci, including Vancomycin Resistant *Enterococcus*- VRE, in marine ecosystems and hospital waste is an indication of fecal contamination. According to the Centers for Disease Control and Prevention- CDC (2002), resistance to this drug is relatively recent, occurring primarily by the production of peptidoglycan precursors in the cell wall associated with vancomycin, preventing its action in blocking cell wall synthesis (Lai et al., 1998).

The high levels of multidrug resistance (n=47; 58%) (Table 3) in the present study should be highlighted. These results are similar to the ones of Costa et al. (2006), who found multidrug resistant characteristics in 46% of *Enterococcus* isolates derived from 14 treatment plants in Portugal. In addition, Arvanitidou et al. (2001) found multidrug resistance values in 33 (5%) of *Enterococcus* isolates from coastal waters in northwestern Greece, and 20 distinct multidrug resistance profiles. For Xu et al. (2007), selective pressure experienced by bacteria force them to adapt quickly, especially by horizontal gene transfer, and their resistance to several antimicrobials is now widely recognized.

Hayes et al. (2004) found similar results with the ones in this study, distributed as follows: Cli+Str+Tcy and Cli+Str+Pen+Tcy. Similar to the results presented here, the species with the most frequent resistance phenotype were: *E. faecalis* (53.2%) and *E faecium* (31.4%). These statements suggest that enterococci isolated from the marine environment may become multi-resistant to antibiotics used in human medicine.

MAR index obtained in this study (Table 3) indicate that multi resistance to drugs was predominant in all isolates. Similar data was verified by Son et al. (1999), who reported MAR rates ranging from 0.2 to 0.9.

Another finding worth mentioning is the frequency of isolates (37%) with plasmid-mediated resistance (Table 4). From 31 strains with plasmid resistance expression (phenotypic detection), 24 were derived from the station corresponding to the raw sewage (RS). This suggests that environments polluted by sewage, that is, rich in organic matter, may be vehicles of bacteria carrying antibiotic-resistant plasmids. For McBride et al. (2007), the presence of this type of mobile genetic element is common in enterococci, as they make up a substantial fraction of their genome, and responsible for much of the horizontal gene transfer. The same authors found seven resistance profiles related to plasmids in 88 *E. faecalis* isolates.

It is noted that the isolates from the same sampling site showed different profiles before and after the curing (Table 4). According to Boehm et al. (2002), the transportation of fecal bacteria in the sewage to the surf zone depends on the following conditions: oceanographic (stratification of the water column), wave, tidal range and currents. Thus, this contamination in marine waters might contribute to the establishment of resistance routes for environmental bacteria (Meirelles-Pereira et al. 2002).

Enterococci are prone to acquiring resistance to antibiotics, either by mutation or by horizontal transfer of mobile genetic elements (plasmids and transposons) (Hasmann et al., 2005). The reasons for the high number of plasmids in the resistance levels among several species of *Enterococcus* are still unknown (Jensen et al., 2010). Therefore plasmid detection in environmental bacteria may represent a tool to compare a large number of aquatic environments, promoting a better understanding of their development and ecology (Meireles-Pereira et al., 2002).

The data reported in this study should serve as an alert to public health authorities, since results suggest that the area near the submarine outfall in Fortaleza may contribute to antimicrobial-resistant enterococci spread. Furthermore, it is worth noting, the relationship between the content of the sewages disposal thrown in marine waters and the occurrence of plasmid-carrying, antibiotic-resistant *Enterecoccus* in the same environment.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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REFERENCES

Arvanitidou M, Katsouyannopoulos V, Tsakris A (2001). Antibiotic resistance patterns of enterococci isolated from coastal bathing waters. J. Med. Microbiol. 50: 1001-1005.

Boehm AB, Grant SB, Kim JH, Mowbray SL, McGee CD, Clark CD, Foley DM, Wellman DE (2002). Decadal and shorter period variability of surf zone water quality at Huntington Beach, California. Environ. Sci. Technol. 36: 3885–3892.

Carvalho G, Steigerwalt G, Morey E, Shewmaker L, Teixeira M, Facklam R (2004). Characterization of three new enterococcal species, *Enterococcus* sp. nov. CDC PNS-E1, *Enterococcus* sp. nov. CDC PNS-E2, and *Enterococcus* sp. nov. CDC PNS-E3, isolated from human clinical specimens. J. Clin. Microbiol. 42: 1192–119.

CDC-Centers for Disease Control and Prevention (2002). *Staphylococcus aureus* resistant to vancomycin - United States 51: 565-567.

CLSI-Clinical and Laboratory Standards Institute/NCCLS (2010). Performance standards for antimicrobial susceptibility testing: 17th

ed. CLSI/NCCLS M100-S17. Clinical and Laboratory Standards Institute, Wayne, PA.
Dufour AP (1994). Bacterial indicators of recreational water quality. Can. J. Public Health 75: 49-56.
Erdem-Kimiran A, Arslan EO, Yurudu NOS, Zeybek Z, Dogruoz N, Cotuk A (2007). Isolation and identification of enterococci from seawater samples: Assessment of their resistance to antibiotics and heavy metals. Environ. Monit. Assess. 125: 219–228.
Gersberg R, Tiedge J, Gottstein D, Altmann S, Watanabe K, Lüderitz, V (2008). Effect of the South Bay Ocean Outfall (SBOO) on ocean beach water quality near the USA-Mexico border. Int. J. Environ. Health Res. 18: 149-158.
Graves AK, Weaver, RW (2010). Characterization of Enterococci populations collected from a subsurface flow constructed wetland. J. Appl. Microbiol. 108: 1226-1234.
Gubitoso S, Duleba W, Teodoro AC, Prada SM, Rocha MM, Lamparelli CC, Bevilacqua JE, Moura DO (2008). Estudo geoambiental da região circunjacente ao emissário submarino de esgoto do Araçá, São Sebastião, SP. Rev. Bras. Geocienc. 38: 467-475.
Hasmann H, Villadsen AG, Aarestrup FM (2005). Diversity and stability of plasmids from glycopeptide resistant *Enterococcus faecium* (GRE) isolated from pigs in Denmark. Microb. Drug Resist. 11: 178-84.
Hayes JR, English LL, Carr LE, Wagner, DD, Joseph SW (2004). Multiple-Antibiotic Resistance of *Enterococcus* spp. isolated from commercial poultry production environments. Appl. Environ. Microbiol. 70: 6005–6011.
Hayes JR, English LL, Carter PJ, Proescholdt T, Lee KY, Wagner DD, White DG (2003). Prevalence and antimicrobial resistance of *Enterococcus* species isolated from retail meats. Appl. Environ. Microbiol. 69: 7153-7160.
Iversen A, Kühn I, Franklin A, Roland M (2002). High Prevalence of Vancomycin-Resistant Enterococci in Swedish Sewage. Appl. Environ. Microbiol. 68: 2838–2842.
Iversen A, Kühn I, Rahman M, Franklin A, Burman LG, Olsson-Lijequist T (2004). Evidence for a transmission between humans and the environment of nosocomial strain of *Enterococcus faecium*. Environ. Microbiol. 6: 55-59.
Jensen LB, Garcia-Migura L, Valenzuela AJ, Løhr M, Hasman H, Aarestrup FM (2010). A classification system for plasmids from enterococci and other Gram-positive bacteria. J. Microbiol. Methods 80: 25-43.
Krumperman PN (1983). Multiple antibiotic indexing of *Escherichia coli* to identify high risk sources of fecal contamination of foods. Appl. Environ. Microbiol. 46: 165-170.
Lai KK, Kelley AL, Melvin ZS, Belliveau PP, Fontecchio SA (1998). Failure to eradicate Vancomycin resistant enterococci in a University hospital and the cost of barrier precautions. Infect. Control Hosp. Epidemiol. 19: 647-652.
Lüthje P, Schwarz S (2007). Molecular basis of resistance to macrolides and lincosamides among staphylococcus and streptococci from various animal sources collected in the resistance monitoring program BfT-GermanVet. Int. J. Antimicrob. Agents 29: 528–535.
McBride SM, Fischetti VA, Leblanc DJ, Moellering RC Jr, Gilmore MS (2007). Genetic diversity among *Enterococcus faecalis.* PLoS One 2(7): e582.
Meirelles-Pereira F, Pereira AMS, Silva MCG, Gonçalves VD, Brum PR, Castro EAR, Pereira AA, Esteves FA, Pereira JAA (2002). Ecological aspects of the antimicrobial resistance in bacteria of importance to human infections. Braz. J. Microbiol. 33: 287-293.
Mendonça A, Losada MA, Reis MT, Neves MG (2013). Risk assessment in submarine outfall projects: The case of Portugal. J. Environ. Manage. 116: 186-195.
Moellering RC Jr (1991). The Garrod Lecture. The *Enterococcus*: a classic example of the impact of antimicrobial resistance on therapeutic options. J. Antimicrob. Chemother. 28: 1–12.
Molina-Aja A, Garcia-Gasca A, Abreu-Grobois A, Mejía-Bolán C, Roque A, Gomez-Gil B. (2002). Plasmid profiling and antibiotic resistance of *Vibrio* strains isolated from cultured penaeid shrimp. FEMS Microbiol. Lett. 213: 7-12.
Moore JE, Rao JR, Moore PJA, Millar BCCE, Goldsmith AL, Rooney PJ (2010). Determination of total antibiotic resistance in waterborne bacteria in rivers and streams in Northern Ireland: Can antibiotic-resistant bacteria be an indicator of ecological change? Aquat. Ecol. 44: 349–358.
Mudryk Z, Perlinski P, Skórczewski P (2010). Detection of antibiotic resistant bacteria inhabiting the sandof non-recreational marine beach. Mar. Pollut. Bull. 60: 207–214.
Oliveira AJFC, Pinhata JMW (2008). Antimicrobial resistance and species composition of *Enterococcus* spp. isolated from waters and sands of marinen recreational beaches in Southeastern Brazil. Water Res. 42: 2242 – 2250.
Schmitz FJ, Verhoef J, Fluit Ad C, The Sentry Participants Group (1999). Prevalence of resistance to MLS antibiotics in 20 European university hospitals participating in the European SENTRY surveillance programme. J. Antimicrob. Chemother. 43: 783-792.
Scroccaro I, Ostoich M, Umgiesser G, De Pascalis F, Colugnati L, Mattassi G, Vazzoler M, Cuomo M (2010). Submarine wastewater discharges: dispersion modelling in the Northern Adriatic Sea. Environ. Sci. Pollut. Res. Int. 17: 844-855.
Son R, Nimita F, Rusul G, Nasreldin E, Samuel L, Nishibuchi M (1999). Isolation and molecular characterization of vancomycinresistant *Enterococcus faecium* in Malaysia. Lett. Appl. Microbiol. 29: 118–122.
Whitman RL, Shively DA, Nevers HPMB, Byappanahalli MN (2003). Occurrence of *Escherichia coli* and enterococci in *Cladophora* (*Chlorophyta*) in nearshore water and beach sand of Lake Michigan. Appl. Environ. Microbiol. 69: 4714-4719.
Xu J, Gallert C, Winter J (2007). Multiple antibiotic resistances of *Enterococcus* isolates from raw or sand-filtered sewage. Appl. Microbiol. Biotechnol. 74: 493–500.

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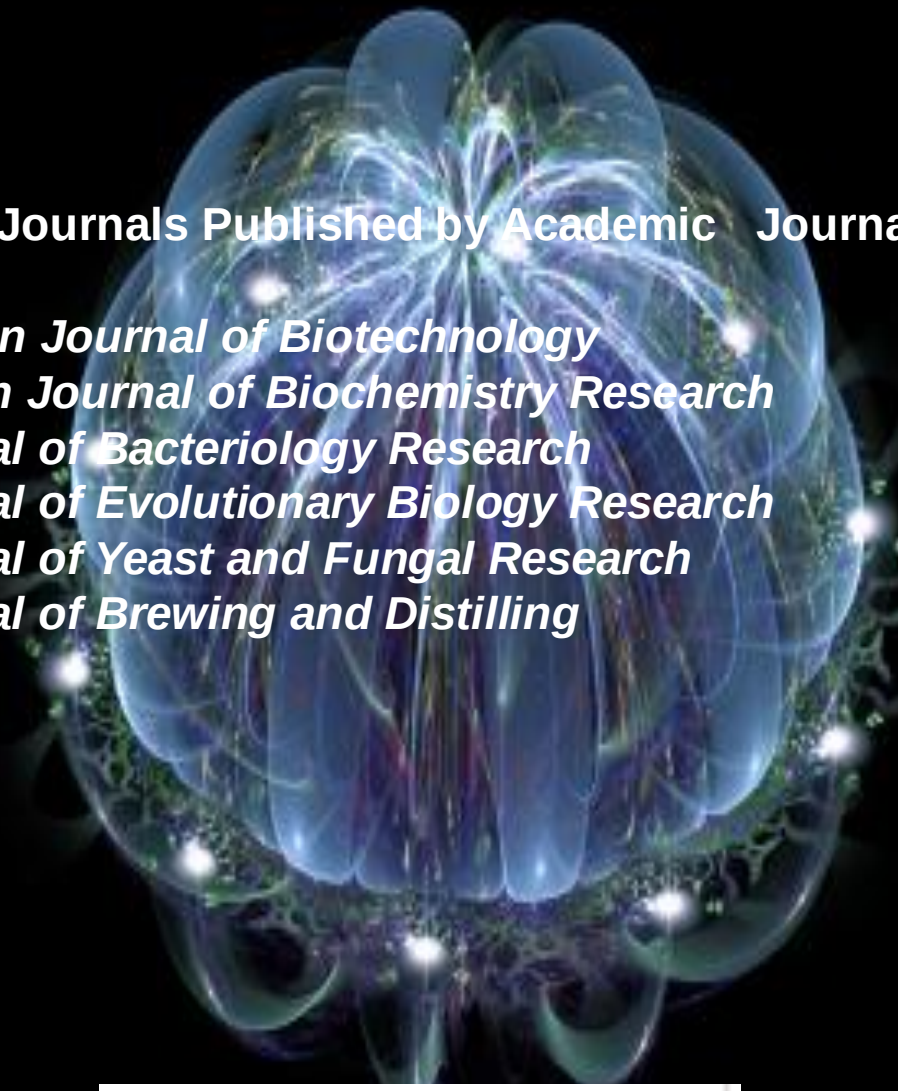

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