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Full Length Research Paper

Developmental biology and infection cycle of *Sclerotinia sclerotiorum* causing stem rot of carnation in India

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Carnation (*Dianthus caryophyllus* L.) is a cut flower with greater stipulation in the world cut flower market. In India, carnations are cultivated under polyhouses in Nilgiris and Kodaikanal districts in the state of Tamil Nadu. Carnations cultivation is impeded by various diseases, among them stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary was found to be predominant in all varieties. Survey among commercially cultivated varieties of carnation during 2013, revealed the occurrence of stem rot incited by *Sclerotinia sclerotiorum*, for the first time in India. The pathogen was identified as *Sclerotinia sclerotiorum* on the basis of phenotypic and genotypic characteristics. Carpogenic germination was induced artificially, and life cycle of the fungi was studied. Microscopic studies of the apothecium revealed the presence of spermatia, croziers, paraphyses as well as monomorphic ascospores specific to *S. sclerotiorum*.

Key words: Apothecium, carnation, croziers, India, *Sclerotinia,* spermatia.

INTRODUCTION

Floriculture is a persuasive field in trade with high potential returns per unit area. Because of this large number of farmers are attracted towards cut flower cultivation. In India, conditions prevailing in hilly regions are highly favorable for the cultivation of various cut flowers. Area under cut flower production is increasing constantly (Indian Horticulture Database, 2015). Carnations are one of the most preferred cut flower varieties next to rose owing to their shelf life and greater degree of available colours. Karnataka is the leading state in India accounting for carnation production. Total production of carnation all over India is 800 MT (Indian Horticulture Database, 2015). Even though carnation cultivation is increasing, it's development is hampered by various diseases. Among them, stem rot incited by *S. sclerotiorum* was found to be highly destructive. *Sclerotinia* has been previously reported as a polyphagous fungi infecting more than 148 plant species

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(Saharan and Mehta, 2008). *Sclerotinia* is a facultative parasitic fungus belonging to *Ascomycota* (Kirk et al., 2008). It is a robust pathogen having greater rate of survival even under adverse environmental conditions and survives up to eight years in soil as sclerotial bodies (Adams and Ayers, 1979). Yield losses have been reported to be hundred per cent in susceptible crops (Purdy, 1979). Three well documented species of *Sclerotinia* (*S. sclerotiorum*, *S. minor* and *S. trifoliorum*) are considered to be much important and likely to have wide host range and reported with huge economic loss (Saharan and Mehta, 2008). In India, stem rot of carnation was identified for the first time in 2013. (Vinod Kumar et al., 2015). The results of our study on survey, morphological, molecular characterization and apothecium induction in *S. sclerotiorum* are presented in this communication.

MATERIALS AND METHODS

Survey

Commercially cultivated carnation varieties like Charmant pink, Pudding yellow, Castor purple, Baltico white, White liberty, Golem purple, Yellow liberty, Bizet, Gaudina red and Farida were surveyed for the occurrence of stem rot in kothagiri, kodumudi, kunnur regions of Nilgiris district in Tamil Nadu, India. The disease incidence was calculated by using the formula proposed by Wheeler (1969).

$$\text{Percent Disease Incidence} = \frac{\text{No .of infected plants}}{\text{Total No .of plants}} \times 100$$

Isolation

The pathogen was isolated from both infected stem tissue and sclerotial bodies. Infected stem tissues and sclerotial bodies were surface sterlized with 0.1% mercuric chloride ($HgCl_2$) for 60 s and then the chemical traces were removed by rinsing thrice in sterile distilled water. Surface sterlized tissues and sclerotia were plated on sterile Petri plates containing potato dextrose agar (PDA) medium, amended with 100 µg/ml of streptomycin sulphate and incubated at 20 ± 2°C for 7 days. After emergence of fungal growth, the pathogen was pure cultured by single hyphal tip technique (Tutte, 1969).

Pathogenicity

Pathogenicity experiments were conducted on 30 days old potted cuttings of carnation variety, Charmant pink as per method described by Kim and Cho (2003). The cuttings were grown in pots with sterilized pot mixture. Mycelial discs and sclerotial bodies of the pathogen were inoculated to cuttings separately. Mycelial discs (9 mm) were placed in stem portion, 2 cm above ground and covered with moist cotton. Sclerotial bodies (3-5) covered in muslin cloth were buried in soil in proximity with the collar region of the healthy cuttings. Both the tests were replicated thrice with three cuttings per replication. After inoculation the pots were covered with polybags and incubated, ambient inside the polyhouse maintained at Elkhil Agrotech Pvt Ltd at Udhagamandalam. Healthy control was also maintained subsequently. After expression of symptoms the pathogen was re- isolated to confirm pathogenicity.

Identification

The pathogen was subjected to both morphological and molecular characterization. The study fungus was compared with well documented species of *Sclerotinia viz*., *S. sclerotiorum*, *S. minor* and *S. trifoliorum.* Morphological discrimination was based on ascospore morphology and size of sclerotia. Molecular identification was carried out by sequencing the 18S-28S rRNA gene.

Morphological characterization

Species differentiation was possibly made between them by studying the colony character, sclerotia and ascospore morphology (Ekins et al., 2005). Apothecium was induced as per the procedure proposed by Cobb and Dillard (2004).

Induction of apothecium

Before induction of apothecium the sclerotial bodies were subjected for a conditioning process. Medium to large sclerotial bodies (7.4 to 15.5 mm) were selected for better per cent rate of carpogenic germination. Sclerotial bodies were tied in cheese cloth bags and immersed in a container filled with tap water and incubated at 6^0 C. Fresh air was continuously circulated by using an aquarium pump. In this conditioning process, water was replenished once in a week till stipe initiation.

After conditioning process, sclerotia with stipe initials were transferred to Petri plates containing clean, sterilized, dry sand. Sand was moistened and plates were overlaid with lids in order to prevent desiccation. Moisture was maintained with sterile distilled water. Plates were incubated at 20°C in alternate dark and light periods with Bright Boost cool white fluorescent light-18W/840 (Phillips, China make), till apothecia were produced. The conditioning treatment was replicated thrice and a control was also maintained. Apothecium was subjected for ultramicrotome, and observed under microscope to study the ascus and ascospore morphology.

Microtome sectioning

In order to study the ultra-structures, apotheium was subjected to ultra microtome studies as demonstrated by Johanson (1940). The specimen was soaked for a minimum of 12 h in Formalin: Alcohol: Acetic acid: Water in the ratio of 10:50:5:35. Later the specimen was washed in a series of 60, 70, 80, 90 and 100 per cent Teritary Butly Alcohol. Then the specimens were embedded in molten wax at 52-54°C. Then after drying 12 micron thick sections were made with Spencers rotary microtome. After sectioning the specimens were subjected to de waxing with xylene: ethanol (1:1) for 30 min. After de waxing, the specimens were stained with safranin. Excess stains were washed with series of 50, 70 and 90 per cent ethanol. Later the specimens were dried and then mounted on glass slides with DPX mount.

Molecular characterization

The fungus was cultured on potato dextrose broth at 20 ± 2^0C for 4-5 days. Then the mycelium was collected, dried and powdered by freezing in liquid nitrogen. The genomic DNA was extracted by Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Chakraborty et al. (2010).

PCR amplification of 18S-28S rRNA gene

The genomic DNA was used as a template and subjected for the

Table 1. Prevalence of stem rot in different varieties of carnation in Nilgiris district of Tamil Nadu.

Variety	Colour	Percentage of stem rot incidence		
		Kothagiri	Kodumudi	Kunnur
Yellow liberty	Yellow	12.60	3.60	4.3
Gaudina red	Red	8.60	2.90	3.70
Farida	Pink	11.5	3.80	3.50
Bizet	Light pink	14.70	4.40	4.70
Castor purple	Purple	24.60	6.60	6.80
Golem purple	Purple	18.30	5.50	5.60
White liberty	White	13.50	4.50	5.00
Charmant pink	Light Pink	38.50	9.60	10.4
Pudding yellow	Yellow	22.20	7.60	7.20
Baltico white	White	28.40	8.60	8.30
Mean incidence		18.29	5.81	5.95

PCR amplification of 18S-28S rRNA gene using the primer pairs, ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The PCR reaction was performed with ready to use Taq DNA Polymerase 2x master mix supplied from Ampliqon, comprising Tris-HCl pH 8.5, $(NH_4)_2So_4$, 3mM $MgCl_2$, 0.2% Tween 20□, 0.4mM dNTPs, 0.2units/µl Ampliqon Taq DNA polymerase and inert red dye. The programming cycle was accomplished as follows: initial denaturation at 94^0C for 2 min, 30 cycles of denaturation at 94^0C for 30 s, annealing at 57^0C for 30 s, extension at 72^0C for 1 min and final extension at 72^0C for 7 min (Saitoh et al., 2006).

Life cycle assessment

Different stages of the pathogen associated with its life cycle were studied by inoculating mycelium and sclerotia of the pathogen on seedlings. Subsequently, the developmental biology of apothecium and their components were studied.

RESULTS AND DISCUSSION

Survey

This is the first record of *sclerotinia* rot of carnations in India. Survey results revealed that the variety Charmant pink was highly susceptible to stem rot. In survey the stem rot incidence was relatively greater in Kothagiri compared to that of Kunnur and Kodumudi. Among the varieties surveyed, pink coloured variety Charmant pink was highly susceptible with 38.50 per cent disease incidence followed by Baltico white (28.40%) and Castor purple (24.60%). Least incidence was observed in the variety Gaudina red (8.60%). However the same was severely susceptible to fairy ring spot and blossom blight. Mean stem rot incidence in Kothagiri was 18.29 per cent whereas in kunnur and kodumudi stem rot incidence was comparatively less *viz.,* 5.81 and 5.94, respectively (Table 1). Consistent relative humidity and relatively lower temperature, prevailing in Kothagiri region have resulted in greater loss compared to other regions.

Symptomatology

Symptoms were found to be associated from seedling to maturity stage. However, the plants are highly susceptible at seedling stage. Initial symptoms include paleness of the plant accompanied with drooping leaves (Figure 1a). However, the most typical symptom is the presence of cottony white mycelial growth on root zone (Figure 1b), as well as collar region of the plant. Subsequently, the plant dries to straw yellow and finally dies. Besides, longitudinal splitting of the infected stems revealed plenty of dark, black, sclerotial bodies (12-20) of varying shapes and sizes (Figure 1c). Saharan and Mehta (2008) described that symptom includes, cottony white mycelium on the root zone and collar region accompanied with dark, black, irregular sclerotia inside the stem, followed by, complete drying of the plant to straw yellow. Similar descriptions were made by Purdy (1979).

Isolation

On PDA, pure cultured study fungus produced fluffy white mycelial growth. The hyphae was hyaline, septate and metamorphosed into irregular, black sclerotial bodies of various size following mycelia aggregation as per the previous reports (Colotelo, 1974; Purdy, 1979). In this study, the pathogen produced dense cottony white mycelium on PDA and fungal mass covered the entire Petriplate within 5 days. After 8 days large, dark, black, irregular sclerotial bodies appeared in a circular fashion along the corners of the Petri plate (Figure 2a). The hyphae were hyaline and septate. Sclerotia were produced, superseding the accumulation of nutrients in

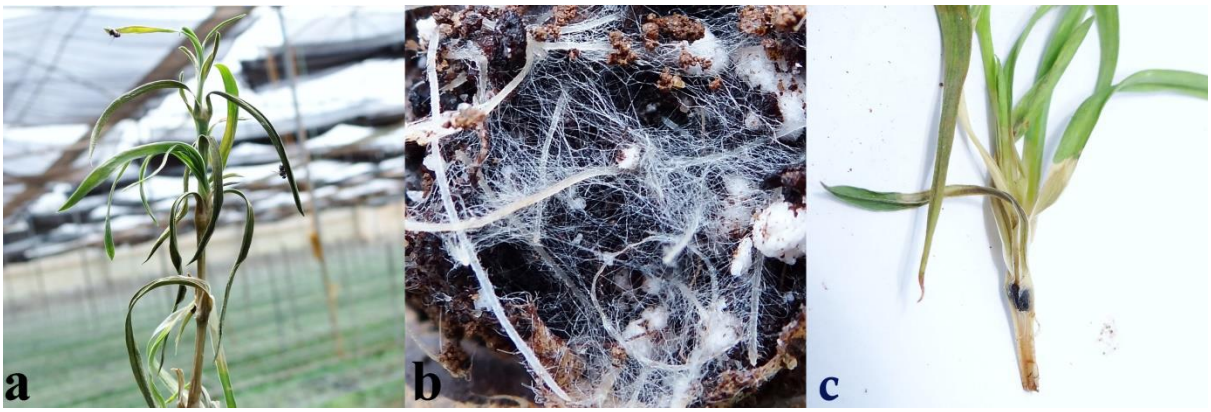

Figure 1. (a) Pale drooping infected plant, **(b)** Root zone colonized by mycelium of *S. sclerotiorum*, **(c)** Sclerotial bodies inside the infected stem.

Figure 2 . (a) Colony morphology of *S. sclerotiorum,* **(b)** Dew drops on developing sclerotia.

Figure 3. Pathogenicity with typical drooping and sclerotial bodies in stem.

the form of dew drops (Figure 2b). The morphology and development of our isolate resembled *S. sclerotiorum* as described by Kohn (1979) and Wang et al. (2008). Sclerotial size and number were greater in Petri plates exposed to light, compared to that incubated under complete darkness.

Pathogenicity

Inoculation of mustard plants with 6 mm mycelial disc of the pathogen *S. sclerotiorum* expressed the symptom of stem rot disease after 10 days when incubated at 100% relative humidity at 22^{o}C (Kim and Cho, 2003). Pathogenicity in *Brassica* sp, Canola, peanut and Bell pepper has been established successfully (Young et al., 2012; Khangura and Macleod, 2013; Faske et al., 2014; Gonzalez et al., 1998). Similarly, in our study pathogenicity was proved by inoculating mycelial discs and sclerotial bodies. The typical symptoms were expressed by the plants after 25 days of inoculation. The plant turned pale and showed drooping symptom (Figure 3a) and, typical rot was observed in the stem portion accompanied with small, black sclerotial bodies (Figure 3b). Hundred per cent infection was observed in the plants inoculated with mycelia disc and sclerotial bodies. The pathogen was re-isolated several times and morphological characters were akin to that of the previously isolated pathogen. Thus Koch postulates were established.

Developmental biology of *S. sclerotiorum* and morphological characterization

Identity of the pathogen was confirmed by morphological characterization mainly based on sclerotial size and ascospore morphology. Morphology of study fungus was compared with three well documented commonly occurring species of *Sclerotinia*. Species differentiation was confirmed among three plant pathogenic species *viz., S. sclerotiorum, S. minor* and *S. trifoliorum* by morphologically characterization as proposed by Ekins et al. (2005). The most distinguishing parameter was the

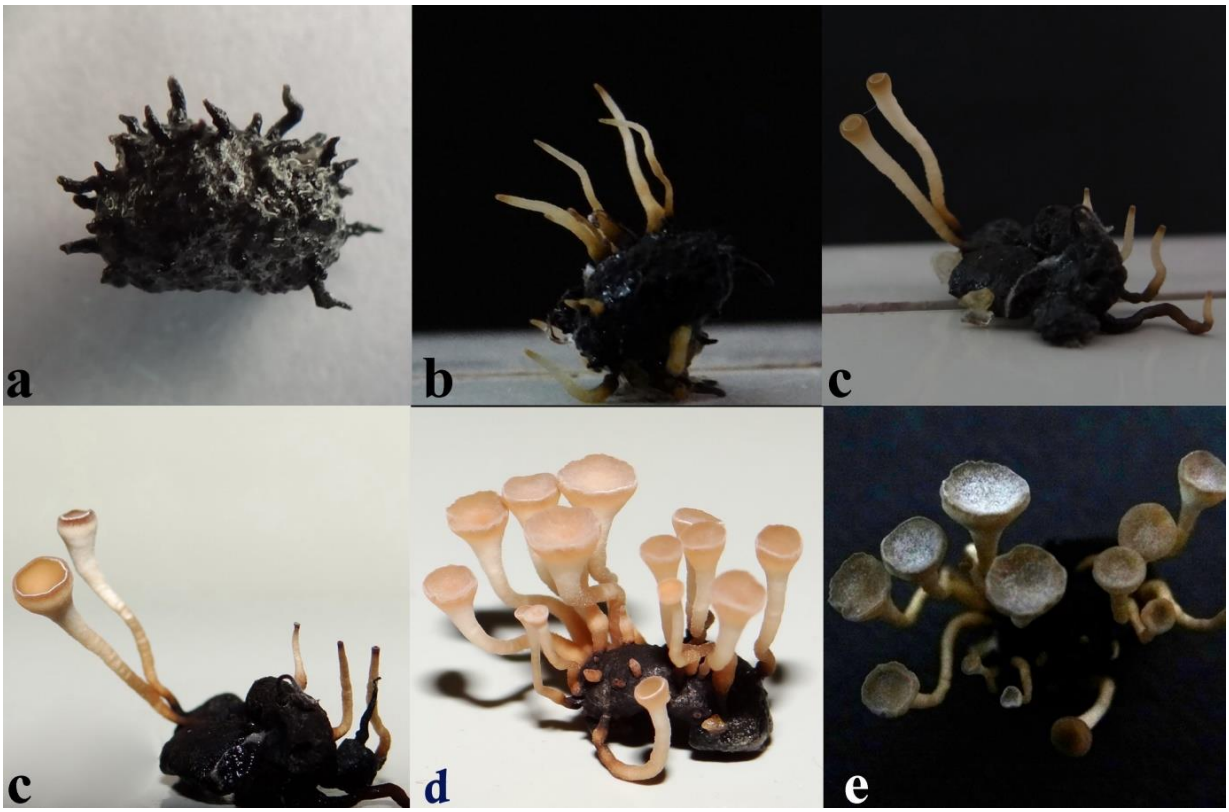

Figure 4. (a) Sclerotia with stipe initials, **(b)** Melanization and elongation of stipe initials, **(c)** Initiation of disc differentiation, **(d)** Growing disc, **(e)** Completely matured apothecium, **(f)** Apothecial disc loaded with ascospores.

sclerotial size that differentiated *S. minor* (1-2 mm dia) from other two species *viz., S. trifoliorum* and *S. sclerotiorum* that produced larger sclerotial bodies (3-100 mm dia). *S. minor* with comparatively smaller sclerotia enables separation from *S. trifoliorum* and *S. sclerotiorum* (Jagger, 1920; Willets and Wong, 1971; Wong, 1979; Tariq et al., 1985). Ascospore morphology enabled further separation between the other two species *viz., S. sclerotiorum* and *S. trifoliorum*. Monomorphic ascospores were observed in *S. sclerotiorum*, whereas *S. trifoliorum* is known to produce dimorphic ascospores (Kohn, 1979; Uhm and Fujii, 1983; Ekins et al., 2005). According to previous studies (Saharan and Mehta, 2008; Pellegrini et al., 1989) *S. sclerotiorum* produced larger sclerotia compared to other species and produced monomorphic ascospores. In our study, the pathogen produced, large, dark, black irregular, sclerotial bodies ranging from 7x4 to 15x5 mm under *in vitro*. This confirmed the identity between *S. sclerotiorum* and *S. trifoliorum* eliminating the chance to be *S. minor*.

In order to study the ascospore morphology, sclerotia were subjected for carpogenic germination. Sanogo and Puppala (2007) reported that *S. sclerotiorum* produced tan to beige coloured apothecial discs. In our study, conditioned sclerotia produced stipe initials after 2 months. After fifteen days of incubation, the black stipe initials got melanised, grew in length and cup shaped; ochraceous apothecium were produced from the stipe initials at the rate of 10-20 per sclerotia. The apothecia formed were of varying sizes ranging from 1.0 to 1.5 cm. The developmental stages of apothecium *viz.,* stipe initiation, melanisation and elongation of stipe initials, disc differentiation and maturation were observed and recorded (Figure 4). Disc differentiation of apothecium was observed only under the exposure of stipe initials to light. Under darkness, length of the stipe increased without disc differentiation. This revealed the importance of cool white fluorescent light in the development of apothecium. All the three replications subjected to conditioning process produced apothecium @ 10-15 apothecium/sclerotia; however, control remained the same.

The ascocarp was made up of four layers *viz*., hymenium, subhymenium, medullary excipulum and ectal excipulum. A row of vertically arranged asci constituted the fertile hymenial layer (Figure5a) which is the outer most and open part of apothecial disc. The subhymenium was composed of closely packed longitudinal cells immediately below hymenium (Figure 5b). Balloon like elongated parenchyma formed the medullary excipulum (Figure 5c). The ectal excipulum was composed of compactly arranged globose pseudoparenchymatous

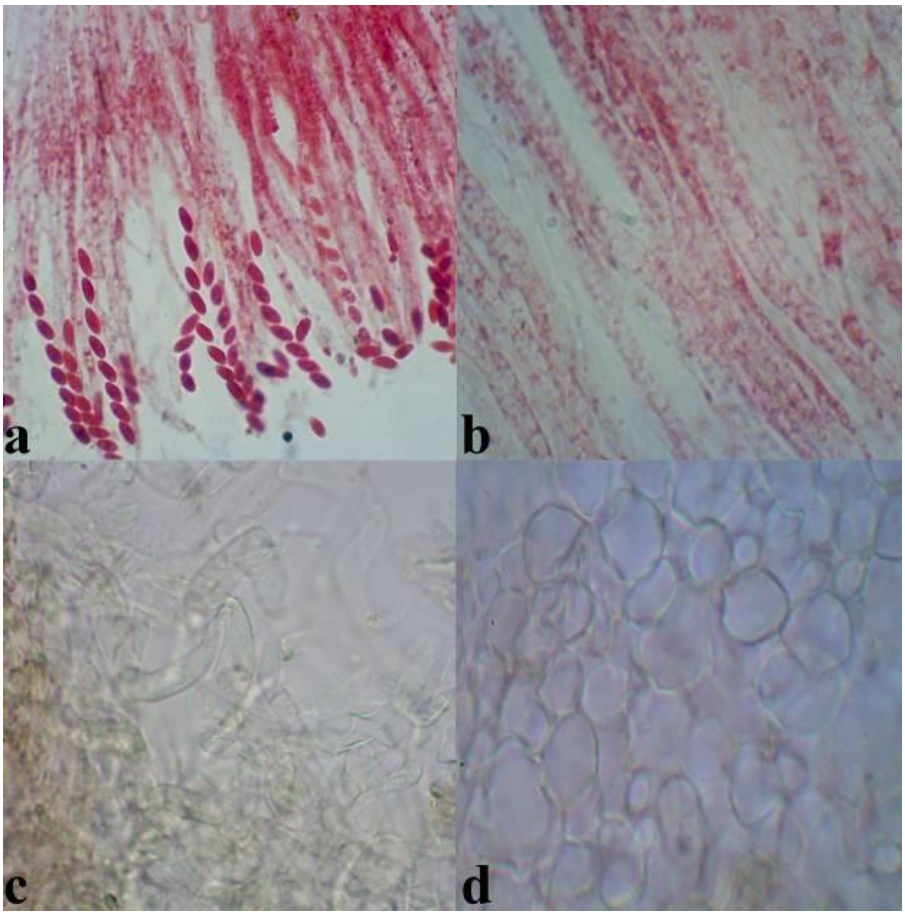

Figure 5a. Hymenial layer with ascus arranged in a row (450X magnification), **b.** Subhymenium (1000X magnification), **c.** Medullary excipulum (1000X magnification), **d.** Ectal excipulum (1000X magnification).

cells that fabricate the stroma (Figure 5d).

Ascus and ascospore

Ascospore morphology enables the separation between *S. sclerotiorum* and *S.trifoliorum*. *S. sclerotiorum* and *S. trifoliorum* are reported to produce monomorphic and dimorphic ascospores, respectively (Evans et al., 2008; Ekins et al., 2005). In the present study, asci were cylindrical, sac like, elongated, with truncated apex and measured 179.39 µm x 8.65 µm at 1000X magnification (Figure 6a). The lateral wall was thin (1.89 µm) and, apex region was thick (5.28 µm) when measured under 1000x magnification (Figure 6b). Each ascus contained eight, ellipsoidal, hyaline, monomorphic ascospores measuring 16.84 µm x 8.65 µm at 1000x magnification. Monomorphic ascospores confirmed the identity as *S. sclerotiorum* distinguishing from *S. trifoliorum*. These results confirmed the identity of the pathogen as *S. sclerotiorum.* Asci were intercepted with supporting sterile hyphae called paraphyses (Figure 6c). The paraphyses were hyaline, filliform with clavate apex. Ascospores were monomorphic and uniform as per the distinctive character of *S. sclerotiorum* (Figure 6a). Ascospores measured 16-19 µ in length and 8-9 µm in width. Ascospores that germinated at both ends were also observed (Figure 6d). Developmental stages of ascospores like empty ascus and immature ascus with under developed ascospores were also observed and recorded (Figures 6e and 6f).

Spermatia and croziers

Besides ascospores, spermatia or microconidia were also observed. Small, hyaline, globose spermatia were singly attached to phialides borne laterally on the hyphae (Figure 6g). Even though spermatia were observed, their exact role in reproduction remains unclear. Small hook like projections with bulged apex resembling croziers were observed in the apothecium (Figure 6h). The croziers later developed into ascus through meiosis and mitosis.

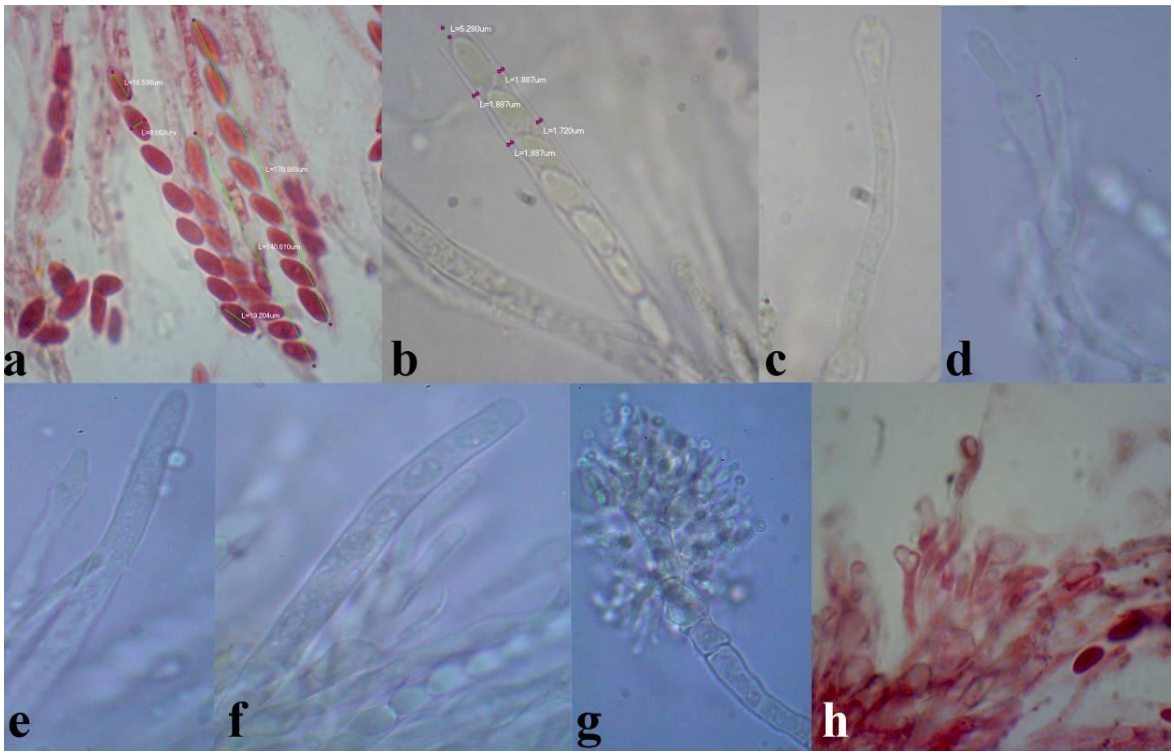

Figure 6. (a) Ascus with ascospore (1000X magnification), **(b)** Ascus wall (1000X magnification), (**c)** Paraphyses with bulged apex, **(d)**Germinating ascospore (1000X magnification), (**e)** Immature empty ascus, **(f)** Underdevloped ascospores inside ascus, **(g)** Spermatia (1000X magnification), **(h)** Croziers (1000X magnification).

The phenotypic characterization of apothecium revealed the components of apothecium identical to previous reports *viz*., ascospore (Saharan and Mehta, 2008), germinating ascospores (Saharan and Mehta, 2008), paraphyses (Saharan and Mehta, 2008), spermatia (Saharan and Mehta, 2008; Rollins, 2007) and crozier formation (Pellegrini et al., 1989). Thus developmental biology of the pathogen was comparatively studied.

Molecular characterization

The ITS region encompassing 18S rRNA, ITS1, 5.8S rRNA, ITS 2 and 28S rRNA can be regarded as an environmental barcodes for the identification of fungi (Bellemain, 2010). The 18S-28S rRNA gene has been reported to have approximately 600 bp (Wang et al., 2008; Jeon et al., 2006). In this study, the PCR product produced an amplicon length of ~600 bp (Figure 7) and the same was sequenced by Sanger dideoxy sequencing method at Excelris genomics, Ahmedabad. Sequencing was carried out using ABI 3730xl Genetic Analyzer (Applied Biosystems, USA). After sequencing the sequence was analysed with BLAST in NCBI (KP676452). The nucleotide sequence of 18S-28S rRNA gene acquired from the study fungus had 99% match with all the three species *viz*., *S. sclerotiorum* (KM272350), *S. trifoliorum* (JQ743329) and *S. minor* (KC836493). Under this predicament, morphological characterization gave a strong distinctive identification of the pathogen. This confirmed the identity of the pathogen as *S. sclerotiorum*.

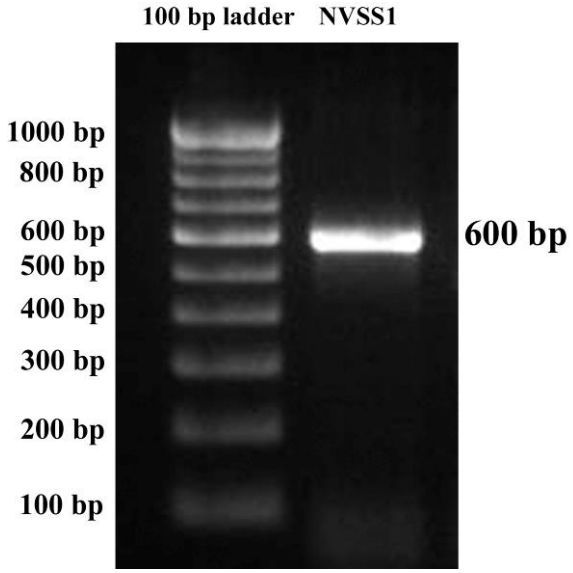

Figure 7. PCR amplification of 18S - 28S rRNA gene of *S. sclerotiorum.*

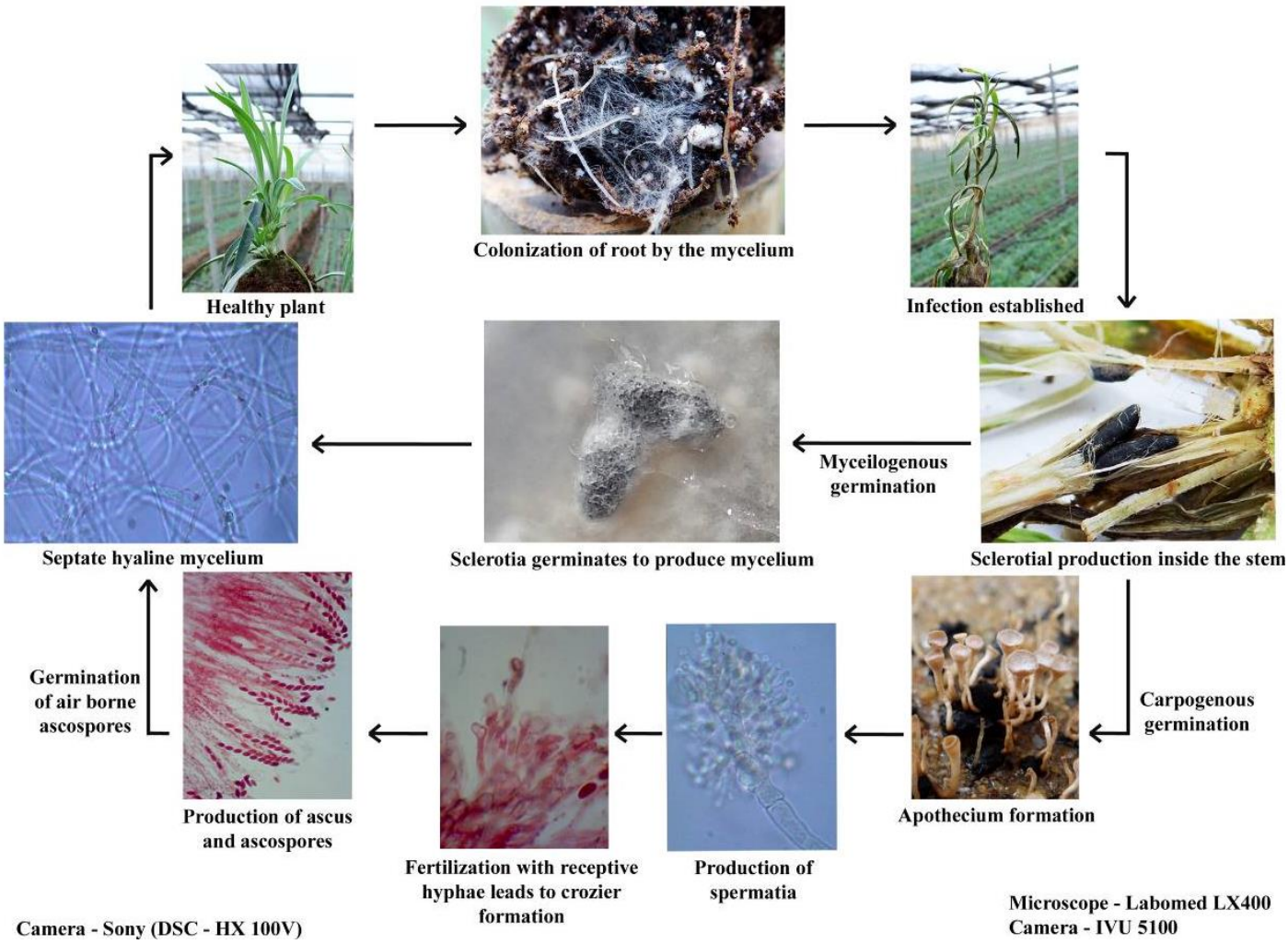

Figure 8. Life cycle of *S. sclerotiorum* in carnation.

Life cycle assessment

The life cycle and different stages of the pathogen were studied. The soil was rich in inoculum due to off season cultivation of leguminous plants. The pathogen resides in soil in the form of sclerotial bodies. The sclerotial bodies are hard and resistant to adverse environmental conditions. Sclerotia play a major role in the disease cycle, since they resist adverse environmental condition and provide required inoculum potential for disease establishment (Willets and Wong, 1980). Conducive environment favours stem rot in carnation, superseding myceliogenic/carpogenic germination of sclerotia (Bolton et al., 2006).

In the present study sclerotial bodies served as primary source of inoculum. Carpogenic germination of sclerotia and airborne dissemination of ascospores have been previously well documented (Heffer Link and Johnson, 2007; Saharan and Mehta, 2008; Purdy, 1979). Under carpogenic germination, apothecia were produced, inside which fertilization occurs and leads to the production of ascospores. The airborne ascospores gets drifted in wind and fall over the healthy flowers. Germinated ascospores gain access to colonize the root zone when the flowers fall off to ground following senescence. Carpogenic germination of sclerotia into apothecium was not observed till now in the field. However, an assumption was made and a lifecycle was designed based on previous studies (Figure 8).

Conclusion

Survey on the distribution of the pathogen *S. sclerotiorum* revealed that, stem rot disease is highly prevalent in Kothagri, Tamil Nadu. Developmental biology of the pathogen was studied in detail. Life cycle assessment shows the mode of infection of the pathogen as mycelium

colonizing root zone and advancing collar region. This provides basic information for future studies to control the disease by breaking developmental biology and infection cycle of the fungus.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Enhanced plant growth and/or nitrogen fixation by leguminous and non-leguminous crops after single or dual inoculation of *Streptomyces griseoflavus* P4 with *Bradyhizobium* strains

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Plant growth-promoting endophytic bacteria have been widely used during crop production for either single inoculation or co-inoculation with nitrogen-fixing bacteria. The effect of *Streptomyces griseoflavus* P4 on growth of various crops was studied and the effects of co-inoculation on plant growth, nodulation and nitrogen fixation by soybean was investigated. Pot experiments were conducted in an environmentally controlled room. Nitrogen fixation was evaluated based on acetylene reduction activity (ARA). Significant increases in soybean and maize growth performance were observed after inoculation with *S. griseoflavus* P4, although some growth parameters were not significant in some crops. *S. griseoflavus* P4 was co-inoculated with high nitrogen-fixing strains. Co-inoculation of *Bradyrhizobium elkanii* AHY3-1 with P4 into the Yezin-6 (non-*Rj*) soybean cultivar resulted in significantly increased root dry weight. Nitrogenase activity also increased due to co-inoculation of *B. elkanii* AHY3-1 with P4 when compared with single inoculation of this strain. Co-inoculation of *Bradyrhizobium japonicum* SAY3-7 and *B. elkanii* AHY3-1 with *S. griseoflavus* P4 into the Yezin-11 (Rj_4) soybean cultivar increased nitrogen fixation significantly by 45 and 31%, respectively, when compared with single inoculation of these strains. Moreover, single or dual inoculation of bradyrhziobial strains resulted in significantly higher shoot biomass than that in the control and P4 alone. These results show that plant growth was promoted by *S. griseoflavus* P4 and that plant growth and nitrogen fixation were enhanced in soybean after co-inoculation of *S. griseoflavus* P4 with the *Bradyrhizobium* strains, *B. japonicum* SAY3-7 and *B. elkanii* AHY3-1.

Key words: Co-inoculation, growth, nitrogenase activity, single inoculation, soybean, *Streptomyces griseoflavus* P4.

INTRODUCTION

Soils have been the predominant reservoir for the isolation of actinomycetes, particularly the genus Streptomyces and soil microbes have been regarded as important sources of biologically active compounds (Balachandran et al., 2015). Endophytic bacteria live in the internal tissues of plants, as symbionts without

causing visible harmful effects (Hallmann et al., 1997). Plant growth-promoting rhizobacteria have been used widely in agriculture. They promote plant growth by producing plant growth hormones, such as auxins (Tien et al., 1979), gibberellins (Bottini et al., 1989), cytokinins (Strzelczyk et al., 1994; Tien et al., 1979), ethylene (Strzelczyk et al., 1994) and indole-3-acetic acid (IAA) to improve root growth (Leong, 1996; Merckx et al., 1987; Nimnoi et al., 2010), induce plant resistance to disease and stressors (Wang et al., 2009) and enhance biological nitrogen fixation (Ashraf et al., 2011).

The symbiosis between the nodulating bacteria and legumes results in the fixation of atmospheric nitrogen in the nodules. Bio-fertilizer promotes nodulation efficiency and increases yield by 16-60% (DAR, 2004). Moreover, symbiotic nitrogen fixation by soybean provides 65 to greater than 160 kg fixed N ha^{-1} (Klubeck et al., 1988), accounting for 40–70% of the total nitrogen requirement. Therefore, maintaining this significant nitrogen input is important for economical and sustainable soybean yields, particularly in soils with low available soil nitrogen (Zablotowicz and Reddy, 2004). Therefore, the symbiosis between leguminuous plants and rhizobia is of considerable importance for the environment and agriculture (Ogutcu et al., 2009).

Co-inoculation of rhizobia with plant growth-promoting bacteria rather than a single inoculation of rhizobia has become popular because co-inoculation improves soybean yield and contributes to sustainable agriculture (Hungria et al., 2015). Co-inoculation of *Bradyrhizobium japonicum* with an *Azospirillum brasilense* strain improved nitrogen fixation in soybean (14.1%) as compared to that of the control (Hungria et al., 2013). Hungria et al. (2015) demonstrated that co-inoculation of *Bradyrhizobium* spp. with an *A. brasilense* strain increases soybean yield without additional nitrogen application to soils with an indigenous rhizobial population.

The endophytic actinomycete strain, P4, was isolated from sweet pea root at Kurima, Tsu-City, Japan by Thapanapongworakul (2003). Tang-um and Niamsup (2012b) reported that the P4 16S rRNA sequence has 99.7% sequence similarity with that of *Streptomyces griseoflavus* (Accession number: EU741217). The production of plant growth-promoting hydrolytic enzymes, such as chitinase, amylase (Tang-um and Niamsup, 2012a) and IAA (Soe, 2013) from *S. griseoflavus* P4 has been reported. N uptake by adzuki beans and Thai sweet pea occurred due to co-inoculation of *Streptomyces* spp. P4 with nitrogen-fixing rhizobia (Thapanapongworakul, 2003). A P4 symbiotic interaction similar to that with Myanmar rhizobia has been reported by Soe et al. (2012) and Soe and Yamakawa (2013a, 2013b) who stated that the synergistic effect of P4 with soybean-nodulating rhizobia increases nodulation and nitrogen fixation in Myanmar soybean cultivars. Moreover, Soe (2013) reported that *S. griseoflavus* P4 increases growth performance in leguminous and non-leguminous cereal and vegetable crops.

Using this endophytic strain as a single inoculant in non-leguminous crops or as a co-inoculant with indigenous rhizobial strains to increase plant growth and nitrogen fixation rates of leguminous crops would be of interest. Therefore, this study was conducted to test the effect of a single inoculation of *S. griseoflavus* P4 on various crops and to evaluate the co-inoculation effects of *S. griseoflavus* P4 with various *Bradyrhizobium* strains on plant growth, nodulation and nitrogen fixation in soybean cultivars.

MATERIALS AND METHODS

Inoculum preparation

S. griseoflavus P4 was cultured in Inhibitory Mold Agar (IMA)-2 liquid medium on a rotary shaker (100 rpm) at 30°C for 5 days. IMA-2 medium was prepared by mixing the following reagents (glucose; 5.0 g, soluble starch; 5.0 g, beef extract; 1.0 g, yeast extract; 1.0 g, NZ-case; 2.0 g, NaCl; 2.0 g, $CaCO_3$;1.0 g) with 1000 mL of sterilized distilled water as described in Shimizu et al. (2000). The P4 bacterial suspension was prepared to obtain 10^5 cells mL^{-1}. The bradyrhizobial strains *B. japonicum* SAY3-7, *B. elkanii* AHY3-1 and *B. liaoningense* SMY3-1 were cultured in A1E liquid media (Kuykendall, 1979) and incubated on rotary shaker at 30°C for 7 days. One millimeter of liquid culture medium from each isolate was diluted with 99 mL half-strength Modified Hoagland Nutrient (MHN) solution (Nakano et al., 1997) to prepare bacterial suspensions of approximately 10^7 cells mL^{-1}. These three strains were isolated from Myanmar (Htwe et al., 2015a). Htwe et al. (2015c) previously reported that these three strains showed higher nitrogen fixing efficiencies on non-*Rj* and Rj_4 soybean cultivars.

S. griseoflavus P4 symbiosis with various crops

Two cereal crops, rice (*Oryza sativa* L.) cv. Manawthuka and maize (*Zea mays* L.) cv. Kakuteru (Nihon nousan shubyo Co., Ltd.); two horticultural crops, spinach (*Spinacia oleracea* L.) cv. O-rai hourennsou (Nihon nousan shubyo Co., Ltd.) and Japanease radish (*Raphanus sativus*) cv. Tokinashi-daikon (Nihon nousan shubyo Co., Ltd.); and two leguminous plants, common bean (*Phaseolus vulgaris* (L.)) cv. Suzinashi-saitou (Nihon nousan shubyo Co., Ltd.) and soybean (*Glycine max* (L.) Merr.) cv. Yezin-6 were used to evaluate the effect of *S. griseoflavus* P4 on growth under nitrogen-free conditions. The seeds were surface sterilized by soaking in 2.5% sodium hypochlorite solution for 5 min, rinsed five times in 10 mL 99.5% ethanol, and washed five times in sterilized MHN solution to remove any trace of sodium hypochlorite and ethanol. Ten seeds per pot were grown in prepared culture pots (1 L volume) filled with 1 L vermiculite and 0.6 L MHN solution and autoclaved at 120°C for 20 min. Vermiculite was sieved

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through a 4-mm sieve to obtain fine growth medium for the crops. *S. griseoflavus* P4 was inoculated at 2 mL per seed. The plants were cultivated in a Phytotron (25°C and 75% relative humidity) for 2 weeks. Autoclaved deionized water was used for watering. This experiment was conducted during July of 2015. Five replicate plants were used from pots of the various crops to measure the growth parameters.

Symbiosis of the *Bradyrhizobium* strains and *S. griseoflavus* P4

Growth conditions occurred under nitrogen-free conditions. The treatments were single inoculations of P4, *B. japonicum* SAY3-7, *B. elkanii* AHY3-1 and *B. liaoningense* SMY3-1 and co-inoculation of P4 with each of the *Bradyrhizobium* strains. Six seeds per pot were grown in prepared culture pots (1 L volume) filled with 1 L vermiculite and 0.6 L MHN solution and autoclaved at 120°C for 20 min. A control un-inoculated treatment was also provided. *S. griseoflavus* P4 was inoculated at 2 mL per seed. *Bradyrhizobium* strain was inoculated at 5 mL per seed. The effectiveness of the single and co-inoculations was determined using the *Rj* cultivars Yezin-6 (non-*Rj*) and Yezin-11 (Rj_4). *Rj* genes of these two varieties and their symbiotic effectiveness with *B. japonicum* USDA110 were described in Htwe et al. (2015b). This experiment was conducted from July to August of 2015. Three replicate plants of the two soybean varieties were used to measure acetylene reduction activity (ARA).

ARA was measured by flame ionization gas chromatography (GC-14A; Shimadzu, Kyoto, Japan) after 4 weeks of cultivation as described by Soe and Yamakawa (2013a). Nodules were removed from the roots after the assay, and the number of nodules was recorded. Shoots, roots and nodules were collected separately and oven dried at 70°C for 24 h to obtain dry weights.

Data analysis

The data were analyzed statistically using STATISTIX 8 analytical software (Tallahassee, FL, USA) and means were compared using Tukey's HSD test at $P < 0.05$.

RESULTS

Symbiosis of *S. griseoflavus* P4 with various crops under nitrogen-free condition

The growth performance of the leguminous plants is presented in Tables 1 and 2. Soybean growth parameters, such as shoot and root length and shoot and root dry weight, differed significantly between the plants inoculated with *S. griseoflavus* P4 and the control plants (Table 1). Shoot length in common bean was affected significantly by inoculation with *S. griseoflavus* P4 (Table 2) although none of the other growth parameters differed from the control.

The growth performance of the cereal crops is presented in Tables 3 and 4. Maize shoot length and shoot and root biomass were significantly higher than those of the un-inoculated control (Table 3). In rice, no differences were observed in any of the growth parameters between the inoculated treatment and the control (Table 4).

Table 1. The effect of P4 on soybean growth at two weeks after sowing.

Treatment	SL (cm)	RL (cm)	SDW (mg plant^{-1})	RDW (mg plant^{-1})
Control	17.84^{b}	20.08^{b}	152.00^{b}	64.00^{b}
P4	19.66^{a}	24.08^{a}	182.00^{a}	88.00^{a}
P value	0.0111	0.0059	0.0127	0.0209
CV%	4.67	7.70	8.88	17.41

Means in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). SL, RL, SDW, and RDW are shoot length, root length, shoot dry weight, and root dry weight, respectively.

Table 2. The effect of P4 on common bean growth at two weeks after sowing.

Treatment	SL (cm)	RL (cm)	SDW (mg plant^{-1})	RDW (mg plant^{-1})
Control	13.82^{b}	24.78^{a}	154.00^{a}	76.00^{a}
P4	17.16^{a}	27.70^{a}	202.00^{a}	104.00^{a}
P value	0.0032	0.3363	0.0641	0.0528
CV%	8.20	17.20	19.86	21.66

Means in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). SL, RL, SDW, and RDW are shoot length, root length, shoot dry weight, and root dry weight, respectively.

Table 3. The effect of P4 on rice growth at two weeks after sowing.

Treatment	SL (cm)	RL (cm)	SDW (mg plant^{-1})	RDW (mg plant^{-1})
Control	11.78^{a}	16.16^{a}	7.38^{a}	12.50^{a}
P4	12.16^{a}	17.86^{a}	7.88^{a}	14.00^{a}
P value	0.4064	0.1156	0.2831	0.1974
CV%	5.73	8.96	9.00	12.23

Means in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). SL, RL, SDW and RDW are shoot length, root length, shoot dry weight, and root dry weight, respectively.

The growth performance of the vegetable crops is presented in Tables 5 and 6. Spinach biomass did not increase after inoculation with *S. griseoflavus* P4 (Table 5); however, root and shoot lengths differed significantly from the control. Radish root biomass was affected significantly by inoculation with *S. griseoflavus* P4 (Table 6), although none of the other growth parameters differed from the control.

Taken together, the bacterial inoculation treatments increased shoot length, root length, shoot biomass and root biomass when compared with the control.

Table 4. The effect of P4 on maize growth at two weeks after sowing.

Treatment	SL (cm)	RL (cm)	SDW (mg plant^{-1})	RDW (mg plant^{-1})
Control	17.64^{b}	31.34^{a}	45.90^{b}	79.60^{b}
P4	22.30^{a}	33.68^{a}	84.70^{a}	116.30^{a}
P value	0.0032	0.4619	0.002	0.0422
CV%	8.2	14.73	20.81	24.5

Means in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). SL, RL, SDW, and RDW are shoot length, root length, shoot dry weight, and root dry weight, respectively.

Table 5. The effect of P4 on spinach growth at two weeks after sowing.

Treatment	SL (cm)	RL (cm)	SDW (mg plant^{-1})	RDW (mg plant^{-1})
Control	1.96^{b}	8.46^{b}	5.70^{a}	3.88^{a}
P4	3.42^{a}	13.90^{a}	7.64^{a}	4.40^{a}
P value	0.01	0.0016	0.3524	0.3499
CV%	25.55	16.53	46.58	20.01

Means in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). SL, RL, SDW, and RDW are shoot length, root length, shoot dry weight, and root dry weight, respectively.

Table 6. The effect of P4 on radish growth at two weeks after sowing.

Treatment	SL (cm)	RL (cm)	SDW (mg plant^{-1})	RDW (mg plant^{-1})
Control	4.94^{a}	12.84^{a}	23.50^{a}	3.56^{b}
P4	5.22^{a}	16.12^{a}	22.90^{a}	7.44^{a}
P value	0.697	0.1248	0.08748	0.0017
CV%	21.59	20.89	24.33	24.12

Means in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). SL, RL, SDW, and RDW are shoot length, root length, shoot dry weight, and root dry weight, respectively.

Symbiosis of three selected *Bradyrhizobium* strains and *S. griseoflavus* P4

The performance of the *Bradyrhizobium* strains and *S. griseoflavus* P4 on two different soybean varieties is shown in Tables 7 and 8.

The numbers of nodules and the nodule dry weights in the Yezin-6 (non-*Rj*) cultivar differed significantly among treatments but were not significantly different between single and dual inoculations of each strain. Shoot dry weight did not increase among the treatments; however, root dry weight differed significantly among treatments. A single inoculation of *B. japonicum* SAY3-7 resulted in the highest root dry weight but the difference was not significant from those of a single inoculation of P4, single and dual inoculation of *B. liaoningense* SMY3-1, or a dual inoculation of *B. elkanii* AHY3-1. Co-inoculation of *B. elkanii* AHY3-1 with P4 resulted in significantly higher root dry weight when compared with a single inoculation of this strain. Nitrogen fixation as indicated by the ARA value differed significantly between the P4 co-inoculation and single inoculation treatments, and the control. Co-inoculation of P4 with either *B. japonicm* SAY3-7 or *B. liaoningense* SMY3-1 did not increase the nitrogen fixation rate when compared with a single inoculation of these strains. Nitrogenase activity increased approximately 24% over the control after co-inoculation of P4 with *B. elkanii* AHY3-1, although no difference was found between single and dual inoculation. These results show that co-inoculation of *B. elkanii* AHY3-1 with P4 promotes nitrogenase activity and root growth in the Yezin-6 (non-*Rj*) cultivar.

Table 7. Effect of single and coinoculation of bradyrhizobial strains on plant growth, nodulation and acetylene reduction activity of Yezin-6 soybean cultivar at 28 DAS.

Treatment	NN (No. plant^{-1})	NDW (mg plant^{-1})	SDW (g plant^{-1})	RDW (g plant^{-1})	ARA (µmol C_2H_4 h^{-1} plant^{-1})
Control	0.00^{c}	0.00d	0.39^{a}	0.25^{b}	0.00^{b}
P4	0.00^{c}	0.00d	0.47^{a}	0.32^{ab}	0.00^{b}
SAY3-7	10.33^{ab}	51.40^{a}	0.48^{a}	0.36^{a}	1.24^{a}
AHY3-1	16.00^{a}	40.00^{abc}	0.47^{a}	0.25^{b}	0.88^{ab}
SMY3-1	10.67^{ab}	22.20^{bc}d	0.46^{a}	0.27^{ab}	1.60^{a}
SAY3-7 + P4	6.33^{b}	17.20^{c}d	0.42^{ab}	0.25^{b}	1.15^{ab}
AHY3-1 + P4	16.33^{a}	42.70^{ab}	0.49^{a}	0.28^{ab}	1.09^{ab}
SMY3-1 + P4	11.67^{ab}	26.00^{bc}	0.43^{a}	0.27^{ab}	1.61^{a}
P value	0.0000	0.0000	0.4471	0.0135	0.0010
CV%	24.97	35.05	12.87	12.38	48.83

Mean in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). NN, NDW, SDW, and RDW indicate nodule number, nodule dry weight, shoot dry weight, and root dry weight, respectively. DAS means days after sowing.

Table 8. Effect of single and coinoculation of bradyrhizobial strains on plant growth, nodulation and acetylene reduction activity of Yezin-11 soybean cultivar at 28 DAS.

Treatment	NN (No. plant^{-1})	NDW (mg plant^{-1})	SDW (g plant^{-1})	RDW (g plant^{-1})	ARA (µmol C_2H_4 h^{-1} plant^{-1}
Control	0.00^{c}	0.00^{b}	0.36^{c}	0.25^{a}	0.00^{c}
P4	0.00^{c}	0.00^{b}	0.45^{bc}	0.33^{a}	0.00^{c}
SAY3-7	8.00^{bc}	32.60^{a}	0.46^{abc}	0.27^{a}	0.77^{b}
AHY3-1	24.67^{a}	33.20^{a}	0.53^{ab}	0.28^{a}	0.75^{b}
SMY3-1	18.00^{ab}	27.70^{a}	0.48^{abc}	0.31^{a}	0.95^{ab}
SAY3-7 + P4	8.00^{bc}	38.20^{a}	0.46^{abc}	0.29^{a}	1.39^{a}
AHY3-1 + P4	18.67^{ab}	36.20^{a}	0.56^{ab}	0.29^{a}	1.08^{ab}
SMY3-1 + P4	13.33^{abc}	31.40^{a}	0.57^{a}	0.27^{a}	0.97^{ab}
P value	0.0001	0.0000	0.0002	0.4269	0.0000
CV%	43.67	22.85	8.43	13.37	24.30

Mean in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). NN, NDW, SDW, and RDW indicate nodule number, nodule dry weight, shoot dry weight, and root dry weight, respectively. DAS means days after sowing.

The nodule dry weight of the Yezin-11 (Rj_4) cultivar did not differ between the co-inoculation and single inoculation treatments with the *Bradyrhizobium* strains. The number of nodules differed significantly among treatments but it was not significantly different between single and dual inoculations of each strain. Root dry weight did not change among treatments. However, shoot dry weight was significantly different among the treatments. Both single and dual inoculation of the tested strains resulted in a significant increase in shoot growth when compared with the control and P4 alone. A significant difference in the nitrogenase activity of the Yezin-11 (Rj_4) cultivar was observed between the single and dual inoculation treatments (Table 8). The highest ARA per plant was found after dual inoculation of *B. japonicum* SAY3-7, followed by dual inoculation of *B. elkanii* AHY3-1, but these ARA values were not significantly different from those of single or dual inoculation of *B. liaoningense* SMY3-1. These results show that co-inoculation of *B. japonicum* SAY3-7 and *B. elkanii* AHY3-1 with P4 increased nitrogen fixation significantly by approximately 45 and 31%, respectively, when compared with single inoculation of these strains.

DISCUSSION

In this study, the growth and plant biomass of soybean was enhanced after inoculation with *S. griseoflavus* P4, which agrees with the results of Soe (2013) who reported that root and shoot biomass was significantly higher in plants inoculated with *S. griseoflavus* P4 than in un-inoculated controls. Soe et al. (2010) reported improved shoot growth of the SJ5 Thailand soybean cultivar after inoculation with P4. Moreover, maize shoot and root biomass amounts were significantly higher than those of

un-inoculated controls. These findings agree with those of Soe (2013) who reported that maize shoot and root dry weights increased significantly after inoculation with *S. griseoflavus* P4 when compared with the control. Radish root biomass increased significantly after inoculation with *S. griseoflavus* P4, although other growth parameters did change relative to the un-inoculated control. These results support the findings of Soe (2009, 2013), who found that the root biomass of sweet pea inoculated with P4 was significantly higher than that of the un-inoculated control, although the shoot biomass did not differ significantly (Soe, 2013). Moreover, inoculation of *S. griseoflavus* P4 increased the root dry weight of Myanmar soybean, Hinthada by approximately 63% over the control (Soe, 2009).

Soybean, common bean, maize and spinach shoots and roots elongated significantly after inoculation with the P4 strain. The plant growth-promoting effects of *S. griseoflavus* P4 on leguminous and non-leguminous crops may be due to the production of plant growth-promoting compounds, such as IAA. Soe (2013) reported that *S. griseoflavus* P4 can produce IAA. Meguro et al. (2006) reported that an endophytic strain of *Streptomyces* spp. MBR-52 caused pronounced enhancement of emergence and elongation of plant adventitious roots. IAA-producing microorganisms are known to stimulate root elongation and enhance plant growth (Patten and Glick, 2002).

In this study, the symbiotic interaction between P4 and *B. elkanii* AHY3-1 improved root growth significantly in the Yezin-6 (non-*Rj*) cultivar as compared to that of a single inoculation of a *Bradyrhizobium* strain and *S. griseoflavus* P4. Co-inoculating P4 with the selected indigenous bradyrhizobial strains *B. japonicum* SAY3-7 and *B. elkanii* AHY3-1 in the Yezin-11 (Rj_4) cultivar significantly increased shoot dry weight as compared to a single inoculation of a *Bradyrhizobium* strain. Bai et al. (2002) reported that the endophytic *Bacillus subtilis* strains NEB4 and NEB5 and *Bacillus thuringiensis* strain NEB17 have excellent potential as plant growth promoters in soybean. They stated that the weight of soybean increased after co-inoculation of *B. japonicum* with another strain under nitrogen-free conditions when compared with plants inoculated with *B. japonicum* alone. The symbiotic interaction between P4 and *B. elkanii* AHY3-1 improved nitrogen fixation significantly as compared to a single inoculation of a *Bradyrhizobium* strain and *S. griseoflavus* P4 in the Yezin-6 (non-*Rj*) cultivar. The symbiotic interaction between P4 and the selected indigenous bradyrhizobial strains *B. japonicum* SAY3-7 and *B. elkanii* AHY3-1 increased nitrogen fixation significantly in the Yezin-11 (Rj_4) cultivar as compared to a single inoculation of a *Bradyrhizobium* strain. This result was supported by findings of others that dual inoculation of a bradyrhizobial strain and an endophytic actinomycete (*Streptomyces* spp. P4) increases nodulation and the nitrogen fixation rate in various soybean varieties (Soe et al., 2012; Soe and Yamakaw, 2013a, b). Thapanapongworakul (2003) reported that dual inoculation of P4 *S. griseoflavus* and the *B. japonicum* USDA 110 bradyrhizobial strain increases N uptake by SJ5 (Thailand soybean cultivar) with approximately 44.3% as compared to single inoculation of the same strain.

The *Rj* genes play roles in adaptation and preference for specific rhizobial strains (Ishizuka et al., 1991; Saeki et al., 2000). In addition, non-*Rj* is compatible with all bradyrhizobial strains, but Rj_4 has unique features that restrict nodulation with specific *Bradyrhizobium* strains (Vest and Caldwell, 1972). Soe and Yamakawa (2013a) reported that symbioses were observed after dual inoculation of P4 and MAS34 and dual inoculation of P4 and MAS23 in Yezin-3 (Rj_4) and Yezin-6 (*non-Rj*), respectively. The MAS34 and MAS23 strains have been isolated from Rj_4- and non-*Rj*-genotype cultivars, respectively (Soe et al., 2013b). In this study, dual inoculation of P4 with *B. japonicum* SAY3-7 and *B. elkanii* AHY3-1 isolated from Rj_4 cultivars increased the symbiotic nitrogen fixation rates in Yezin-11 (Rj_4) cultivars. No difference was found for the Yezin-6 (*non-Rj*) cultivar.

Streptomyces strains are frequently reported to be plant growth-promoting microbes (Nassar et al., 2003; El-Tarabily, 2008). Plant growth-promoting traits such as IAA production and ACC deaminase, cellulase and nitrogenase activities by *Bacillus megaterium* LNL6 and *Methylobatcterium oryzae* CBMB20, contribute to the improvement of the overall symbiosis of nitrogen fixation (Subramanian et al., 2015). Plant growth-promoting traits, including the activity of hydrolytic enzymes such as chitinase and amylase (Tang-um and Niamsup, 2012a and 2012b) and IAA production (Soe, 2013), have been documented for *S. griseoflavus* P4. Therefore, the enhancement of nitrogen fixation observed in this study may be related to the induction of plant growth hormones by *S. griseoflavus* P4. Sturz et al. (2003) and Sarr et al. (2010) reported that beneficial, detrimental and neutral effects of endophytic bacteria depend on the co-inoculated strains of nitrogen-fixing bacteria. In this study, co-inoculation of P4 with either *B. japonicum* SAY3-7 or *B. elkanii* AHY3-1 had a synergistic effect on plant growth and nitrogen fixation in the Yezin-11 cultivar; however, co-inoculation of P4 with *B. liaoningense* SMY3-1 was not beneficial or detrimental to growth or nitrogen fixation rates in either tested cultivar. Therefore, co-inoculation of this endophytic bacterium with *B. japonicum* SAY3-7 or *B. elkanii* AHY3-1 may be useful for producing bio-fertilizer and reduce the need for nitrogen fertilizer by enhancing growth and nitrogen fixation rates in soybean.

Conclusion

A beneficial effect of *S. griseoflavus* P4 on the growth of leguminous and non-leguminous crops was observed in

this study. It was shown that *S. griseoflavus* P4 was effective in both leguminous and non-leguminous crops. The root dry weight of the Yezin-6 (non-*Rj*) cultivar increased significantly after co-inoculation of *B. elkanii* AHY3-1 with P4 when compared with a single inoculation of this strain. Moreover, nitrogenase activity increased (24%) after inoculating *B. elkanii* AHY3-1 with P4 when compared with a single inoculation of this strain, although no difference among treatments was observed. Co-inoculation of *B. japonicum* SAY3-7 and *B. elkanii* AHY3-1 with P4 increased nitrogen fixation in the Yezin-11 (Rj_4) cultivar by approximately 45 and 31%, respectively, when compared with single inoculation of these strains. The results demonstrate that *S. griseoflavus* P4 promotes growth and nitrogen fixation in soybean after co-inoculation with the *Bradyrhizobium* strains *B. japonicum* SAY3-7 or *B. elkanii* AHY3-1. This study was conducted in pots under controlled conditions. Further studies should be conducted under open field conditions.

Conflict of Interests

The authors have not declared any conflict of interests.

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Symbiotic effectiveness of different indigenous *Bradyrhizobium* strains on selected *Rj*-genes harboring Myanmar soybean cultivars

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Many scientists are working to identify effective strains of rhizobia to increase nitrogen fixation and reduce nitrogen fertilizer application. Symbiotic nitrogen fixation of leguminous crops has become an alternative to nitrogenous fertilizer, due to its higher efficiency for nitrogen fixation. This experiment was conducted to select strains for improved nitrogen fixation of soybean (*Glycine max* L.). *Bradyrhizobium* strains isolated from Myanmar were evaluated for symbiotic efficacy, using the cultivar Yezin-6 (*non-Rj*). Five *Bradyrhizobium* strains, *B. japonicum* SAY3-7, *B. elkanii* AHY3-1, *B. liaoningense* SMY3-1, *Bradyrhizobium* spp. AHY3-6 and *B. yuanmingense* SMY6-10 were shown to have superior nitrogenase activity. These five strains were evaluated for their effectiveness on different *Rj*-genes in soybean cultivars. The nitrogen fixation of *B. japonicum* SAY3-7, *B. elkanii* AHY3-1 and *B. liaoningense* SMY3-1 were higher than other indigenous strains and the standard strain, *B. japonicum* USDA110. This was particularly the case for the cultivars Yezin-6 (*non-Rj*) and Yezin-11 (Rj_4), but not for the cultivarsYezin-9 (Rj_3) and Yezin-10 (Rj_2Rj_3). *Bradyrhizobium japonicum* SAY3-7, *B. elkanii* AHY3-1 and *B. liaoningense* SMY3-1 were also evaluated on Yezin-8 (*non-Rj*) and Yezin-3 (Rj_4). *Bradyrhizobium japonicum* SAY3-7 had significantly higher nitrogenase activity on Yezin-8 (*non-Rj*), although *B. japonicum* SAY3-7 was not significantly different than *B. elkanii* AHY3-1 and *B. liaoningense* SMY3-1 on Yezin-3 (Rj_4). Therefore, *B. japonicum* SAY3-7, which was the most effective nitrogen fixing strain in all the experiments, was selected for inoculant production. According to this study, it can be concluded that the strains were specific to cultivars and,thus, selection of a strain compatible to a specific cultivar is necessary to increase symbiotic nitrogen fixation.

Key words: *Bradyrhizobium* strains, Myanmar, nitrogenase activity, *Rj*-genes, soybean.

INTRODUCTION

Soybean (*Glycine max* L.) has become an important crop in Myanmar, as it plays a significant role in human, animal and plant nutrition. It can be grown in various parts of the country. The majority of soybean production

is located in the Shan State, followed by the Mandalay, Sagaing, Ayeyarwady, and Bago regions (CSO, 2006). Soybean acreage has gradually increased since 1995 to 1996, but the productivity of soybean (1.51 tons ha^{-1}) is still low compared to the world average yield (2.52 tons ha^{-1}) (MOAI, 2013).

In Myanmar, the Department of Agricultural Research (DAR) has been producing improved soybean cultivars, such as Yezin-3 and Yezin-6, to replace local varieties and to increase soybean production. Moreover, *Bradyrhizobium* strains such as TAL 377, TAL 379 and TAL 102, produced by Nitrogen Fixation for Tropical Agricultural Legumes (NifTAL), are being used as inoculants. The Plant Pathology Section of the DAR initiated rhizobial inoculant research and production. However, effective, locally adapted *Rhizobium* strains are not readily available on the Myanmar market. Therefore, exotic strains must be replaced by those that are indigenous, as they have adapted to local environmental conditions and are readily available. The symbiotic effectiveness of native *Bradyrhizobium* strains on different Myanmar soybean cultivars has been reported (Aung, 2007; Soe and Yamakawa, 2013a).

The responses of soybean cultivars vary with the *Rhizobium* strains. While some cultivars are fully compatible with other *Rhizobium*, others cannot form nodules even though the *Rhizobium* belongs to certain serogroups of *Bradyrhizobium* (Van et al., 2007). This might be due to nodulation regulatory genes called *Rj* genes found in cultivars. The *Rj* genotypes (non-*Rj*, rj_1, Rj_2, Rj_3 and Rj_4) have been found to exist in nature (Devine and Kuykendall, 1996). The nitrogen fixation rate varies among both cultivars and inoculant strains. Yamakawa et al. (1999, 2003) reported on the higher nodulation ability of $Rj_2Rj_3Rj_4$. Soe and Yamakawa (2013a, 2013c) reported that indigenous strains are effective on Yezin-6 (*non-Rj*) and Yezin-3 (Rj_4). Therefore, it is necessary to select strains that are compatible with different *Rj*-genotypes to promote soybean production through enhancing symbiotic nitrogen fixation.

Inoculation with symbiotic rhizobia for higher biological nitrogen fixation is a common agronomic practice for agricultural production in other countries. In Myanmar, the majority of farmers rely on nitrogenous fertilizer for crop production, although it is very expensive (Than and Han, 1988). An alternative to nitrogenous fertilizer is the use of effective and efficient strains of N-fixing bacteria. *Rhizobium* inoculant production is relatively inexpensive and affordable to most farmers (Than et al., 1987). Inoculation of soybean with *Rhizobium* is essential to increase productivity.

In Myanmar, researchers have been emphasizing the selection of inoculants to promote soybean production. However, the selection of strains compatible with cultivars grown in Myanmar is still limited. To foster soybean productivity, it is necessary to select strains compatible with cultivars. Therefore, the goal of the present study was to screen strains for nitrogen fixation by using Yezin-6 (*non-Rj*) and to evaluate the symbiotic effectiveness of selected *Bradyrhizobium* strains on different *Rj*-gene harboring Myanmar soybean cultivars.

MATERIALS AND METHODS

Origin of *Bradyrhizobium* strains

The *Bradyrhizobium japonicum* strain USDA110 was obtained from the Laboratory of Plant Nutrition, Kyushu University. Indigenous *Bradyrhizobium* strains were obtained from a previous experiment **(**Htwe et al., 2015a). The strains were selected from different groups identified in a phylogenetic tree. The origins of the isolates are provided in Table 1.

Origin of soybean varieties

Soybean varieties

Yezin-3 (Rj_4), Yezin-6 (*non-Rj*), Yezin-8 (*non-Rj*), Yezin-9 (Rj_3), Yezin-10 (Rj_2Rj_3) and Yezin-11 (Rj_4) were obtained from the Food Legume Section, Department of Agricultural Research, Yezin, Myanmar. Nodulation regulatory genes (*Rj* genes) indicated in parentheses were identified by Soe et al. (2013b) and Htwe et al. (2015b).

Cultivation

The 1 L pots were filled with vermiculite and 0.6 L of half-strength modified Hoagland nutrient (MHN) solution (Nakano et al., 1997). The pots were autoclaved at 120°C for 20 min. Control pots were also prepared to check for contamination. For surface sterilization, the seeds were soaked in 2.5% sodium hypochlorite solution for 5 min, rinsed with 10 mL of 99.5% ethanol five times and washed with sterilized MHN solution five times to remove the traces of sodium hypochlorite and ethanol. Five surface sterilized seeds were sown in the pots.

The single pure colony of the *B. japonicum* strain USDA110, and indigenous strains from an A1E plate, were cultured in A1E liquid media (Kuykendall, 1979) and incubated on a rotary shaker at 30°C for 7 days. One milliliter of liquid culture of each isolate was diluted with 99 mL of sterilized Hoagland solution to prepare abacterial suspension of about 10^7cells mL^{-1}. Each seed was inoculated with 5 mL of bacterial suspension. The plants were cultivated in an environmentally controlled room (25°C and 75% relative humidity) for 4 weeks. Watering was done as necessary. Autoclaved deionized water was used in this study. The first screening experiment was performed from February 2015 to March 2015. From first screening experiment, five effective strains showing the higher nitrogenase activity compared to *B. japonicum* USDA110 and other indigenous strains in Yezin-6 (*non-Rj*) soybean cultivar were selected for second screening experiment. These selected five strains were evaluated for symbiotic efficacy on different *Rj*-

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Table 1. The origins of Bradyrhizobium strains isolated from Myanmar.

Isolate (Accession number)	Species name	Isolated area	Location
AHY6-1 (LC037244)	*B. liaoningense*	Aungban	Shan State
SMY3-1 (LC037334)	*B. liaoningense*	Myaung	Sagaing Region
MMY3-2 (LC037285)	*B. liaoningense*	Madaya	Madalay Region
MMY6-9 (LC037292)	*B. liaoningense*	Madaya	Madalay Region
SAY6-1 (LC037304)	*B. japonicum*	Aungban	Shan State
SHY3-10 (LC037323)	*B. japonicum*	Heho	Shan State
SAY3-7 (LC037300)	*B. japonicum*	Aungban	Shan State
SMY6-4 (LC037347)	*B. yuanmingense*	Myaung	Sagaing Region
SMY6-10 (LC037353)	*B. yuanmingense*	Myaung	Sagaing Region
SAY3-5 (LC037298)	*B. elkanii*	Aungban	Shan State
SAY6-2 (LC037305)	*B. elkanii*	Aungban	Shan State
AHY3-1 (LC037234)	*B. elkanii*	Hinthada	Ayeyawaddy Region
BLY6-1 (LC037264)	*B. elkanii*	Letpandan	Bago Region
BLY3-1 (LC037254)	*B. elkanii*	Letpandan	Bago Region
MMY6-1 (LC037284)	*B. elkanii*	Madaya	Madalay Region
SAY3-4 (LC037297)	*B. elkanii*	Aungban	Shan State
SAY6-5 (LC037308)	*B. elkanii*	Aungban	Shan State
AHY6-8 (LC037251)	*B. elkanii*	Hinthada	Ayeyawaddy Region
SMY3-5 (LC037338)	*B. elkanii*	Myaung	Sagaing Region
SHY3-1 (LC037314)	*Bradyrhizobium* spp.	Heho	Shan State
SHY6-1 (LC037324)	*Bradyrhizobium* spp.	Heho	Shan State
MMY3-5 (LC037278)	*Bradyrhizobium* spp.	Madaya	Madalay Region
MMY3-7 (LC037280)	*Bradyrhizobium* spp.	Madaya	Madalay Region
AHY3-6 (LC037239)	*Bradyrhizobium* spp.	Hinthada	Ayeyawaddy Region
AHY3-9 (LC037242)	*Bradyrhizobium* spp.	Hinthada	Ayeyawaddy Region

Source: Htwe et al. (2015a).

gene-harboring soybean cultivars designated as Yezin-6 (*non-Rj*), Yezin-9 (Rj_3), Yezin-10 (Rj_2Rj_3) and Yezin-11 (Rj_4) from June 2015 to July 2015. Htwe et al. (2015b) stated that Yezin-6 (*non-Rj*), Yezin-9 (Rj_3), Yezin-10 (Rj_2Rj_3) and Yezin-11 (Rj_4) showed the higher nitrogen fixation and nodulation due to inoculation with *B. japonicum* USDA110. Therefore, these four efficient soybean cultivars for nitrogen fixation were used to evaluate the symbiotic effective of selected strains for second screening experiment. From second screening experiment, three different strains showing higher nitrogenase activity in Yezin-6 (*non-Rj*) and Yezin-11 (Rj_4) were selected for further experiment. Symbiotic effectiveness of these three selected strains was tested on Yezin-3 (Rj_4) and Yezin-8 (*non-Rj*) in August 2015. This experiment aimed to check the effectiveness of these selected strains on other Rj_4 and *non-Rj* genotypes soybean cultivars. Nodulation, nitrogenase activity, shoot dry weight and root dry weight were measured from three plants per pot at 28 days after sowing.

Acetylene reduction assay

The soybean plants were cut at the cotyledonary nodes and the roots with intact nodules placed in 100 mL conical flasks. Flasks were sealed with a serum stopper and injected with 12 mL of (C_2H_2) gas to replace air with acetylene. One-mL subsamples were analyzed for ethylene (C_2H_4) concentration at 5 and 65 min after injecting with C_2H_2 gas, using a flame ionization gas chromatograph (GC-14A, Shimadzu, Kyoto, Japan) equipped with a stainless steel column (3 mm diameter, 0.5 m long) as described by Soe and Yamakawa (2013a). After the assay, nodules were counted. Shoots, roots and nodules were collected separately and oven dried at 70°C for 24 h to record dry weights.

Statistical analysis

Data were analyzed using the STATISTIX 8 software package (Analytical Software, Tallahassee, FL, USA) and the means were compared by Tukey's HSD test with a P value < 0.05 taken to indicate statistical significance.

RESULTS

Screening of effective bacterial strains by Yezin-6 (*non-Rj*) for N fixation

The cultivar Yezin-6 (*non-Rj*) was inoculated with 25 *Bradyrhizobium* strains isolated in Myanmar and the strain USDA110, and compared for nodulation and nitrogenase activity. They were not significantly different in terms of shoot or root dry weight (Table 2). The highest nodule counts were found in the plants inoculated with *B. japonicum* USDA110. The highest nodule dry weights

Table 2. Effect of Myanmar Bradyrhizobial strains on the acetylene-reducing activity, nodulation and plant growth of Yezin-6 soybean cultivar at 28 days after sowing.

Strain	NN (No. plant^{-1})	NDW (mg plant^{-1})	SDW (g plant^{-1})	RDW (g plant^{-1})	ARA (µmol C_2H_4 h^{-1} plant^{-1})
AHY6-1	10.00^{abcd}	28.60^{ab}	0.39^{a}	0.18^{a}	1.23^{ab}
SMY3-1	8.33^{abcd}	24.00^{ab}	0.41^{a}	0.17^{a}	**1.95^{ab}**
MMY3-2	10.67^{abcd}	24.70^{ab}	0.33^{a}	0.15^{a}	1.46^{ab}
MMY6-9	8.67^{abcd}	23.70^{ab}	0.34^{a}	0.17^{a}	1.45^{ab}
SAY6-1	10.33^{abcd}	26.60^{ab}	0.33^{a}	0.14^{a}	0.70^{ab}
SHY3-10	9.67^{abcd}	30.90^{ab}	0.37^{a}	0.15^{a}	1.12^{ab}
SAY3-7	5.67^{cd}	33.50^{ab}	0.34^{a}	0.17^{a}	**2.21^{a}**
SMY6-4	9.67^{abcd}	24.80^{ab}	0.38^{a}	0.17^{a}	1.29^{ab}
SMY6-10	8.00^{bcd}	25.00^{ab}	0.38^{a}	0.17^{a}	**1.45^{ab}**
SAY3-5	13.67^{abc}	35.80^{ab}	0.40^{a}	0.17^{a}	1.22^{ab}
SAY6-2	10.33^{abcd}	27.90^{ab}	0.34^{a}	0.16^{a}	1.15^{ab}
AHY3-1	10.33^{abcd}	36.10^{ab}	0.33^{a}	0.15^{a}	**2.00^{ab}**
BLY6-1	7.67^{bcd}	28.90^{ab}	0.38^{a}	0.16^{a}	1.48^{ab}
BLY3-1	9.00^{abcd}	32.60^{ab}	0.35^{a}	0.15^{a}	1.35^{ab}
MMY6-1	7.33^{bcd}	36.50^{ab}	0.35^{a}	0.15^{a}	1.32^{ab}
SAY3-4	15.67^{ab}	40.20^{a}	0.33^{a}	0.16^{a}	1.21^{ab}
SAY6-5	10.67^{abcd}	31.00^{ab}	0.29^{a}	0.13^{a}	1.05^{ab}
AHY6-8	10.67^{abcd}	29.90^{ab}	0.26^{a}	0.15^{a}	1.22^{ab}
SMY3-5	11.67^{abcd}	35.40^{ab}	0.30^{a}	0.13^{a}	1.34^{ab}
SHY3-1	12.67^{abcd}	38.20^{ab}	0.32^{a}	0.13^{a}	0.90^{ab}
SHY6-1	8.00^{bcd}	40.40^{a}	0.37^{a}	0.15^{a}	1.22^{ab}
MMY3-5	5.67^{cd}	25.90^{ab}	0.34^{a}	0.16^{a}	0.81^{b}
MMY3-7	6.33^{cd}	27.40^{ab}	0.33^{a}	0.19^{a}	1.47^{ab}
AHY3-6	8.00^{bcd}	23.60^{ab}	0.30^{a}	0.17^{a}	**1.57^{ab}**
AHY3-9	4.67^{d}	19.30^{b}	0.28^{a}	0.15^{a}	1.00^{ab}
USDA110	17.00^{a}	21.90^{ab}	0.30^{a}	0.14^{a}	0.86^{ab}

Mean values in each column followed by the same letters are not significantly different at $P<0.05$ (Tukey's test). NN, nodule number; NDW, nodule dry weight; SDW, shoot dry weight; RDW, root dry weight; ARA, acetylene reduction activity. The selection of strains was based on nitrogenase activity. Isolates in bold were selected for second screening experiment.

were obtained from the plants inoculated with *Bradyrhizobium* spp. SHY6-1 and *B. elkanii* SAY3-4, but they were only significantly different from the strain *Bradyrhizobium* spp. AHY3-9.

In this screening experiment, the strains were compared for their ability to fix N, in terms of acetylene reduction activity (ARA) per plant. The nitrogenase activity of each bacterial strain on the cultivar Yezin-6 (non-*Rj*) is described in Table 2. The highest ARA per plant was observed for the *B. japonicum* strain SAY3-7. The top four *Bradyrhizobium* strains, designated as *B. japonicum* SAY3-7, *B. elkanii* AHY3-1, *B. liaoningense* SMY3-1 and *Bradyrhizobium* spp. AHY3-6, had superior N fixing ability compared to the other strains. Nitrogenase activities of *B. liaoningense* MMY3-2, *B. elkanii* BLY6-1 and *Bradyrhizobium* spp. MMY3-7 were higher than that of *B. yuanmingense* SMY6-10, but they were not significantly different. Therefore, we selected the *B. yuanmingense* SMY6-10 because it belonged to different species from top four strains. Moreover, *B. yuanmingense* SMY6-10 was superior in terms of N fixation ability among the *B. yuanmingense* strains. Therefore, these five strains were selected to study their symbiotic effectiveness on different *Rj* genotypes.

Evaluation of the effectiveness of selected *Bradyrhizobium* strains on different *Rj*-gene soybean cultivars

Five *Bradyrhizobium* strains, designated as *B. japonicum* SAY3-7, *B. elkanii* AHY3-1, *B. liaoningense* SMY3-1, *Bradyrhizobium* spp. AHY3-6 and *B. yuanmingense* SMY6-10, and the control strain *B. japonicum* USDA110, were compared for symbiotic effectiveness on four soybean cultivars. For the cultivar Yezin-6 (non-*Rj*) inoculated with different *Bradyrhizobium* strains, the nitrogenase activity and nodule number per plant differed significantly among the strains (Table 3). Similarly, inoculation with *B. liaoningense* SMY3-1 showed

Table 3. Effect of six different bradyrhizobial strains on acetylene reduction activity, nodulation and plant growth of Yezin-6 soybean cultivar at 28 DAS.

Strain	NN (No. plant^{-1})	NDW (mg plant^{-1})	SDW (g plant^{-1})	RDW (g plant^{-1})	ARA (µmol C_2H_4 h^{-1} plant^{-1})
SAY3-7	3.67^{c}	26.00^{a}	0.45^{a}	0.22^{a}	1.25^{ab}
AHY3-1	17.33^{a}	38.40^{a}	0.51^{a}	0.26^{a}	0.90^{b}
SMY3-1	13.00^{ab}	23.80^{a}	0.46^{a}	0.22^{a}	1.67^{a}
SMY6-10	10.33^{abc}	20.60^{a}	0.44^{a}	0.17^{a}	0.82^{b}
AHY3-6	6.33^{bc}	23.50^{a}	0.48^{a}	0.22^{a}	0.82^{b}
USDA110	15.00^{a}	17.90^{a}	0.37^{a}	0.19^{a}	0.64^{b}

Mean values in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). NN, nodule number; NDW, nodule dry weight; SDW, shoot dry weight; RDW, root dry weight; ARA, acetylene reduction activity.

Table 4. Effect of six different bradyrhizobial strains on acetylene reduction activity, nodulation and plant growth of Yezin-9 soybean cultivar at 28 DAS.

Strain	NN (No. plant^{-1})	NDW (mg plant^{-1})	SDW (g plant^{-1})	RDW (g plant^{-1})	ARA (µmol C_2H_4 h^{-1} plant^{-1})
SAY3-7	8.33^{a}	31.10^{a}	0.43^{a}	0.20^{a}	1.13^{a}
AHY3-1	7.67^{a}	24.50^{b}	0.37^{a}	0.18^{a}	0.82^{a}
SMY3-1	7.67^{a}	13.40^{d}	0.33^{a}	0.17^{a}	1.01^{a}
SMY6-10	6.00^{a}	19.20^{bc}	0.39^{a}	0.18^{a}	0.97^{a}
AHY3-6	7.00^{a}	18.60^{cd}	0.36^{a}	0.15^{a}	0.63^{a}
USDA110	6.67^{a}	21.20^{bc}	0.40^{a}	0.19^{a}	0.97^{a}

Mean values in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). NN, nodule number; NDW, nodule dry weight; SDW, shoot dry weight; RDW, root dry weight; ARA, acetylene reduction activity.

significantly higher nitrogenase activity among tested strains, but the activity was not significantly different from *B. japonicum* SAY3-7. *Bradyrhizobium elkanii* AHY3-1 and *B. japonicum* USDA110 produced a significantly greater number of nodules than the other strains, but nodule production was not significantly different from that of *B. liaoningense* SMY3-1 and *B. yuanmingense* SMY6-10. In the cultivar Yezin-6 (non-*Rj*), dry weights of nodules, shoots and roots were not significantly different among the *Bradyrhizobium* strains (Table 3). These results confirmed that *B. liaoningense* SMY3-1 and *B. japonicum* SAY3-7 were superior in terms of the ability to fixN, and *B. elkanii* AHY3-1 was superior for nodulation.

In cultivar Yezin-9 (Rj_3), nodule dry weight differed significantly among the inoculated *Bradyrhizobium* strains (Table 4), although nodule number, shoot dry weight and root dry weight N activity were not significantly different. Among the tested strains, *B. japonicum* SAY3-7 produced a significantly greater nodule mass. *Bradyrhizobium japonicum* SAY3-7 had the highest N fixing ability among inoculated strains, although it was not superior for nitrogenase activity. These results indicated that the *B. Japonicum* strain SAY3-7 was more effective in terms of nodulation, N fixation and plant growth for the cultivar Yezin-9 (Rj_3).

There was significant difference in nodule number and nodule dry weight for the cultivar Yezin-10 (Rj_2Rj_3), inoculated with *Bradyrhizobium* strains. *Bradyrhizobium elkanii* AHY3-1 produced a sgnificantly greater number of nodules and higher nodule dry weight, although there were no significant differences with *B. liaoningense* SMY3-1. Nitrogenase activity and the shoot and root dry weights were not significantly different among the inoculated strains (Table 5). These results confirmed that *B. elkanii* AHY3-1 was more effective in nodule formation on the roots of Yezin-10 (Rj_2Rj_3).

For Yezin-11 (Rj_4), the nitrogenase activity, nodule number and nodule dry weight were significantly different among the *Bradyrhizobium* strains (Table 6). A higher level of N fixation, in term of nitrogenase activity, was observed for Yezin-11 (Rj_4) inoculated with *B. japonicum* SAY3-7and *B. elkanii* AHY3-1, although they were not significantly different from Yezin-11 (Rj_4) inoculated with *B. liaoningense* SMY3-1, *B. yuanmingense* SMY6-10 or *B. japonicum* USDA110. *B. elkanii* AHY3-1 produced a significantly greater number of nodules compared to the other strains, but it was not significantly different from *B. japonicum* USDA110. Moreover, *B. elkanii* AHY3-1 produced a significantly higher nodule dry weight compared with the other strains. Shoot and root dry weights were not significantly different among the *Bradyrhizobium* strains (Table 6). These results demonstrated that the strains *B. elkanii* AHY3-1 and *B. japonicum* SAY3-7 were more effective in terms of

Table 5. Effect of six different bradyrhizobial strains on acetylene reduction activity, nodulation and plant growth of Yezin-10 soybean cultivar at 28 DAS.

Strain	NN (No. plant^{-1})	NDW (mg plant^{-1})	SDW (g plant^{-1})	RDW(g plant^{-1})	ARA (µmol C_2H_4 h^{-1} plant^{-1})
SAY3-7	4.33^{c}	11.20^{b}	0.35^{a}	0.15^{a}	0.44^{a}
AHY3-1	15.67^{a}	23.60^{a}	0.42^{a}	0.19^{a}	0.54^{a}
SMY3-1	13.33^{ab}	16.40^{ab}	0.43^{a}	0.18^{a}	0.77^{a}
SMY6-10	8.67^{bc}	11.80^{b}	0.41^{a}	0.17^{a}	0.48^{a}
AHY3-6	10.33^{bc}	11.00^{b}	0.41^{a}	0.17^{a}	0.59^{a}
USDA110	9.00^{bc}	13.70^{b}	0.36^{a}	0.17^{a}	0.56^{a}

Mean values in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). NN, nodule number; NDW, nodule dry weight; SDW, shoot dry weight; RDW, root dry weight; ARA, acetylene reduction activity.

Table 6. Effect of six different bradyrhizobial strains on acetylene reduction activity, nodulation and plant growth of Yezin-11 soybean cultivar at 28 DAS.

Strain	NN (No. plant^{-1})	NDW (mg plant^{-1})	SDW (g plant^{-1})	RDW (g plant^{-1})	ARA (µmol C_2H_4 h^{-1} plant^{-1})
SAY3-7	5.67^{b}	27.20^{ab}	0.39^{a}	0.20^{a}	1.41^{a}
AHY3-1	19.00^{a}	33.50^{a}	0.46^{a}	0.24^{a}	1.41^{a}
SMY3-1	11.67^{ab}	23.20^{abc}	0.43^{a}	0.22^{a}	1.03^{ab}
SMY6-10	7.33^{b}	11.90^{c}	0.48^{a}	0.24^{a}	0.67^{ab}
AHY3-6	5.67^{b}	18.60^{bc}	0.45^{a}	0.20^{a}	0.59^{b}
USDA110	14.00^{ab}	25.10^{abc}	0.42^{a}	0.22^{a}	1.23^{ab}

Mean values in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). NN, nodule number; NDW, nodule dry weight; SDW, shoot dry weight; RDW, root dry weight; ARA, acetylene reduction activity.

Table 7. Effect of three different bradyrhizobial strains on acetylene reduction activity, nodulation and plant growth of Yezin-3 soybean cultivar at 28 DAS.

Treatment	NN (No. plant^{-1})	NDW (mg plant^{-1})	SDW (g plant^{-1})	RDW (g plant^{-1})	ARA (µmol C_2H_4 h^{-1} plant^{-1})
SAY3-7	9.33^{b}	28.00^{a}	0.57^{a}	0.23^{a}	0.85^{a}
AHY3-1	27.00^{a}	39.70^{a}	0.75^{a}	0.32^{a}	0.53^{a}
SMY3-1	14.33^{ab}	31.50^{a}	0.65^{a}	0.24^{a}	0.95^{a}

Mean values in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). NN, nodule number; NDW, nodule dry weight; SDW, shoot dry weight; RDW, root dry weight; ARA, acetylene reduction activity.

nitrogen fixation on Yezin-11 (Rj_4). Moreover, it was found that *B. elkanii* AHY3-1 has very efficientnodulation, as indicated by superior nodule counts and higher nodule dry weight.

Effectiveness of selected *Bradyrhizbium* strains on Yezin-3 (Rj_4) and Yezin-8 (*non-Rj*)

Three *Bradyrhizobium* strains, designated as *B. japonicum* SAY3-7, *B. elkanii* AHY3-1, and *B. liaoningense* SMY3-1, were evaluated on the soybean cultivars Yezin-3 (Rj_4) and Yezin-8 (*non-Rj*) for symbiotic effectiveness and compatibility. On Yezin-3 (Rj_4), nodule number was significantly different among these inoculated *Bradyrhizobium* strains (Table 7). Nodule dry weight, shoot dry weight, root dry weight and nitrogenase activity did not differ significantly among the tested isolates. *B. elkanii* AHY3-1 produced more nodules than the other strains, but nodule production was not significantly different from *B. liaoningense* SMY3-1. *B. elkanii* AHY3-1 had greater nodule dry weight compared to the other strains. These results indicated that *B. elkanii* AHY3-1 is superior for nodule production efficiency on Yezin-3 (Rj_4).The nitrogenase activities of *B. liaoningense* SMY3-1 and *B. japonicum* SAY3-7 was relatively greater than for *B. elkanii* AHY3-1, although they were not significantly different. These results suggest that *B. liaoningense* SMY3-1 and *B. japonicum* SAY3-7 are more effective, compared to the other strains, in terms of N

Table 8. Effect of three different bradyrhizobial strains on acetylene reduction activity, nodulation and plant growth of Yezin-8 soybean cultivar at 28 DAS.

Treatment	NN (No. plant^{-1})	NDW (mg plant^{-1})	SDW (g plant^{-1})	RDW (g plant^{-1})	ARA (µmol C_2H_4 h^{-1} plant^{-1})
SAY3-7	17.00^{a}	50.30^{a}	0.54^{a}	0.29^{a}	1.31^{a}
AHY3-1	19.00^{a}	41.70^{a}	0.46^{ab}	0.27^{a}	0.55^{b}
SMY3-1	19.67^{a}	23.10^{b}	0.42^{b}	0.25^{a}	0.74^{b}

Mean values in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). NN, nodule number; NDW, nodule dry weight; SDW, shoot dry weight; RDW, root dry weight; ARA, acetylene reduction activity.

fixation on Yezin-3 (*Rj*$_4$).

For Yezin-8 (non-*Rj*), nodule dry weight, shoot dry weight and nitrogenase activity were different among the *Bradyrhizobium* strains (Table 7). Inoculation with *B. japonicum* SAY3-7 resulted in significantly higher nitrogenase activity among the tested strains. Significantly greater nodule dry weight and shoot weight were obtained from the plants inoculated with *B. japonicum* SAY3-7. Nodule number and root dry weight were not significantly different among the *Bradyrhizobium* strains (Table 8). These results highlighted *B. japonicum* SAY3-7 as having greater N fixing ability.

DISCUSSION

Nitrogen is an essential nutrient for plant growth. However, its availability is one of the major limiting factors in plant growth and development (Newbould, 1989). Therefore, current agricultural practices primarily rely on chemical fertilizers to increase productivity (Peoples et al., 2009; Jensen et al., 2012). The heavy use of chemical fertilizers in agriculture is of global concern: alternatives to chemical fertilizers are urgently needed (Sharma and Kumawat, 2011). Biological nitrogen fixation technology is now one such alternative, as it reduces the need for chemical nitrogenous fertilizer (Stacey et al., 2006). Therefore, rhizobia have been used extensively in agriculture to enhance nitrogen fixation (Elkan, 1992).

Soybean, in symbiosis with *Bradyrhizobium*, has the ability to fix nitrogen at a rate of up to 300 kg N ha^{-1} under favorable conditions (Smith and Hume, 1987). Optimizing this symbiosis can both increase yields and improve soil fertility, thus reducing the costs and environmental impacts caused by nitrogen fertilizer application (Peoples et al., 2009; Canfield et al., 2010). Therefore, increasing the use of legume crops and identifying important factors related to nodulation are needed to reduce the use of nitrogenous fertilizers and improve the sustainability of agriculture (Peoples et al., 2009; Canfield et al., 2010; Jensen et al., 2012). Selection of an efficient strain is considered to be one of the major factors affecting symbiotic N fixation.

Selection of *Rhizobium* strains suitable for each legume cultivar is essential in inoculant production. To produce inoculants for each recommended cultivar of soybean, several strains and isolates of rhizobia were initially screened in the greenhouse for their symbiotic effectiveness and nitrogen fixation ability. Then, selected strains and isolates were produced as inoculants and tested for nodulation, nitrogen fixation ability and yield response (Boonkerd and Singleton, 2002). Screening experiments were performed in a greenhouse, under controlled conditions, to select the most effective strains for each cultivar.

The symbiotic effectiveness of 25 *Bradyrhizobium* strains on Yezin-6 (non-*Rj*) was determined, as cultivars harboring non-*Rj* genes are compatible with all types of *Bradyrhizobial* strains (Ishizuka et al., 1991). The top four strains showing higher nitrogenase activity were *B. japonicum* SAY3-7, *B. elkanii* AHY3-1, *B. liaoningense* SMY3-1 and *Bradyrhizobium* spp. AHY3-6. Moreover, *B. yuanmingense* SMY6-10 belonged to different species from top four strains was also selected. Since the effectiveness of *Rhizobium* strains varies, the first step for inoculant production is to obtain the most effective strain for N fixation for the legumes to be inoculated (Kucey et al., 1988). Therefore, these five strains were selected for the next experiment.

Five strains were evaluated for their symbiotic effectiveness on four soybean cultivars: Yezin-6 (*non-Rj*), Yezin-9 (*Rj*$_3$), Yezin-10 (*Rj*$_2$*Rj*$_3$) and Yezin-11 (*Rj*$_4$). When they were compared fornitrogenase activity, it was found that all tested Myanmar *Bradyrhizobial* strains were more effective on Yezin-6 (*non-Rj*)and Yezin-11 (*Rj*$_4$), but not on Yezin-9 (*Rj*$_3$) and Yezin-10 (*Rj*$_2$*Rj*$_3$). This might be due to regulatory genes, as *Rj* genes have the ability to control the compatibility of hosts and rhizobia (Ishizuka et al., 1991; Saeki et al., 2000). In this study, *Bradyrhizobium* spp. AHY3-6 and *B. yuanmingense* SMY6-10 strains showed lower N fixation efficiency, in terms of ARA, on all cultivars when compared with other strains. It was clear that the N fixation rates depended on not only cultivars and inoculated bacterial strains, but also on the interaction between cultivar and strain. Graham (2000) showed that nitrogen fixation levels vary among legume cultivars; therefore, the higher efficiency with respect to nitrogen fixation is also dependent on particular combinations of strains and cultivarsin some crops. It has

also been reported that the effectiveness of *Bradyrhizobium* strains varies with soybean genotype (Okereke et al., 2001; Tien et al., 2002).

The top three strains, *B. japonicum* SAY3-7, *B. liaoningense* SMY3-1 and *B. elkanii* AHY3-1,which were shown to be more effective on Yezin-6 (*non-Rj*) and Yezin-11 (Rj_4) cultivars, were tested on other Rj_4 and *non-Rj* cultivars, identified as Yezin-3 and Yezin-8, respectively, to determine their compatibility and effectiveness. These results indicated that *B. japonicum* SAY3-7 had the highest nitrogen fixing ability on Yezin-8 (*non-Rj*), but the nitrogenase activity of that strain was not significantly different from those of *B. liaoningense* SMY3-1 and *B. elkanii* AHY3-1 strains on Yezin-3 (Rj_4). In Yezin-3 (Rj_4) soybean cultivar, *B. elkanii* AHY3-1 produced a greater number of nodules and nodule mass although nitrogenase activity did not differ among inoculated strains. Adhikari et al. (2013) also stated that strains producing greater nodule mass do not increase N fixation, in terms of ARA.

B. japonicum SAY3-7 had consistently higher nitrogenase activity, especially on *non-Rj*- and Rj_4- gene harboring soybean cultivars was selected for *Rhizobium* inoculant production. Israel et al. (1986) reported that selection of host cultivar-compatible inoculantsis important for increasing nitrogen fixation in soybean. The extent of nitrogen fixation by soybean cultivars may vary depending on the symbiotic effectiveness of *Rhizobium* strains and their compatibility. Our results supported the findings of other studies which is clearly stated that increases in biological nitrogen fixation in soybean production can be obtained by selecting effective strains and efficient soybean cultivars as cultivar-strain pairs (Duong et al., 1984; Kucey et al., 1988; Thi, 2007; Soe and Yamakawa, 2013a).

Conclusion

In this study, 25 *Bradyrhizobium* strains were selected and studied for their symbiotic effectiveness on the cultivar Yezin-6 (non-*Rj*).The five *Bradyrhizobium* strains, *B. japonicum* SAY3-7, *B. elkanii* AHY3-1, *B. liaoningense* SMY3-1, *Bradyrhizobium* spp. AHY3-6 and *B. yuanmingense* SMY6-10, were selected for their greater nitrogen fixing capacity. They were tested on different *Rj*-gene-harboring Myanmar soybean cultivars. *B. japonicum* SAY3-7, *B. liaoningense* SMY3-1, and *B. elkanii* AHY3-1 were more effective on Yezin-6 (*non-Rj*) and Yezin-11 (Rj_4), whereas they were not effective on Yezin-9 (Rj_3) or Yezin-10 (Rj_2Rj_3). These three strains were superior in terms of nitrogen fixing ability compared with other indigenous strains and the most widely used exotic strain, *B. japonicum* USDA110. They were tested on Yezin-8 (*non-Rj*) and Yezin-3 (Rj_4) to evaluate their effectiveness and compatibility. In this experiment, *B. japonicum* SAY3-7 was found to be the most effective strain for N fixationon Yezin-8 (non-*Rj*). Over all of the experiments, *B. japonicum* SAY3-7 was the most effective strain for N fixation, as it showed superior nitrogenase activity on Yezin-6 (non-*Rj*) in the first screening experiment, on Yezin-6 (*non-Rj*), Yezin-9 (Rj_3) and Yezin-11 (Rj_4) cultivars in the second experiment, and on Yezin-8 (non-*Rj*) in the third experiment. According to the results of this study, the most effective strain, *B. japonicum* SAY3-7, was selected to be used as an inoculant for specific cultivars to increase soybean production in Myanmar. This study was conducted in pots under controlled conditions. *B. japonicum* SAY3-7 should also be evaluated on Yezin-6 (*non-Rj*), Yezin-8 (*non-Rj*) and Yezin-11 (Rj_4) and Yezin-3 (Rj_4) for symbiotic effectiveness under field conditions.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Chromogenic agar media for rapid detection of Enterobacteriaceae in food samples

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Recently, chromogenic media were used for rapid detection of pathogenic agents isolated from different materials (food, water and stool). Results of the present study indicated that specific and rapid identification of Enterobactiriaceae isolates from 46 food samples (egg, dairy products, meat, salads and cooked rice) as compared to conventional methods of biochemical test and plating selective media consume times, are expensive , autoclaving, and less selective. Both media ECC Agar (HIMEDIA) and Salmonella differential agar (Raj Hans Medium, HIMEDIA) are powerful for screening isolates from food samples: *Esherichia coli, Salmonella* spp., *Klebsiella pneumoniea.*

Key words: Chromogenic media, Enterobacteriaceae, detection, food.

INTRODUCTION

Enterobacteriaceae, *Esherichia coli* coliform are the most infectious bacteria of foodstuff products. The detection and quantification of this emerging pathogen is therefore an important task for microbiological food and clinical diagnostic laboratories. Traditional methods for bacterial detection like biochemical test have been used for long. These methods consume time and materials. Previous study on Coliforms and specially *E. coli* made it possible to identify them as microbial contaminants marker in food and water. Their presence in drinking water and food indicates that these materials are contaminated with other enteric pathogens. So their isolation and enumeration have great importance in the determination of food hygiene (Muller et al., 2001). Standard ISO 6579 2003 (Microbiology of food and animal feeding stuffs – Horizontal method for detection of *Salmonella* spp.) includes four stages of the detection process and depending on the need to obtain confirmations, it lasts for 5 to 7 days: 1. Pre-enrichment in non-selective liquid medium; 2. Selective enrichment in liquid media; 3. Plating on selective media; 4. Serological and biochemical identification of suspected colonies

For the detection of *Salmonella* spp. as a frequent cause of gastroenteritis, there is need to isolate the pathogen from stool samples. Media containing lactose, plus a pH indicator, have been traditionally used for differentiation of Salmonella (a non-fermenter) from Commensals such as *Escherichia coli*. However, it is frequently necessary to screen many other commensals that also fail to ferment lactose (e.g. *Proteus* spp.) to

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exclude the presence of *Salmonella*. The screening of commensal bacteria to exclude pathogens is time consuming and can be costly in terms of serological or biochemical reagents (Perry and Freydie`re, 2007).

Analysis of naturally contaminated food samples showed that enumeration and isolation of colonies was much easier on the chromogenic plating media, in particular on *Bacillus cereus* group plating medium (BCM), than on the standard plating media (Martina et al., 2008). Chromogenic media contain synthetic chromogenic substrates that are cleaved by specific enzymatic activities of certain micro-organisms (Manafi, 1996, 2000; Reissbrodt, 2005).

Over the last 30 years, a range of chromogenic media has been developed that are designed to target pathogens with high specificity. Such media exploit enzyme substrates that release coloured dyes upon hydrolysis, thus resulting in pathogens forming coloured colonies that can easily be differentiated from commensal flora. Ideally, commensal bacteria should either be inhibited completely by selective agents or form colourless colonies thus allowing pathogens to 'stand out' against background flora. This allows clear differentiation of microbes producing the target enzyme from those that do not. This is especially important when attempting to detect specific pathogens within polymicrobial cultures. The substrate and products of hydrolysis should be no inhibitory to microbial growth (Ledeboer et al., 2007). The goal of this study was to evaluate the efficiency of two chromogenic media in detecting and isolating Enterobacteriaceae from food samples.

MATERIALS AND METHODS

Media preparation

Brain heart infusion broth (BHI; DifcoLabrotaries, USA), selective media were used for Gram negative bacteria, Mac Conkey Agar (FlukaBioChemika), EMB Agar (HiMedia Laboratories) and Tetrathionate Broth (OXOID) were used for coliform and enteric pathogens, whilr Bismuth Sulphite Agar (HiMedia), Hekton Enteric Agar (OXOID), XLD Agar (Becton Dickinson), and SS Agar (Biolife) were used for isolation of *Salmonella* spp.

Two chomogenic media were prepared for detection of *E. coli* and other coliforms bacteria: Hicrome ECC Agar (HIMEDIA) and Salmonella Differential Agar (Raj Hans Medium, HIMEDIA). Each medium was prepared by boiling without autoclaving.

Food sampling

Forty six samples of different foods (eggs, salad, dairy products, meet and cooked rice) were purchased from local markets in Baghdad capital in Iraq.

Bacterial isolation on different media

25 g of each sample was added to 225 ml normal saline solution; after three dilutions of 0.1ml of each food suspension were cultured separately in the different media described above .The plates were incubated at 37°C for 24 h for full color development of chromogenic media.

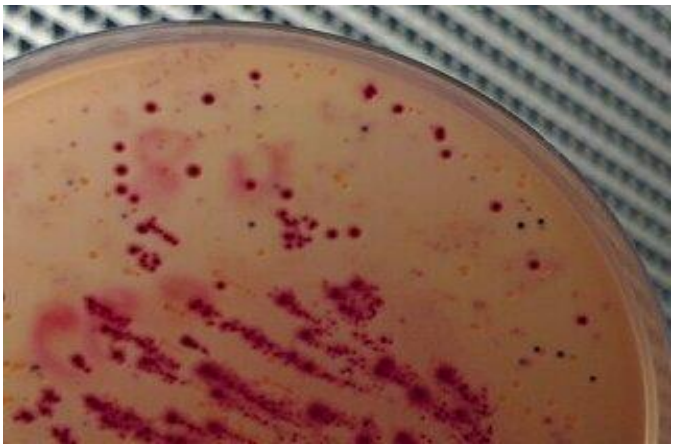

Figure 1. Stock solution of food sample on ECC Agar media.

Biochemical analysis

Exponentially growing cells of the various isolates were used for biochemical analysis in API 50 CHL kit (Biomerieux) following the manufacturer's instruction (Ramli et al., 2014).

RESULTS AND DISCUSSION

Forty six samples of different foods were examined for their bacterial micro-flora. All 46 samples contained mixtures of Enterobacteriasae, *E. coli* (n=28), *Klebsiella pneumonia* (n=7), *Salmonella* spp. (n=30). Every isolate appears with specific color on ECC & Salmonella differential agar. Stock solution of food samples contained many types of bacteria differentiated with the colors shown in Figure 1. *E. coli* isolates appear as blue and violet on ECC (Figure 2).

Salmonella spp. on two chromogenic media: *Salmonella* enteritidis is a colorless colony on ECC(A), *Salmonella* typhi & *Salmonella* typhimurium are pink colony on Salmonella Differential Agar (B) (Figure 3).

K. pneumonia is a blue colony on *Salmonella* Differential agar and green to torques on ECC (Figure 4). Preliminary identification of the isolate was based on the Gram stain. Enterobacteriaceae were detected on Mac Conkey Agar morphologically and culturally. Plated enrichment cultures from food samples grown overnight on Brain heart infusion broth, Mac Conkey Agar, EMB Agar, Bismuth Sulphite Agar, Hekton Enteric Agar, Tetrathionate Broth, XLD Agar, SS Agar media resulted in massive growth of Enterobacteriaceae, which made it difficult to isolate suspected bacteria. When comparing the chromogenic media for Enterobacteriaceae detection, ECC and *Salmonella* differential agar were found most suitable for Enterobactereiaceae members and tested

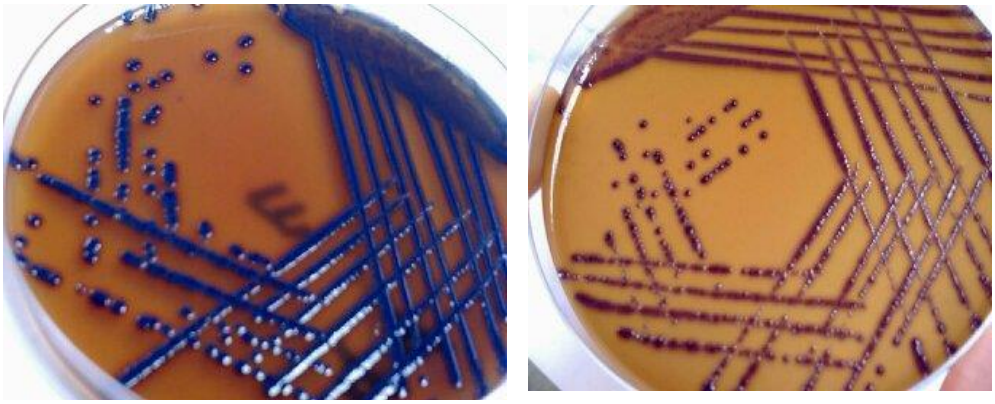

Figure 2. *E. coli* on ECC Agar media.

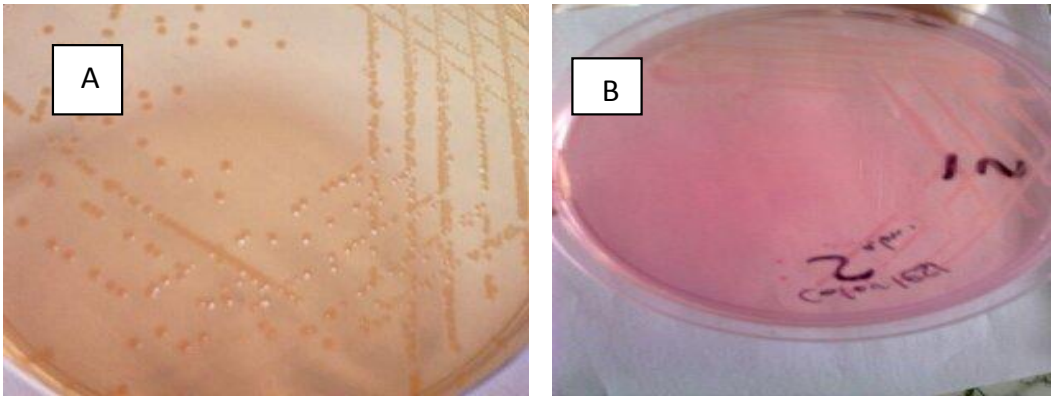

Figure 3. *Salmonella* spp. on *Salmonella* differential agar.

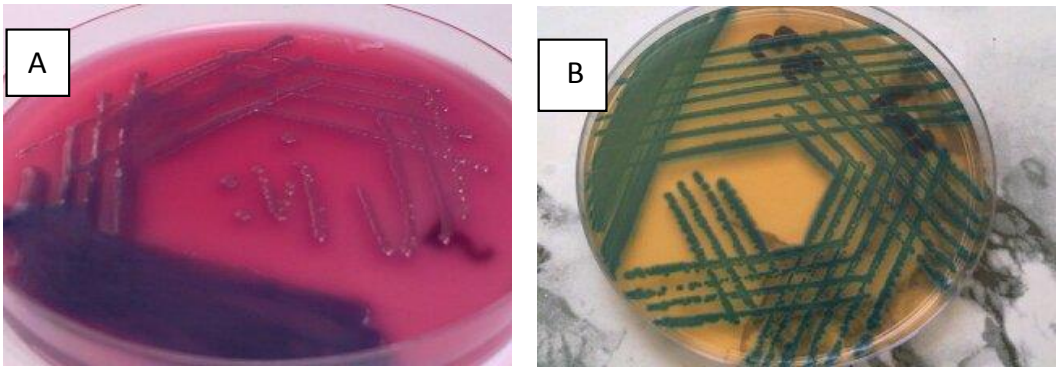

Figure 4. *K. pneumonia* (A) on Salmonella differential agar; (B) on ECC.

indicator media for the detection of Enterobacteriaceae from food samples. Replacement of Mac Conkey Agar and EMB Agarmedia by chromogenic media (ECC, Salmonella differential agar) resulted in an approximately 50% reduction of the natural bacterial background flora without decreasing numbers of detected bacterial isolates.

Interestingly, the bacterial background and flora from food sample were strongly inhibited. Salmonella colonies were isolated using chromogenic media and were

confirmed for their serotypes which is polyvalent *Salmonella* antiserum followed by specific O and H. Definitive identification of the isolates was undertaken using API 50 kits to characterize the carbohydrates – fermenting ability of the Enterobacteriaceae members. It indicated that sugars present in kit had potential to serve as discriminative markers for the selective isolation of the Enterobacteriaceae bacteria (Ramli et al., 2014).

In recent years, biotechnology advance has led to change in food test technique, and today, we benefit from methods that are more specific, faster and often more sensitive compared to conventional method (Stampi et al., 2004; Ten et al., 2004). Media containing lactose, plus a pH indicator, have been traditionally used for differentiation of *Salmonella* (a non-fermenter) from commensals such as *E. coli* (Tavakoli et al., 2008; Huang et al., 2010). The principle of chromogenic media is based on fermentation of carbohydrate that produces a localized pH drop initiating a color change in the indicator present in media. Overall we showed that two types of chromogenic media are good for rapid detection of food born bacteria; they are highly selective, need no autoclaving when compared with the conventional media that consume time, are expensive and poorly selective. These results are associated with other reports (Obeng-Nkruumah et al., 2013). Isolation and identification of food born bacteria like Enterobacteriaceae on chromogenic media were most suitable for the selection of all isolates that contribute to the color of the colonies due to the metabolic activity of these bacteria that reacted with the substrate of this media. In many studies chromogenic media demonstrate a proven advantage over conventional culture media due to a superior detection rate for target pathogens or a superior differentiation of mixed cultures. Media containing chromogenic substrates are invariably more expensive than conventional media but this can be offset by a reduced need for complementary reagents and less labor time associated with the processing of culture plates and suspect pathogens. Due to these factors, the use of chromogenic media in diagnostic laboratories is increasingly widespread. It is likely in the next few years that a wider range of pathogens will be targeted to continue the rapid expansion of the range of chromogenic media available for isolation (Paniagua et al., 2010; Ramli et al., 2014). Hicrome ECC Agar and *Salmonella* differential agar were useful media for rapid detection of food born bacteria that cause many diseases.

Conclusion

From this study, ECC, Salmonella differential agar showed high specificity and sensitivity as in other reports. However, the drawback in this study was limited to two chromogenic media used for screening Enterobacteriaceae. Nevertheless, they can be alternative for cheap routine Enterobacteriaceae to prompt the initiation of infection control measures.

Conflict of Interests

The authors have not declared any conflict of interests.

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African Journal of Microbiology Research

Full Length Research Paper

Serogroups and antibiotics susceptibility pattern of avian pathogenic *Escherichia coli* strains responsible for colibacillosis in broiler breeding farms in the east of Algeria

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In East Algeria, 100 *Escherichia coli* strains were isolated from livers and spleens of 120 broiler carcasses that presented colibacillosis lesions at autopsy. Serogrouping of these strains demonstrated that 83% of their serogroups belong to the most pathogenic serogroups of Avian Pathogenic *Escherichia coli* (APEC): O1 (14%), O2 (53%) and O78 (16%). *In vitro* susceptibility to antimicrobials of veterinary significance was determined by disc diffusion test. Antibiogram demonstrated a high level of resistance to enrofloxacin (82%), trimethoprim-sulfmethoxazole (82%), ampicillin (89%) and amoxicillin/ Ac clavulanic (90%), nalidixic acid (99%) and tetracycline (100%). There were moderate levels of resistance to neomycin (49%) and nitrofurantoin (53%). There were low levels of resistance to gentamicin (2%), chloramphenicol (13%) and no resistance to colistin sulfate (0%). All strains were multi-drug resistant and more than half (58%) of the isolates were resistant to seven antibiotics at least. Thus, thirty one antibiotic resistant patterns of *E. coli* strains were detected, of which 11 were present significantly. Co-resistance was found in eight of the eleven most common multidrug resistant patterns, 62% of the strains expressed this co-resistance. *E. coli* strains isolated expressed resistances to molecules that are routinely used in the field. It is clear that these are less effective against colibacillosis. It is more than ever necessary to perform antibiotic susceptibility testing prior to treatment in order to prescribe the molecule of choice, and set up a monitoring program in Algeria to monitor antimicrobial resistance in pathogenic bacteria that could be potentially transmitted to humans from animal food.

Key words: Algeria, antibiotic resistance, colibacillosis, *Escherichia coli*, serogroups.

INTRODUCTION

Changes in the poultry industry over the past few decades have largely contributed to its economic success. However, they have also created conditions favourable to contagious disease outbreaks. The increase in farm size, in regional farm density, and the increase in alternative poultry productions are among risk

factors that have led to the emergence and re-emergence of poultry diseases (Vaillancourt, 2009). Colibacillosis is the most frequently reported disease in surveys of poultry diseases or condemnations at processing (Saif, 2003). Avian colibacillosis is a bacterial disease, and it is referred to any localized or systemic infection caused entirely or partly by Avian Pathogenic *Escherichia coli* (APEC), including colisepticemia, coligranuloma (Hjarre's disease), air sac disease (chronic respiratory disease, CRD), cellulites (inflammatory process), swollen-head syndrome, peritonitis, salpingitis, osteomyelitis/synovitis (turkey osteomyelitis complex), panophthalmitis, and omphalitis/yolk sac infection (Stordeur and Mainil, 2002; Saif, 2003; Robineau and Moalic, 2010). In broiler chickens, colisepticemia and air sacs disease are the most common forms of colibacillosis (Stordeur and Mainil, 2002; Saif, 2003). Several serogroups of APEC strains are known for their virulence. The first studies on avian *E. coli* by Sojka and Carnaghan (1961) showed that the most common serogroups were O1, O2, O35 and O78. More recently, studies of 112 *E. coli* strains isolated from cases of colibacillosis in Canada by Dozois et al. (1992) showed that 16 serogroups were represented including serogroups O78 (52%) and O1 (6%) which were the most frequent. Other significantly represented serogroups associated with avian colibacillosis are: O8, O15, O18, O35, O88, O109, O115 and O116 (Bree et al., 1989; Dho-Moulin et al., 1990; Babai et al., 1997; Blanco et al., 1997; Dho-Moulin and Fairbrother, 1999). Colibacillosis is an economical devastating disease in the poultry industry; it is the primary cause of morbidity, mortality and condemnation of carcasses in Algeria and many parts of the world (Dho-Moulin and Fairbrother, 1999; Delicato et al., 2003; Ewers et al., 2003; Hammoudi and Aggad, 2008). Colibacillosis is likely the primary cause of antibiotic treatment in poultry house, and the emergence of resistant strains is a legitimate concern (Robineau and Moalic, 2010). Antibiotic usage is considered the most important factor promoting the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine (Neu, 1992; Witte, 1998). Resistance to two or more classes of antibiotics is now common in veterinary medicine, and the choice of antibiotic for treatment remains quite arbitrary. In Algeria, very little data are available in epidemiology of antimicrobial resistance of *E. coli* and serogroups from which they belong. Hence, the objective of our study was to get a better knowledge of *E. coli* isolates responsible for colibacillosis in broiler breeding farms. The present study determined serogroups and the sensitivity to 11 antibiotics among a collection of one hundred avian pathogenic *E. coli,* isolates from diseased birds diagnosed with colibacillosis in five cities in the east Algeria.

MATERIALS AND METHODS

Sampling method

From August to October 2014, 120 chicken carcasses were selected from twenty-four poultry houses (five broilers from each poultry house) implemented in the following cities of East Algeria (Setif, Batna, Mila, Constantine and Annaba). Size was 5000 broilers in each poultry house. They were at the end of breeding aged between 49 and 56 day old. The broilers chicken was ISA broiler F15 and Arbor Acres. Autopsies were performed in the slaughter house. Samples were collected weekly every Sunday. Five broilers from each poultry house with growth retardation or generalized carcass congestion were randomly selected and presented to autopsy.

Autopsy of broilers

The liver and spleen of each chicken having colibacillosis lesions (septicemia, airsacculitis, pericarditis and perihepatitis) were removed under sterile conditions. All samples (120 livers and 120 spleens) were placed in separate sterile plastic bags to prevent spilling and cross contamination and immediately transported to the laboratory in a cooler with ice packs (4°C).

Isolation and identification of *E. coli* isolates, media and growth requirements

The organs (liver and spleen) were flamed using a Bunsen burner and cut into small pieces. Enrichment was done by seeding pieces in the tubes of nutrient broth (Pasteur Institute of Algeria) and incubated aerobically at 37°C for 18 to 24 h. A drop of nutrient broth was inoculated with method of exhaustion on Mc Conkey (BiotechLab, Algeria) (selective medium for Gram-negative Enterobacteriaceae) and then incubated aerobically at 37°C for 18 to 24 h. Positive colonies have a diameter of 2-3 mm, light pink color (lactose +) and are surrounded by an opaque halo due to precipitation of bile salts. They are Gram negative, catalase positive and oxidase negative. These colonies were tested by the TSI medium (IPA, Algeria) and urea-indole medium (IPA, Algeria) before being confirmed as *E. coli* by biochemical identification tests using the API 20E system (Bio Mérieux, France).

Serogrouping

Serogrouping was performed by the rapid slide agglutination. The reaction involves the binding of specific antibodies present in the serum with bacterial antigens to form clumps visible to the naked eye. Materials: 1- A clean transparent plate with wells (2x4) (Citoglas, UK), 2– Reagents (Sera): monovalent serum against specific somatic antigen (lipo polysaccharide) of avian *E. coli* strain O1, O2 and O78 (Biovac, France), 3- The tested strains were cultivated on trypto-caseine soja medium (TSA Agar) (labodib, Algeria) overnight at 37°C, 4- Pasteur pipette, 5– Saline (0.9%). All components were brought to room temperature before use, excessive heat was avoided, especially from a light source. The

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Table 1. Antibiotic resistance and serogroups of *E. coli* strains isolated.

Antibiotic	Number of strain (%) n=100				
	O1	O2	O78	Other serogroups	Total
Amoxicillin/Ac clavulanic (AUG,30 µg)	13 (92.8)	48 (90.5)	14 (87.5)	15 (88.2)	90 (90)
Ampicillin (AMP, 10 µg)	11 (78.5)	49 (92.4)	14 (87.5)	15 (88.2)	89 (89)
Tetracyclin (TE, 30 µg)	14 (100)	53 (100)	16 (100)	17 (100)	100 (100)
Nalidixic acid (NA, 30 µg)	14 (100)	52 (98.1)	16 (100)	17 (100)	99 (99)
Enrofloxacin (ENR, 5 µg)	9 (64.2)	44 (83.0)	13 (81.2)	16 (94.1)	82 (82)
Gentamicin (CN, 10 µg)	0 (0)	2 (3.7)	0 (0)	0 (0)	2 (2)
Neomycin (N,30 µg)	5 (35.7)	32 (60.3)	6 (46.1)	6 (35.2)	49 (49)
Colistin Sulfate(CS, 10 µg)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Nitrofurantoin (F, 300 µg)	7 (50)	30 (56.6)	7 (43.7)	9 (52.9)	53 (53)
Trimethoprim-sulfmethoxazole (SXT, 25 µg)	12 (85.7)	42 (79.2)	13 (81.2)	15 (88.2)	82 (82)
Chloramphenicol (C, 30 µg)	2 (14.2)	19 (35.8)	1 (6.2)	1 (5.8)	23 (23)
Total number of isolates	14	53	16	17	100 (100)

bottles of sera were vigorously shaken before use to restore the suspension. Quality control: a - Control of reagents (sera) were deposited in a well with a drop of reagent (about 30 µl). One drop (approximately 30 µl) of saline was added. The reagent is consistent if there is no agglutination reaction after mixing. b - Control of bacterial culture in another well was placed by a drop of saline (about 30 µl). Using a Pasteur pipette or inoculating loop, 3-4 colonies were collected (colonies should be visible to the naked eye). Gently emulsify in the drop of saline. The bacterial culture is compliant if no agglutination occurs after mixing. The test can be done once these two quality controls are compliant.

Antimicrobial susceptibility test

Antibiotic sensitivity was determined by disc diffusion method (Bauer et al., 1966) on solid Mueller-Hinton medium (MH) (Pasteur institute of Algeria) according to the guidelines of the "Clinical Laboratory Standards Institute" CLSI (2008). *E. coli* ATCC 25922 was used as control. Susceptibility was tested against the following antibiotics discs: Ampicillin (AMP, 10 µg), amoxicillin/Ac clavulanic (AUG, 30 µg), chloramphenicol (C, 30 µg), colistin sulfate (CS, 10 µg), Neomycin (N, 30 µg), Nalidixic acid (NA, 30 µg), Enrofloxacin (ENR, 5 µg), tetracycline (TE, 30 µg), Nitrofurantoin (F, 300 µg), Trimethoprim/sulfamethoxazole (SXT, 25 µg) and gentamicin (CN, 10 µg). Commercial antibiotic discs were purchased from Liofilchem, Italy (Ampicillin, amoxicillin/Ac clavulanic, chloramphenicol, colistin sulfate, trimethoprime/sulfaméthoxazole, Nalidixic acid), Bioanalyse, France (Gentamicin, tetracycline, nitrofurantoin), Bio-rad, France (Enrofloxacine and neomycin). The MH plates agar were incubated for 18 to 24 h at 37°C and the diameters of inhibition zones were interpreted by referring to the reading table of *Eenterobacteriaceae* as recommended by the Standardization of Susceptibility to the National Scale Human and Veterinary (2011).

RESULTS

One hundred *E. coli* strains were collected from 120 samples of livers and spleens, the susceptibility of these strains to each antimicrobial agent was tested and their serogroups are shown in Table 1. 83% of the 100 strains isolated in the study are among the most virulent serogroups of APEC: O1 (14%), O2 (53%), O78 (16%). The antibiotics to which there were very high levels of resistance, were in ascending order: enrofloxacin (82%), trimethoprim-sulfmethoxazole (82%), ampicillin (89%) and amoxicillin/ Ac clavulanic (90%), nalidixic acid (99%) and tetyracycline (100%). The antibiotics to which there were moderate levels of resistance, were in ascending order: neomycin (49%) and nitrofurantoin (53%). The antibiotics to which there were low levels of resistance, were in ascending order: Colistin sulfate (0%), gentamicin (2%), chloramphenicol (13%).

All isolated *E. coli* showed multi-drug resistance were resistant to 2 antibiotics or more as shown in Table 2, the highest multi-resistance rates are 26, 24 and 22% were recorded in strains resistant to 7, 6 and 8 antibiotics, respectively. A total of 31 antibiotic resistance patterns were distinguished. The most important are those designated in Table 3 as D, E, F, I and J. The most common multi-drug resistant profile among these isolates was profile I (13%), which was resistant to tetracycline, nalidixic acid, amoxicillin/Ac clavulanic, ampicillin, trimethoprim/sulfaméthoxazole, Enrofloxacine, nitro-furantoin, neomycin. Co-resistance to tetracycline-nalidixic acid- amoxicillin/Ac clavulanic- ampicillin-trimethoprim/sulfaméthoxazole - enrofloxacine was found in eight of the eleven most common multidrug resistant patterns, 62% of our strains express this co-resistance.

DISCUSSION

Among the 120 chicken carcasses autopsied, expressing lesions of colibacillosis, bacteriological analysis of livers and spleens got 100 positive cultures of *E. coli* (83.3%). In the remaining 20 cases (16.7%), bacterial cultures were negative. The negative cultures may result from drug intervention before referring the cases to the

Table 2. Strains of *E. coli* showing multi-drug resistance.

Number of antibiotics	Number of strains (n=100)	Percentage of strains resistant out of 11 tested
0	0	0
1	0	0
2	1	1
3	3	3
4	6	6
5	8	8
6	24	24
7	26	26
8	22	22
9	8	8
10	2	2
11	0	0

Table 3. The most frequent antibiotic resistance patterns of *E. coli* isolates (n=76).

Resistance patterns	Designation	Number of strains (%)
TE-NA-AUG-AMP-SXT	A	5 (5)
TE-NA-AUG-AMP-SXT-N	B	4 (4)
TE-NA-AUG-AMP-ENR-F	C	5 (5)
TE-NA-AUG-AMP-SXT-ENR	D	9 (9)
TE-NA-AUG-AMP-SXT-ENR-N	E	10 (10)
TE-NA-AUG-AMP-SXT-ENR-F	F	12 (12)
TE-NA-AUG-AMP-SXT-ENR-N-C	G	4 (4)
TE-NA-AUG-AMP-SXT-ENR-F-C	H	4 (4)
TE-NA-AUG-AMP-SXT-ENR-F-N	I	13 (13)
TE-NA-AUG-AMP-SXT-ENR-F-N-C	J	8 (8)
TE-NA-AUG-AMP-SXT-ENR-F-N-C-CN	K	2 (2)

TE: Tetracyclin NA: Nalidixic acid AUG: Amoxicillin/Ac clavulanic AMP: Ampicillin SXT: Trimethoprim-sulfmethoxazole F: Nitrofurantoin C: Chloramphenicol N: Neomycin ENR : Enrofloxacin CN: Gentamicin

laboratory, probably the chickens may be on treatment so there's no importance for the timeout of antibiotic treatment like that reported by Saberfar et al. (2008). The APEC are responsible for many forms of colibacillosis in chickens, and it is increasingly recognized that the possession of certain genes chromosomal or plasmid encoding the virulence factors gives APEC strains pathogenicity own due to their ability to survive in the host like that reported by Blanco et al. (1997), Stordeur and Mainil (2002) and Stordeur et al. (2003). Among the 100 *E. coli* isolated in our study, 83% belong to three serogroups O1, O2, O78 most virulent among the APEC, with the dominance of O2 with a rate of 53% followed by O78 (16%) and O1 (14%) while (17%) of our strains belong to other serogroups. Our observations are correlated with those reported by Blanco et al. (1997) and Dozois et al. (2000) and Mellata et al. (2003). Lafont et al. (1984) and Chulasiri and Suthienkul (1989) reported that characteristics of virulent *E. coli* in birds and other animals are often shared, and avian strains potentially can be a source of genes and plasmids that encode for antimicrobial resistance and virulence factors.

For the antibiotic resistance, these high rates of resistance to amoxicillin/clavulanic acid (90%) and ampicillin (89%) are probably related to the excessive and indiscriminate use of β-lactams antibiotics, and the diversity of mechanisms of resistance of *E. coli* as reported by Quintiliani and Courvalin (1995). Tetracycline (100%) is the oldest drug used, especially in therapy preventively and even as "growth factors", resulting in very high resistance in poultry as reported by Abdennebi (2006). For quinolone: Nalidixic acid (99%) and enrofloxacin (82%), these very high levels of resistance can be explained on one hand by the extensive use of

these molecules due to their large availability on the Algerian market, and especially by the presence of generics at affordable prices, and on the other hand with the fact that the quinolones share one and the same mechanism of action. Therefore, acquired resistance to one automatically confers resistance to other members of this family (cross-resistance). Baucheron et al. (2003) reported that two mutations in the gyrA gene and one or two mutations in the parC gene at the quinolone resistance determining region (QRDR) in *E. coli* strains of avian origin, give a high level of resistance to nalidixic acid and enrofloxacin. For sulfonamides, Trimethoprim/sulfamethoxazole (82%) is probably the consequence of the very important requirement of this anti-infective, used especially in the prevention of salmonella, and also during coccidiosis almost systematically used in combination with the anticoccidial treatment and prevention of the latter, leading to ineffectiveness against coliform. The abusive and anarchic use of the whole range of antibiotics available in Algeria is probably the major cause of the high percentages of resistance. In addition, lack of legislative restrictions of their use for therapy, prophylaxis, or growth promotion can be seen in the study of Hammoudi and Aggad (2008). Despite the fact that administration of chloramphenicol (13%) and nitrifurantoin (53%) is forbidden in veterinary, resistance to these antibiotics was high. This is probably due to persistence of previous resistances, a "cross" resistance or more likely to illegal use of these agents. The sensitivity of all strains to colistin sulfate could be explained by the moderate use of this molecule in poultry and these characteristics. In effect, this molecule does not cross the intestinal barrier and is inactive orally on systemic colibacillosis. Moreover, the resistance of Gram-negative bacteria is uncommon to colistin, even exceptional, and is chromosome-type. However, Garnacho-Montero et al. (2003) reported that chromosomal mutation is rare. The high sensitivity of *E. coli* strains to gentamicin is due to the non-use of this antibiotic in poultry farms and therefore no selection of resistant strains. In practice, there are constraints on its use, it is not available for poultry in Algeria and is used only for humans injectable form. Uninteresting for the farmers: the administration of this product requires a skilled workforce which can lead to stress and make the situation very delicate. The injections are not tolerated in chickens, especially with colibacillosis, as reported by Saberfar et al. (2008). Resistance was observed in all of the examined strains. This is similar to the findings of previous studies done in other countries by Blanco et al. (1997), Zahraei and Salehi (2006) and Saberfar et al. (2008). But our results were higher than those reported by Hammoudi and Aggad (2008) and Aggad et al. (2010) in Western Algeria.

Multi-drug-resistance appears as a veritable problem, as 100% of *E. coli* strains were resistant to at least two antibiotics, while over three quarters (82%) were resistant to at least 6 antibiotics, more than half (58%) were resistant to at least 7 antibiotics and more than a third (32%) were resistant to at least 8 antibiotics. Multi-resistance is probably due to the self-medication by breeders and alternating molecules before the first treatment gives these results. There is no importance attached to the processing delay. Indeed, numerous antibiotics are administrated often concomitantly for prophylaxis or infections. This indicates that the abusive and indiscriminate use of antibiotic is probably the genesis of the high incidence of antibiotic resistance and multi-resistances of *E. coli* in poultry breeding in Eastern Algeria. Such practices, especially without prior antibiotic sensitivity testing of bacterial isolates, may lead to the development of a pool of antibiotic-resistant genes and to the selection of increasing numbers of resistant *E. coli* clones, as reported by Hamoudi and Aggad (2008). The results of this multi-resistance were treatment failures, and consequently reduced production due to high levels of morbidity and mortality in poultry flocks.

In our study, 31 *E. coli* patterns were isolated, the most important are presented in Table 3 and designated A to K. the danger of *E. coli* strains expressing antibiotic resistant patterns D, E, F, G, H, I, J and K, with rates of 9, 10, 12, 4, 4, 13, 8 and 2%, respectively were must considered. These strains show resistance to 6, 7, 8, 9, 10 antibiotics, even to antibiotics regarded as the most active on *E. coli* strains, such as gentamicin, chloramphenicol and nitrofurans and neomycin. These strains could transfer their wide antibiotic resistant pattern via the exchange of genetic material. Bebora et al. (1994) and Davies (1994) indicated that antibiotic resistances are frequently encoded by conjugative plasmids or transposons, thus *E. coli* of avian origin could act as a possible source for the transfer of antibiotic resistances to other bacterial species, including human pathogens. Thus, an increase in the reservoir of antibiotic resistant bacteria could heavily impair the treatment of human diseases. Indeed, Van Den Boogard et al. (2001) identified similar antibiotic resistant patterns present in *E. coli* isolated from people who worked with these birds, and, in some instances, specific strains were shared among the birds and workers. These findings indicate that transmission of antibiotic resistance by organisms that affect chickens to humans is common.

In this study, co-resistance to tetracycline - nalidixic acid- amoxicillin/Ac clavulanic-ampicillin- trimethoprim/ sulfaméthoxazole - enrofloxacine was found in *E. coli* strains expressing antibiotic resistant patterns D, E, F, G, H, I, J and K. This co-resistance is present in 11 out of 13 most important multidrug resistant profiles and more than half 62% of our strains express it. The consequence of this genetic organization is the co-selection: a class of antibiotics to which the bacteria are resistant may select resistance to unrelated classes of antibiotics, thus generating a wide resistant phenotype of bacterium like that reported by Courvalin (2008).

Conflict of interests

The authors have not declared any conflict of interest.

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Study of resistance mechanism in *Enterobacter spp.* with special reference to antimicrobials ceftazidime, moxifloxacin and nalidixic acid

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Microbial resistance to antimicrobials is spreading all over the world making it difficult to treat diseases effectively. This study aims to understand the mechanism of resistance towards the antimicrobials Ceftazidime, Moxifloxacin and Nalidixic acid, by using resistant *Enterobacter* spp. and sensitive *Enterobacter* spp. While there was complete inhibition of growth of sensitive *Enterobacter* spp. at 8 µg/ml Ceftazidime, 0.125 µg/ml of Moxifloxacin and 16 µg/ml of Nalidixic acid, resistant *Enterobacter* spp. even tolerated 256, 32 and 1536 µg/ml Ceftazidime, Moxifloxacin and Nalidixic acid respectively. Browthdilution method was used to determine the growth of organisms at different concentrations of antibacterial agents. Mechanism of resistance was found to be present in plasmid. Absence of *Beta lactamase* enzyme seems to be an important finding in this sudy. *B. lactamase* enzyme was checked with help of the acidometric and idometric method. Plasmid isolation and analysis was done by agarose gel electrophoresis. Successful curing of plasmid was carried out with 10% sodium dodecyl sulfate (SDS). When colonies after SDS treatment were tested, resistant strains were found which were later converted to sensitive ones. In this study, the resistant Enterobacter spp. executed resistance to three different classes of antimicrobials due to the resistance plasmid. The results obtained in this study support most of the previous study findings who contributed in this field. Therefore, it might be useful to recognize the resistance mechanism, and to determine the correct practicing of drug usage.

Key words: Antimicrobials, enterobacter, sensitive enterobacter, resistance mechanism, inhibition, plasmid.

INTRODUCTION

Pathogenic enteric bacteria that exhibit antimicrobial resistance are a widespread phenomenon and arguably constitute a global epidemic (Chau et al., 2007). Whilst the depth of knowledge regarding antimicrobial-resistant organisms isolated from patients with infection or circulation in the hospital environment is broad, less is known about antimicrobial resistant organisms that are disseminated in the community. Furthermore, little is known about the antimicrobial resistance patterns of community-acquired organisms that circulate in developing countries where antimicrobials are available without prior consultation with a physician. Quinolones

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and fluoroquinolones are groups of antimicrobial compounds that are commonly used for the treatment of many bacterial infections (Strahilevitz et al., 2007). However, multiple studies have highlighted that, in recent years, resistance to fluoroquinolones has increased globally, particularly in members of the *Enterobacteriaceae* (Wang et al., 2008).

Enterobacter species are motile aerobic gram negative bacilli belonging to the family *Enterobacteriaceae*. The major species are *Enterobacter cloacae*, *Enterobacter aerogenes* and *Enterobacter agglomerans*. They first achieved wide notoriety as pathogens in 1976 following a nationwide outbreak of septicemia in 378 patients at 25 hospitals resulting from contaminated intravenous solutions (Maki et al., 1976).

The genus Enterobacter was first proposed by Hormaeche and Edwards (1960). Enterobacter species are found in the natural environment in habitats such as water, sewage, vegetables and soil. Before the widespread use of antibiotics, Enterobacter species were rarely found as pathogens, but these organisms are now increasingly encountered, causing nosocomial infections such as urinary tract infections and bacteremia (Eickhoff et al., 1966). In addition, they occasionally cause community-acquired infections. In 1975 in the United States, Enterobacter species accounted for 4.6% of all pathogens causing infections, and accounted for 5.7% of all cases of primary bacteremia (Center for Disease Control, 1977).

Enterobacter species accounted for 5.9% of all nosocomial infections in U.S. hospitals and 6.3% of all nosocomial bacteremia (Centers for Disease Control, 1984). Enterobacter cloacae occur as a commensal organism in water, sewage, soil, meat, hospital environments, the skin, and in the intestinal tracts of humans and animals. Among 234 patients, the rate of stool carriage of *E. cloacae* on admission to the hospital was 2.6%. This rate increased to 4% after antibiotic therapy (Rose and Schreier, 1968). Like other enteric gram-negative rods, *Enterobacter* species cause a wide variety of nosocomial infections, including those affecting the lungs, urinary tract, intrabdominal cavity and intravascular devices. *Enterobacter sakazakii* causes neonatal sepsis with meningitis (Bar-Oz et al., 2001; Nazarowec-White and Farber, 1997). Sanders et al. (1996) described successful therapy with cefepime of 17 infections due to *Enterobacter* strains resistant to third generation cephalosporins. These patients had infections at a variety of sites. All patients responded clinically, and bacteriologic eradication was documented in 88%.

Enterobacter infections can include bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections (UTIs), endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, central nervous system (CNS) infections and ophthalmic infections. *Enterobacter* infections can necessitate prolonged hospitalization, multiple and varied imaging studies and laboratory tests, various surgical and nonsurgical procedures, and powerful and expensive antimicrobial agents. With few exceptions, the major classes of antibiotics used to manage infections with these bacteria include the beta-lactams, carbapenems, fluoroquinolones, aminoglycosides and sulfamethoxazole and trimethoprim (SMZ-TMP). Because most *Enterobacter* species are either very resistant to many agents or can develop resistance during antimicrobial therapy, the choice of appropriate antimicrobial agents is complicated. Consultation with experts in infectious diseases and microbiology is usually indicated. In 2006, Paterson published a good review of resistance among various Enterobacteriaceae (Paterson, 2006).

Infections caused by antibiotic-resistant bacteria, especially the “ESKAPE” pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aerugenosa and Enterobacter* species), cause significant morbidity and mortality (Rice, 2008; Boucher et al., 2009). The incidence of nosocomial infections due to *Enterobacter* is rising, and broad resistance to third generation cephalosporins, penicillins and quinolones is an increasing problem. A number of agents remain effective for treatment. Among the beta-lactams, the fourth generation cephalosporins and carbapenems are the most attractive options. Aminoglycosides retain good activity but usually require combination with another agent. Quinolones are highly active against most strains, but emerging resistance is a major concern. Trimethoprim-sulfamethoxazole is under-utilized as therapy of *Enterobacter* infections (http://www.antimicrobe.org/b97.asp#r36).

Bacteria may be intrinsically resistant to more than one class of antimicrobial agents, or may acquire resistance by *de novo* mutation or via the acquisition of resistance genes from other organisms. Acquired resistance genes may enable a bacterium to produce enzymes that destroy the antibacterial drug and most resistant strains express efflux systems that prevent the drug from reaching its intracellular target required to kill or inhibit the infecting pathogen (Tenover and MacDonald, 2006). Quinolone resistance in *Enterobacter* is usually due to chromosomal genes that may upregulate efflux pumps (Nikaido, 2001) or confer resistance due to altered DNA gyrase (Dekitsch et al, 1999). Ceftazidime resistance was an inclusion criterion because of the strong association between qnr genes and plasmids carrying cephalosporinase genes. The ciprofloxacin MIC was the minimum expected for *Enterobacteriaceae* containing a qnr gene. (Gay et al., 2006). High prevalence of quinolone resistance determinants was identified, particularly the *qnrS* gene, in both - community and hospital-associated strains (Vien, 2009). The data suggest that intestinal commensal organisms are a significant reservoir for the dissemination of plasmid-mediated quinolone resistance in Ho Chi Minh City.

This study attempted to study the mechanism of

resistance in Enterobacter species in relation with the earlier mentioned work. The phenomenon of resistance was assessed by the finding such as absence of *B* lactamase and resistance plasmid isolation from the resistant species of Enterobacter after the determination of MIC values for sensitive species and the tolerance of resistant species towards the antimicrobials at various concentrations above the MIC level determined for the sensitive species of Enterobacter. During plasmid isolation both the species of Enterobacter species had been processed but the sensitive Enterobacter spp. failed to show any band on the agarose gel when run along with the sample obtained from resistant species and the DNA ladder.

The dissimilar growing concentrations of the sensitive Enterobacter and resistant Enterobacter were found within different concentrations of Ceftazidime, Moxifloxacin and Nalidixic acid.

MATERIALS AND METHODS

Bacterial species and their cultivation

The strains of resistant and sensitive *Enterobacter spp.* were obtained from BAC TEST Laboratory Nashik, Maharashtra and stocked in this laboratory (Microbiology Laboratory, Department of Life Sciences, University of Mumbai). This particular pathogenic microorganism was selected for the study after testing various pathogens for their susceptibility towards the antimicrobials. These organisms were grown on a nutrient agar at 37°C and maintained at 5°C. The inoculum of MacFarland Turbidity standard was prepared in nutrient broth after subculturing, and was incubate for 48 h. The cells of Resistant and sensitive species of *Enterobacter* were cultured on nutrient agar (NA) (HiMedia, India) slants containing Beef extract 0.5 g; Peptone 2.5 g; Sodium chloride 2.5 g; Agar 15 g in a liter of distilled water. pH was maintained at 7.4, slants were incubated at 37°C for 24 h (Lankeshwar and Bagde, 2013) .

Detection of antimicrobial resistance and sensitivity in bacteria

Disc diffusion method

Antimicrobial susceptibility of resistant and sensitive *Enterobacter* Species was determined with the help of disc diffusion method of Kirby et al. (1994) with modification by the NCCLS (1999, 2001). The antimicrobial discs of Ceftazidime (30 mcg), Moxifloxacin (30 mcg), Nalidixic acid (5 mcg), and Muller Hinton (MH) Agar were procured from HiMedia (India). Zone of inhibition was measured after agar plates were incubated overnight and the zone of inhibition were measured with the help of standard scale (Standard strains have been used for reference).

Broth dilution method

The broth dilution method was used to determine the growth of organisms at different concentrations of antibacterial agents according to the guideline given by NCCLS (2001) protocol. The MH broth and the antimicrobials were purchased in powder form from HiMedia (India). The experiments were carried out in tubes and 100 ml Ehrlenmeyer flasks with side arm. These flasks were inoculated with 1.0 ml inoculum (Mac farland standard/10^3 CFU/ml) prepared in nutrient broth after subculturing 48 h incubation. Optical density of culture was measured at 540 nm. The final concentrations of antimicrobials tested were Ceftazidime 1.0, 2.0, 4.0, 8.0 µg/ml, Moxifloxacin 0.016, 0.032, 0.064, 0.125 µg/ml, Nalidixic acid 2.0, 4.0, 8.0, 16.0 µg/ml, for the sensitive strain and ceftazidime 32, 64, 128, 256 µg/ml, Moxifloxacin 4, 8, 16, 32 µg/ml, and Nalidixic acid 192, 384, 768, 1536 µg/ml for the resistant strain, respectively.

β lactamase activity

Detection of *β* lactamase was carried out by tube method and paper strip method of idometric test and acidometric test (Livermore and Brown, 2001), and *B* lactamase activity testing was done by qualitative plate test (George et al., 1983), nitrocefin test was not done due to unavailability of nitrocefin.

Plasmid isolation

Plasmid Isolation was conducted for separation and analysis of nucleic acid by alkaline lysis method (Sambrook et al., 1989). Overnight, bacterial culture in LB broth containing 50 *ug*/ml of ampicillin was chilled for an hour and centrifuged at high speed. Then the suspending/neutralizing buffer, lysis buffer, renaturation buffer, acidic potassium phosphate, phenol: chloroform solution were used to isolate the plasmid by this procedure. It was precipitated with chilled ethanol. The pellet was allowed to air dry for 15 to 20 min and stored with 50 *ul* of $T_{10}E_1$ Buffer at 4°C.

Spectrophotometric method

Qualitative determination of DNA was carried out with help of the spectrophotometric method given by Maniatis et al. (1982).

Agarose gel electrophoresis

Agarose gel electrophoresis of plasmid DNA was done according to the method of Sambrook et al. (1989). Plasmid DNA from *Enterobacter* resistant species was electrophoresed with DNA ladder in 1.0% agarose gel slab at 50 V in Tris Acetate buffer (TAE) running buffer (0.5M Tris base, 0.5M EDTA, 1 M Glacial acetic acid). Ethidium bromide incorporated in the agarose gel stained the plasmid DNA and the bands of plasmid seen under UV illumination were photographed. The size of plasmid was estimated by comparing with standard Supermix DNA ladder obtained from BangloreGenei, (Banglore, India). It consisted of DNA fragments ranging from 500 to 33500-24500 bp generated from number of propriotory plasmids and lambda DNA.

Plasmid curing

Elimination of resistance due to plasmid was carried out by using the sodium dodecyl sulphate (SDS) treatment described by Tomoeda et al. (1968). An overnight culture of resistant (R) cells in penassay broth (5 g/L Peptone, 1.5 g/L Yeast extract, 1.5 g/L Beef extract, 3.5 g/L Sodiuim chloride, 1 g/ Dextrose, 3.86 g/L potassium phosphate dibasic, 1.32g/L potassium phosphate monobasic. pH adjusted to 6.9) containing MIC concentration of antimicrobial (µg/ml) for sensitive species (10^3 cells/ml) diluted in broth, and added to the tubes containing 10% (w/v) SDS and shaken at 37°C. After appropriate dilutions in saline cells were spread on nutrient agar and the sensitivity discs were placed on it.

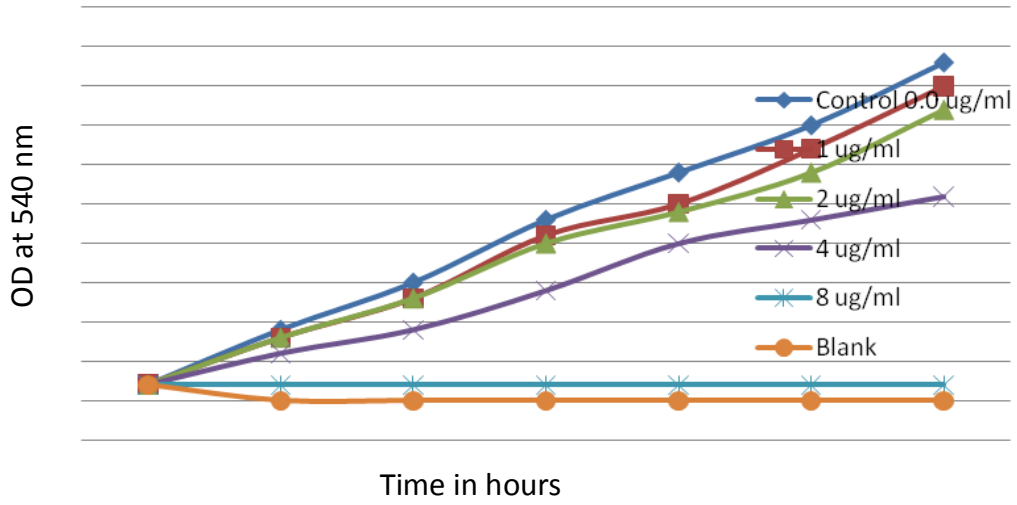

Figure 1. Sensitive *Enterobacter* species showing sensitivity to Ceftazidime.

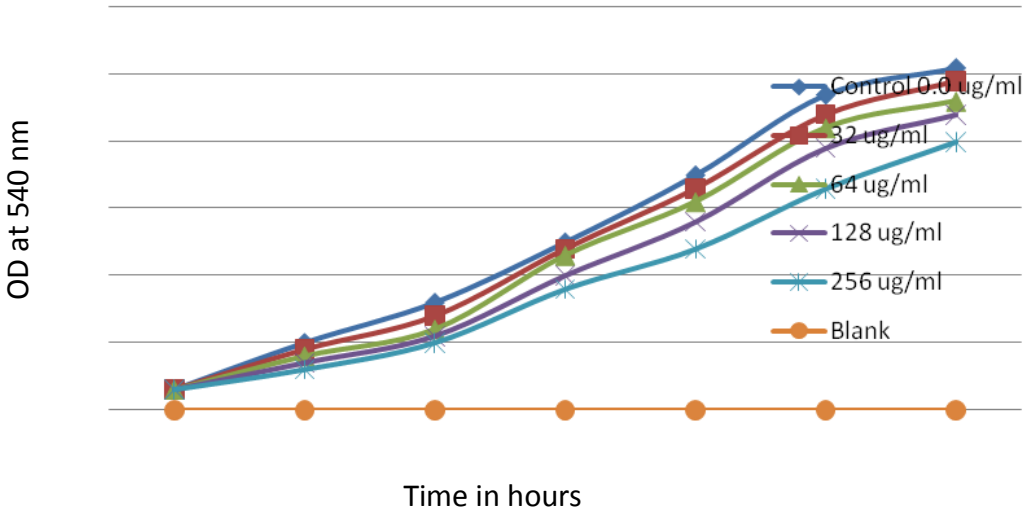

Figure 2. Resistant *Enterobacter* species showing resistance to Ceftazidime.

RESULTS

Effect of antimicrobials on growth of sensitive and resistant species of *Enterobacter*

When concentrations of ceftazidime of 1.0, 2.0, 4.0 and 8.0 µg/ml were tested, sensitive species of *Enterobacter* showed MIC 8 µg/ml for ceftazidime. There was complete inhibition growth of sensitive *Enterobacter* spp. at 8 µg/ml ceftazidime (Figure 1). But when concentrations of ceftazidime 32, 64, 128 and 256 µg/ml were tested, resistant *Enterobacter* spp. tolerated 256 µg/ml Ceftazidime (Figure 2).

When concentrations of moxifloxacin 0.016, 0.032, 0.064 and 0.125 µg/ml were tested for sensitive species of *Enterobacter,* Moxifloxacin MIC was noted as 0.125 µg/ml. There was complete inhibition of growth of sensitive *Enterobacter* spp. at 0.1258 µg/ml of moxifloxacin (Figure 3), but when concentrations of moxifloxacin 4, 8, 16 and 32 µg/ml were tested, resistant *Enterobacter* spp. even tolerated 32 µg/ml moxifloxacin (Figure 4).

When concentrations of nalidixic acid 2.0, 4.0, 8.0 and 16.0 µg/ml were tested in the sensitive species of *Enterobacter,* nalidixic acid MIC was found to be 16 µg/ml. There was complete inhibition growth of sensitive *Enterobacter* spp. at 16 µg/ml of nalidixic acid (Figure 5). But when concentrations of nalidixic acid 192, 384, 768 and1536 µg/ml were tested, resistant *Enterobacter* spp. even tolerated1536 µg/ml Nalidixic acid (Figure 6).

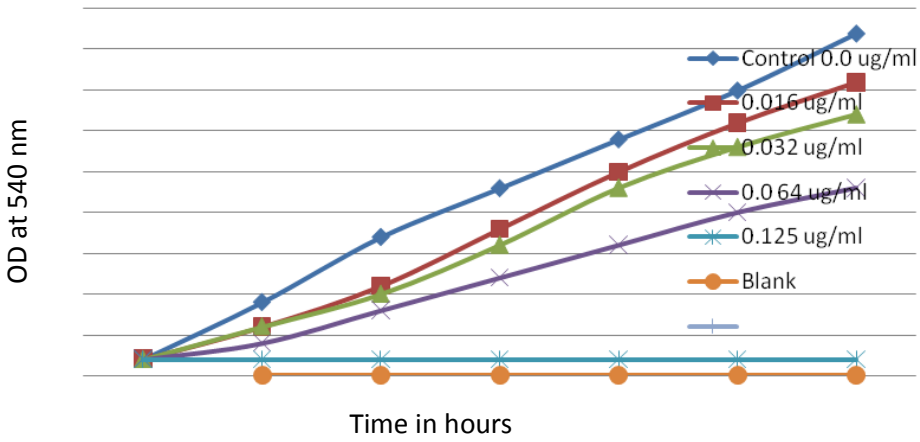

Figure 3. Sensitive *Enterobacter* species showing sensitivity to Moxifloxacin.

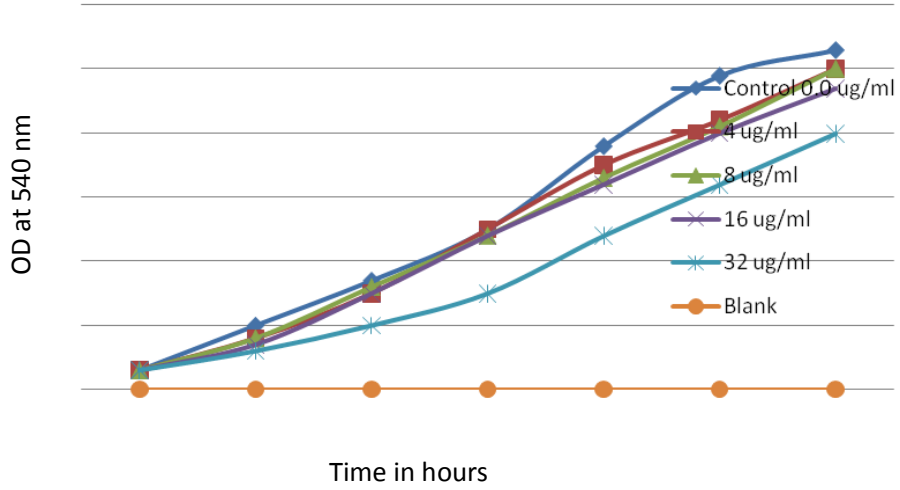

Figure 4. Resistant *Enterobacter* species showing resistance to Moxifloxacin.

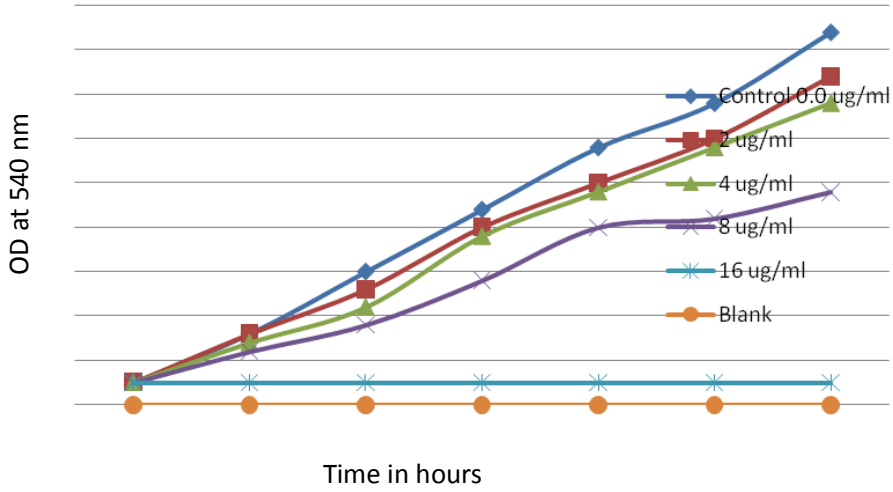

Figure 5. Sensitive *Enterobacter* species showing sensitivity to Nalidixic acid.

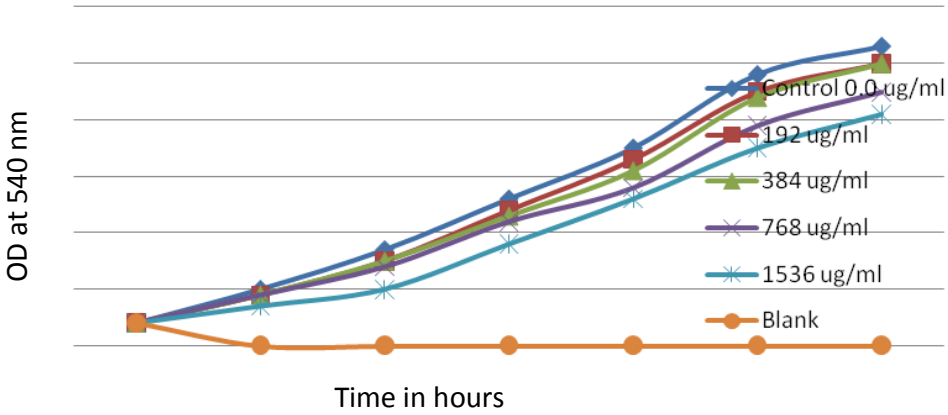

Figure 6. Resistant *Enterobacter* species showing resistance to Nalidixic acid.

Table 1. Activity of resistant and sensitive *Enterobacter spp.* towards the antimicrobials.

S/N	Pathogen	Antimicrobial	Size of inhibition zone (mm)	Tolerance / Inhibition (µg/ml - MIC method)
1	Sensitive *Enterobacter Spp.*	Ceftazidime	25 (30 mcg disc)	8
		Moxifloxacin	30 (5 mcg disc)	0.125
		Nalidixic acid	25 (30 mcg disc)	16
2	Resistant *Enterobacter Spp*.	Ceftazidime	11 (30 mcg disc)	Not inhibited at 256
		Moxifloxacin	12 (5 mcg disc)	Not inhibited at 32
		Nalidixic acid	0 (30 mcg disc)	Not inhibited at 1536

Table 2. Growth percentages of sensitive Enterobacter after 72 h at different concentrations of antimicrobials.

Concentration of moxifloxacin (ug/ml)	OD at 540 nm	% of growth	Concentration of ceftazidime (ug/ml)	OD at 540 nm	% of growth	Concentration of nalidixic acid (ug/ml)	OD at 540 nm	% of growth
0.0	0.35	100	0.0	0.52	100	0.0	0.48	100
0.016	0.31	88.57	1.0	0.41	78.84	2.0	0.43	89.58
0.032	0.28	80	2.0	0.37	71.15	4.0	0.40	83.33
0.063	0.23	65.71	4.0	0.33	63.46	8.0	0.33	68.75
0.125	0.0	0	8.0	0.0	0	16.0	0.0	0

The susceptibility of resistant and sensitive species of *Enterobacter* was noted as size of inhibition zone in mm. The sensitive *Enterobacter* showed zone of inhibition for ceftazidime -25 mm, for moxifloxacin –30 mm, and for Nalidixic acid –25 mm. The resistant species showed 0, 11, 12 and 6 mm, respectively size of inhibition zone in Nalidixic acid, Ceftazidime and moxifloxacin respectively. This clearly indicated the difference between the susceptibility of both species of *Enterobacter* (Table 1).

As shown in Table 2, the growing concentrations of sensitive *Enterobacter (*10^3 CFU/ml)in 4, 0.063, 8, µg/ml of ceftazidime, Moxifloxacin and Nalidixic acid, were found to be 63.46, 65.71 and 68.75% after 72 h. The growing concentrations of resistant *Enterobacter* at 10^3 CFU/mlin 256, 32 and 1536 µg/ml, of ceftazidime, Moxifloxacin and Nalidixic acid respectively, were found about 76.36, 81.63 and 77.77 after 72 h (Table 3). The higher most antimicrobial concentration tolerated by the resistant species is given here.

β Lactamase detection

As to beta lactamase detection, no decolorization occurred in 5 min in Idometric method within the tube or on the strip. Violet to Yellow color change was not

Table 3. Growth percentages of resistant Enterobacter after 72 h at different concentrations of antimicrobials.

Concentration of moxifloxacin (ug/ml)	OD at 540 nm	% of growth	Concentration of ceftazidime (ug/ml)	OD at 540 nm	% of growth	Concentration of Nalidixic Acid (ug/ml)	OD at 540 nm	% of growth
0.0	0.49	100	0	0.55	100	0	0.54	100
4.0	0.42	85.71	32	0.52	94.54	192	0.51	94.44
8.0	0.41	83.67	64	0.50	90.90	384	0.49	90.74
16.0	0.40	81.63	128	0.48	87.27	768	0.46	85.18
32.0	0.40	81.63	256	0.42	76.36	1536	0.42	77.77

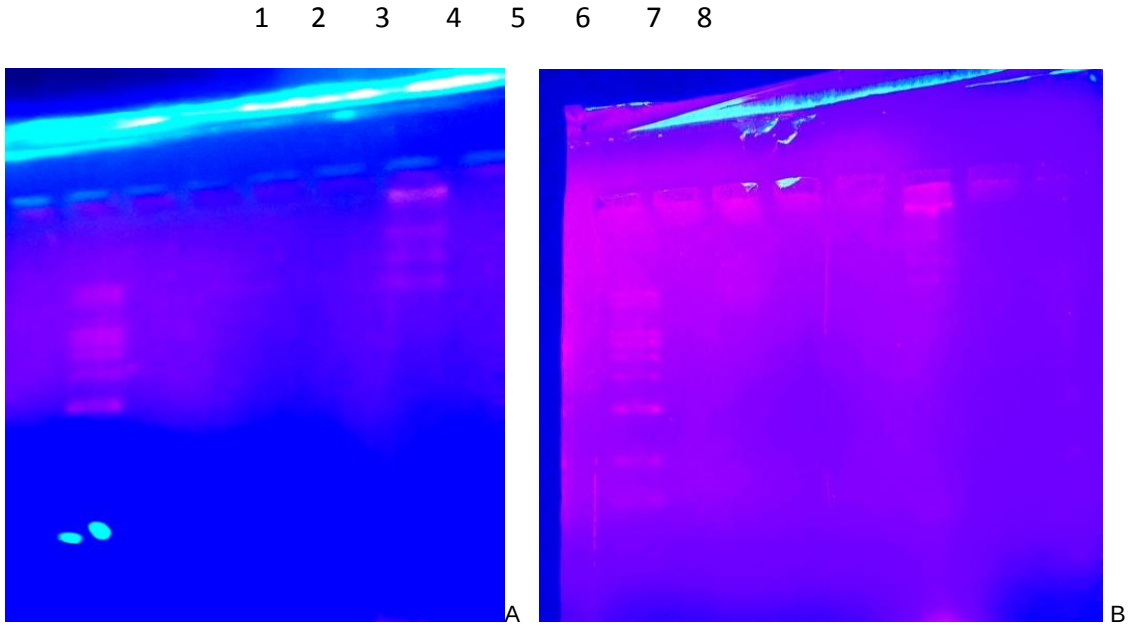

Figure 7. Agarose gel Electrophoresis of plasmid DNA isolated from *Enterobacter* species resistant to Ceftazidime,Moxifloxacin, and Nalidixic acid.

observed within 5 min in Acidimetric method. No yellow color was observed around the colony within 60 min in qualitative plate test. Therefore, *β* Lactamase was not detected.

Plasmid isolation

In this experiment, sensitive and resistant *Enterobacter were* used for plasmid isolation. Plasmid was isolated successfully from the resistant species of *Enterobacter* and electrophoresed on agarose gel. Confirmation of plasmid was done with help of the DNA ladder.

Quantitative estimation of DNA

The purity of the isolated plasmid DNA was calculated as 1.65. The absorbance of plasmid of resistant *Enterobacter* species was 0.982. at 260 nm and 0.595 at 280 nm. The concentration of plasmid DNA from resistance *Enterobacter* calculated was 3928 gm. Therefore, the ratio of protein to extracted DNA was 1.65. Agarose gel electrophoresis of plasmid DNA isolated from *Enterobacter* species resistant to Ceftazidime, Moxifloxacin, Nalidixic acid Showed 4 different bands (Figures 6 and 7) and the Supermix DNA ladder showed bands of different molecular weight, while the sensitive species of *Enterobacter* did not show any band in lane 3, 4 and 6 (Figure 7).

The plasmid DNA has been run along with the DNA ladder for the purpose of conformation only. There are more than 1 plasmid DNA found near the bands of ladder having molecular weight of 15000 bp (at lower side), and molecular weight more than 33500 bp (both at upper side).

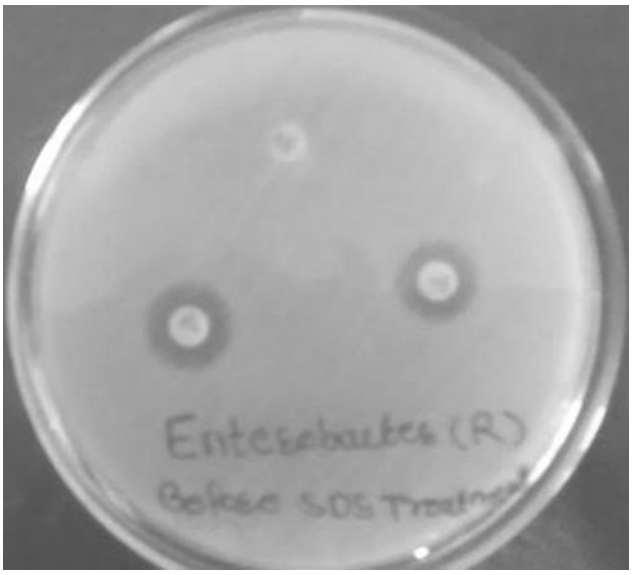

Figure 8. Elimination of resistance after SDS treatment.

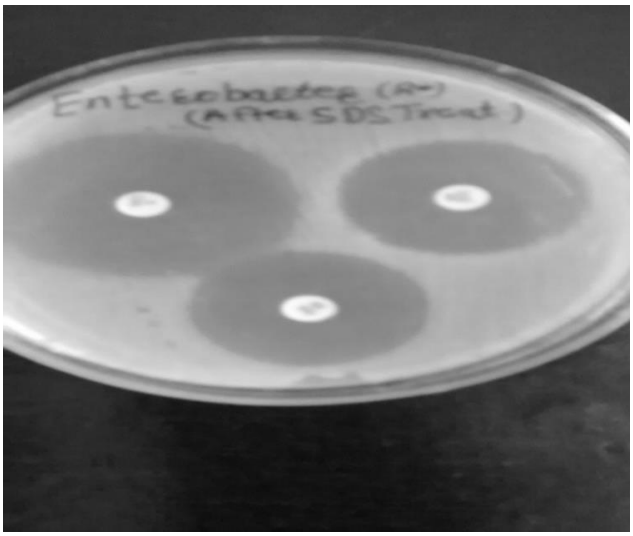

Figure 9. Sensitivity to the antimicrobials before SDS treatment.

Plasmid curing

In this experiment, the cells of *Enterobacter* carrying resistance plasmid were treated with 2 to 10% concentration of sodium dodecyl sulphate. The treatment of sodium dodecyl sulphate was found to be effective to turn the resistant cells into susceptible ones. The resistant species showed up to 51 to 27% growth at 2 to 10% SDS concentration as compared to control set. After SDS treatments, resistant strains were analyzed at interval for the presence or absence of drug resistance against different concentration of antimicrobials. The resistance was lost and the resistant species completely changed into the sensitive species (Figure 8 and 9).

DISCUSSION

The present study has exposed the mechanism of resistance towards the antimicrobials. There was complete inhibition of growth of sensitive *Enterobacter* spp. at 8 µg/ml Ceftazidime, 0.125 µg/ml of Moxifloxacin, and 16 µg/ml of Nalidixic acid, while the resistant *Enterobacter* spp. even tolerated 256, 32 and 1536 µg/ml of Ceftazidime, Moxifloxacin and Nalidixic acid respectively. Different ranges of concentrations of the earlier mentioned antimicrobials have been incorporated in the MIC study after determination of the MIC value of each antimicrobial through several trials of experiment in various batches. Evaluation of a wide range of antibiotics tested against *E. cloacae*, *Enterobacter hormaechei* and *Enterobacter asburiae* strains, have provided a database for their natural susceptibility (Stock et al., 2001).

In this present study, the growing concentrations were found 76.36, 81.63 and 77.77% at 10^3 CFU/ml after 72 h for 256, 32, 16 and 1536 µg/ml of ceftazidime, Moxifloxacin, and Nalidixic acid, respectively in resistant *Enterobacter.*The growing concentrations of sensitive *Enterobacter (* 10^3 CFU/ml) were found up to 63.46, 65.71 and 68.75% after 72 h in 4, 0.063, 4, and 8 µg/ml of ceftazidime, Moxifloxacin, and Nalidixic acid. These findings can be compared with the findings of other studies. The antimicrobial-resistance rates of qnr-positive strains to Ciprofloxacin, Levofloxacin, Norfloxacin, Nalidixic acid, and Moxifloxacin were 51.1, 46.8, 46.8, 74.5 and 53.2%, respectively (Haeng et al., 2011). Most isolates of the *E. cloacae* complex are susceptible to fluoroquinolones, trimethoprim/sulfamethoxazole, chloramphenicol, aminoglycosides, tetracyclines, piperacillin-tazobactam and carbapenems, while they are intrinsically resistant to ampicillin, amoxicillin, amoxicillin–clavulanate, first-generation cephalosporins and cefoxitin owing to the production of constitutive AmpC β-lactamase. In particular, fosfomycin seems to have a different activity against all species, because *E. cloacae* and *E. asburiae* are both naturally susceptible and resistant, while *E. hormaechei* is only naturally sensitive (Stock et al., 2001).

Plasmid was not isolated from sensitive strain, but there was successful isolation of plasmids from resistant species of *Enterobacter* which supported to state that the mechanism of resistance was plasmid mediated. Similar observations were made in earlier studies where successful isolation of one or more plasmids of various molecular sizes was reported from resistant species, and plasmid was not isolated from sensitive strains (Ghosh et al., 1997; Lankeshwar and Bagde, 2004, 2008, 2013).

The result of agarose gel electrophoresis is clearly showing the presence of 3 different bands into the second picture in Figure 9. It could be specified as nicked circle plasmid DNA, linear plasmid DNA and supercoiled

plasmid DNA (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981).

The more recent discovery and rapid dissemination of plasmid-mediated quinolone resistance (PMQR) genes has further highlighted the problem of quinolone (Nalidixic acid) and fluoroquinolone (Moxifloxacin) resistance and increased the study understanding of resistance mechanisms associated with these antibacterial compounds (Robicsek et al., 2006). Although quinolone resistance results mostly from chromosomal mutations in Enterobacteriaceae, it may also be mediated by plasmid-encoded Qnr determinants. Qnr proteins protect DNA from quinolone binding and compromise the efficacy of quinolones such as Nalidixic acid (Nordmann and Laurent, 2005). The experiment done in this study is in agreement with this statement, as the resistant species of *Enterobacter* found to be resistant to different classes of antimicrobials namely, third generation cephalosporin (Ceftazidime), quinolone (Moxifloxacin) and fluroquinolone (Nalidixic acid).

In present study *Enterobacter* carrying resistance plasmid was treated with 2 to 10% concentration of sodium dodecyl sulphate. The treatment of sodium dodecyl sulphate was found to be effective to turn the resistant cells into susceptible ones. The resistance was lost and the resistant species completely changed into the sensitive species. This was very much in agreement to earlier studies of plasmid elimination using SDS (Tomoeda et al., 1968; Pan-Hau et al., 1981; Lankeshwar and Bagde, 2008, 2013). Reportedly also, acriflavine was used for elimination of resistance to penicillin in *S. aureus* (Hashimoto et al., 1964). However, antimicrobial susceptibility testing for clinically significant strains is highly recommended, as resistance to antibacterial agents may be strain dependent (Max et al., 2011).

Conclusion

This study revealed the mechanism of resistance on molecular basis towards the antimicrobials Ceftazidime, Moxifloxacin and Nalidixic acid as the resistance plasmid was isolated in resistant species of *Enterobacter* and not from the Sensitive. The mechanism of resistance to Ceftazidime in two clinical isolates of *Enterobacter cloacae* that emerged during therapy with broad-spectrum beta-lactam antibiotics was studied earlier (Quinn et al., 1987). Plasmid mediated fluroquinolones resistance was also reported by Maria et al. (2012) due to Qnr-mediated topoisom protection with enzyme type Qnr(A, B, S, C, D) in *E. cloacae* and *E. hormaechei*. Ceftazidime resistance was an inclusion criterion because of the strong association between qnr genes and plasmids carrying cephalosporinase genes (Gay et al., 2006).

It is reported that the maximum resistance was seen against Ceftazidime (74.8%) followed by Cefotaxime (70.6%). In ESBL, producing bacteria was (59.6%) mostly in *K. pneumoniae* (68.8. %) followed by *E. coli* (65.0 %). ESBL producing bacteria showed maximum resistance to Ceftazidime (95.4%), followed by Cefotaxime (94.6%), while minimum resistance was seen with Imipenem (0%), followed by Piperacillin/Tazobactam (3.8%) and Cefepime (7.7%) (Ahmed et al., 2013).

In accordance with these findings, the Ceftazidime resistance in this study can be specified with *Enterobacter spp.* Over the past years, the development and application of molecular diagnostic techniques has initiated a revolution in the diagnosis and monitoring of infectious diseases (Yi-Wei et al., 1997). This study might contribute to understand the status and phenomenon of resistance in pathogens, efficacy of drugs, relation with MDR and finally the correct usage of antimicrobials.

Conflict of interest

The authors have not declared any conflict of interest.

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Short Communication

Salmonella Typhimurium, Enteritidis, Infantis and Derby in pasty *dulce de leche*

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In order to evaluate the fate of *Salmonella* Typhimurium, Enteritidis, Infantis and Derby in pasty *dulce de leche*, aliquots of this sweet were experimentally contaminated with these pathogenic microorganisms at 10^2 bacterial cells per gram, and later analysed to evaluate microorganism count after storage for 0, 1, 2, 3, 5, 10 and 20 days. *Salmonella* Enteritidis was able to survive for up to 20 days in *dulce de leche*, while *Salmonella* Typhimurium and Infantis for five and ten days, respectively, and serotype Derby for three days. The *Salmonella* serotypes studied showed different adaptive capabilities regarding population growth in pasty *dulce de leche*, though all representing potential hazard to the consumer's health. The results are a warning regarding the need to take the appropriate hygienic-sanitary practices during *dulce de leche* manufacture, packing and handling.

Key words: *Salmonella*, *dulce de leche*, food safety.

INTRODUCTION

Dulce de leche is the product obtained from concentration and heating of milk with the addition of sucrose (Brasil, 1997). It can be produced in pasty or tablets form. The pasty *dulce de leche* is widely consumed and commercialized in Brazil and other Latin American countries, where the fractioning into portions to be sold by retail in supermarkets and street market stalls is common. In spite of the existence of barriers against microbial growth, such as its low water activity due to the concentration of carbohydrates, the *dulce de leche* can be contaminated by microorganisms, during the manufacturing process, distribution and storage, constituting a potential hazard to people's health (Timm et al., 2007).

Salmonellosis is a food-borne disease of frequent occurrence throughout the world. The causative agent, *Salmonella* spp., is widely distributed in nature and the main reservoir is the gastrointestinal tract of animals and man. The existence of asymptomatic carriers and persistence of *Salmonella* in the environment and in foods contributes to this organism assume a relevant role in public health (Shinohara et al., 2008). The annual occurrence of salmonellosis cases in the United States is estimated in 1.4 million, of which approximately 40,000

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Table 1. *Salmonella* counts (log MPN/g) in experimentally contaminated pasty *dulce de leche*.

Strain	Serotype	Storage days						
		0	1	2	3	5	10	20
LIPOA 2046	Typhimurium	1.5 (0.2)[a]	1.0 (0.9)	**0**[b]	**0**	NR[c]	NR	NR
ATCC 13311	Typhimurium	1.3 (0.5)	0.9 (0.5)	**0.2 (0.3)**	**0.2 (0.4)**	**0.2 (0.4)**	NR	NR
LIPOA 2024	Enteritidis	1.9 (0.2)	0.5 (0.2)	0.2 (0.2)	1.1 (0.4)	NR	NR	NR
ATCC 14028	Enteritidis	1.9 (0.2)	0.7 (0.9)	0.5 (0.5)	2.1 (0.8)	1.3 (1.0)	1.6 (1.1)	**0.1 (0.2)**
LIPOA 2005	Infantis	1.3 (0.7)	2.1 (0.9)	2.6 (2.1)	**1.6 (1.9)**	**0.7 (1.2)**	NR	NR
LIPOA 2039	Infantis	1.5 (0.5)	2.0 (2.0)	1.9 (1.2)	0.9 (1.3)	2.5 (2.2)	**0.1 (0.2)**	NR
LIPOA 2007	Derby	1.6 (0.1)	2.0 (0.4)	1.9 (1.7)	0.3 (0.3)	NR	NR	NR
LIPOA 2035	Derby	2.1 (0.6)	1.6 (0.5)	0.1 (0.1)	**0**	NR	NR	NR

[a] Average of three repetitions (stander deviation). [b] Log 0 = most probable number per g (MPN/g) lesser than 1. [c] NR = *Salmonella* was not recovered from any repetition. Values in bold = *Salmonella* was not recovered from all repetitions.

are confirmed by isolation of *Salmonella* (Centers for Disease Control and Prevention, 2011). In Brazil, 8,663cases of foodborne diseases were reported to the Ministry of Health between 2000 and 2011, and *Salmonella* was the main etiological agent identified (Brasil, 2011).

The ability of *Salmonella* to survive in pasty *dulce de leche* for long periods has been reported by Andrews et al. (2011) but there are no studies about its behavior in this food. The knowledge of the fate of *Salmonella* in the environment provided by *dulce de leche* is important for the adoption of adequate control measures to avoid the infection of the consumers. The aim of this paper was to study the fate of *Salmonella enterica* subsp. *enterica* sorotypes Typhimurium, Enteritidis, Infantis and Derby in pasty *dulce de leche*.

MATERIALS AND METHODS

The *dulce de leche* was prepared from standardised milk containing 3% milk fat with the addition of two hundred gram sucrose to each liter of milk. The ingredients were kept at 100-105°C and stirred for 2 h and 30 min.

Strains of *Salmonella enterica* subsp. *enterica* from serotypes Typhimurium LIPOA 2046, isolated from pasty *dulce de leche*, and ATCC 13311; Enteritidis, LIPOA 2024, from poultry sausage, and ATCC 14028; Infantis, LIPOA 2005, from pork dry-sausage, and LIPOA 2039, from bovine ground beef; Derby, LIPOA 2035, from bovine ground beef, and LIPOA 2007, from poultry ground beef, were used. The isolates were kept at -70°C and recovered when necessary.

Twenty-five gram *dulce de leche* fractions were packed in sterile plastic bags, inoculated with 0.25 mL inoculum in the concentration 10^4 bacterial cells per mL, to obtain final concentrations of about 10^2 bacterial cells per gram of *dulce de leche*. The samples ware homogenized, kept at a temperature between 15 and 20°C, and analyzed after storage for 0, 1, 2, 3, 5, 10 and 20 days. Twenty-five grams of non-inoculated contaminated *dulce de leche* were used for negative control. *Salmonella* were counted by the method of the most probable number (MPN). Serial dilutions of the inoculated experimentally contaminated pasty *dulce de leche* were incubated at 37°C for 20 h in tubes with buffered peptone water (BPW, Acumedia). The presence of *Salmonella* in each tube was performed according to U.S. Food and Drug Administration - FDA recommendations (Andrews et al., 2011). The results were interpreted using the MNP table. The experiment was performed in triplicate.

RESULTS AND DISCUSSION

Several strains of *Salmonella* used to experimentally contaminate the *dulce de leche* were able to survive and to grow in this food (Table 1). The *Salmonella* ability to survive in dairy products has been reported (Hentges et al., 2010; Borges et al., 1990; Modi et. al., 2001), but the present paper is the first study about the fate of *Salmonella* in *dulce de leche*.

Silveira et al. (2012), working with wild strains of the serotypes Typhimurium and Enteritidis, found variable capacity to be able in *dulce de leche*, as our study, although we have worked with ATCC and wild strains. *Salmonella* Typhimurium was able to survive for five days in experimentally contaminated *dulce de leche*, although it was not able to grow in this medium. In despite of the strain LIPOA 2046 had been previously isolated from *dulce de leche* in a study performed by Timm et al. (TIMM), it survived for a lesser period than the strain ATCC 13311, what suggest strains LIPOA 2046 did not developed specials adaptations to this environment. It should be considered that the infective dose of *Salmonella* Typhimurium may be less than 10 bacterial cells (D'Aoust et al., 2001). Therefore, the mere presence of bacteria in food, even at low concentrations, is enough to cause concern about food security.

The two strains of *Salmonella* Enteritidis grew in *dulce de leche*, showed different behaviors as its ability of surviving. The strain ATCC 14028 showed considerable growth on the third day after the contamination, which may have contributed to the permanence of relatively high populations after ten days of storage and to the presence of some bacterial cells viable even after 20 days of the beginning of the experiment. This study suggest that strains of the serotype Enteritidis may have

high adaptability in foods with adverse conditions to its survival such as *dulce de leche*.

Both strain from the Infantis serotype showed adaptability to the environment provided by pasty *dulce de leche*, being able to survive for up to ten days of storage and increased relevantly its population in the early days. This adaptive capability justifies the concern about the presence of *Salmonella* Infantis in *dulce de leche*.

The strains of serotype Derby, showed different behavior. The strain LIPOA 2007 grew on the first day of storage, whereas the strain LIPOA 2035 showed progressive decrease in population density. However, none of the strains were recovered from the food five days after contamination, indicating that it was the serotype with lower survival capability in *dulce the leche* among the studied serotypes. This characteristic of *Salmonella* Derby is not enough to discard the serotype as important to food security, because *dulce de leche* is often consumed shortly after its acquisition in the retail market.

Conclusion

Salmonella Typhimurium, Enteritidis, Infantis and Derby showed different adaptive capabilities regarding population growth and survival in pasty *dulce de leche*, though all represent potential hazard to the consumer's health. The results are a warning regarding the need to take the appropriate hygienic-sanitary practices during *dulce de leche* manufacture, packing and handling.

Conflict of Interests

The authors have not declared any conflict of interests.

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