

**MANAGEMENT OF SORGHUM  
TURCICUM LEAF BLIGHT USING  
BACTERIAL ENDOPHYTES**

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**B. Sc. (Ag.)**

**MASTER OF SCIENCE IN AGRICULTURE  
(PLANT PATHOLOGY)**



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TURCICUM LEAF BLIGHT USING  
BACTERIAL ENDOPHYTES**

**BY**  
**KAVYA NATI**

**B. Sc. (Ag.)**

**THESIS SUBMITTED TO THE  
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**CHAIRPERSON: Dr. V. PRASANNA KUMARI**



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**2021**

## **DECLARATION**

I, **KAVYA NATI** hereby declare that the thesis entitled “**MANAGEMENT OF SORGHUM TURCICUM LEAF BLIGHT USING BACTERIAL ENDOPHYTES**” submitted to **Acharya N. G. Ranga Agricultural University**, for the degree of Master of Science in Agriculture in the main field **PLANT PATHOLOGY** is the result of original research work done by me. I also declare that no material contained in the thesis has been published earlier in any manner.

Place: Bapatla

**(KAVYA NATI)**

Date:

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# **CERTIFICATE**

**Miss. KAVYA NATI** has satisfactorily prosecuted the course of research and the thesis entitled “**MANAGEMENT OF SORGHUM TURCICUM LEAF BLIGHT USING BACTERIAL ENDOPHYTES**” submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that neither the thesis nor its part thereof has been previously submitted by her for a degree of any University.

Date:

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# CERTIFICATE

This is to certify that the thesis entitled “**MANAGEMENT OF SORGHUM TURCICUM LEAF BLIGHT USING BACTERIAL ENDOPHYTES**” submitted in partial fulfillment of the requirements for the degree of ‘**Master of Science in Agriculture**’ of the Acharya N. G. Ranga Agricultural University, Guntur is a record of the bonafide original research work carried out by **Miss. KAVYA NATI** under our guidance and supervision.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part and all assistance received during the course of the investigations have been duly acknowledged by the author of the thesis.

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## LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Per Cent
@	:	At the rate of
°C	:	Degree Celsius
AUDPC	:	Area under disease progress curve
CAS	:	Chrome azurool sulphonate
CD ( $P \leq 0.05$ )	:	Critical difference at 5 per cent level
cm	:	Centimeter
CFU ml <sup>-1</sup>	:	Colony forming units per ml
CRD	:	Completely randomised design
CV	:	Coefficient of variation
DAI	:	Days after inoculation or incubation
DAS	:	Days after sowing
eg	:	Example
<i>et al.</i>	:	and co authors
<i>etc.</i>	:	<i>et cetera</i> (and other things)
Fig	:	Figure (s)
FS	:	Foliar spray
g	:	Gram (s)
g lit <sup>-1</sup>	:	Gram (s) per litre
h	:	Hour (s)
<i>i.e.,</i>	:	<i>id elli</i> (that is)
kg	:	Kilogram (s)
kg ha <sup>-1</sup>	;	Kg per hectare
l	:	Litre (s)
m	:	Metre
M	:	Molar (s)
min	:	Minute (s)

ml	:	Millilitre (s)
mM	:	Milli molar (s)
M ha	:	Million hectares
M T	:	Million tonne
mm	:	Millimetre (s)
mm <sup>2</sup>	:	Square millimetre
NA	:	Nutrient agar
PDI	:	Percent disease index
PDA	:	Potato dextrose agar
pH	:	Hydrogen ion concentration
pg	:	pico grams
RH	:	Relative humidity
sec	:	second (s)
SEm	:	Standard error mean
SMA	:	Skim milk agar
sp., spp.	:	Species (singular or plural)
ST	:	Seed treatment
TSA	:	Tryptic soy agar
U	:	Unit (s)
UV	:	Ultra violet
<i>viz.</i> ,	:	Namely
μM	:	Micro molar (s)
μm	:	Micrometre
μM	:	Micromolar

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## ABSTRACT

Author	: <b>KAVYA NATI</b>
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The present research on “Management of sorghum Turcicum leaf blight using bacterial endophytes” was carried out at the Department of Plant Pathology, Agricultural College, Bapatla. A total of 13 bacterial endophytes were isolated and designated based on the plant part from which they were isolated.

Number of endophyte colonies was consistent in occurrence irrespective to the age of the leaf used for isolation, but was high from roots. The incubation period of 2.0 to 2.8 days was required for bacterial expression in sap inoculated samples. However it extended to 4.6 days with leaf bits.

Morphologically most of the endophytes from the roots and leaf bits were round with few exceptions while most of the isolates from the leaf sap were irregular except SLSE-01 which was round in shape. Culture characters of the isolates had even to undulated colony margins with an exception with SLSE-05 to be lobate. Eight of the isolates were with raised colonies, while five isolates SLBE-02, 03, 05 and SLSE-01, 05 were flat. Most of the colonies had smooth texture with opaque nature except SRSE-02 and 03 which were translucent. Most of the isolates were creamy white while, SRSE-02, SRSE-03, SLBE-01 and SLSE-05 isolates were white colour and, SLBE-04 was cream coloured.

Antagonistic studies revealed endophyte SRSE-01 with 52.75 % inhibition and was found significantly superior over other endophytes isolated from healthy leaf bits, root sap and leaf sap. SLSE-04 (51.65%), SLSE-05 (51.10%) and SLSE-03 (50.00%) isolates of leaf sap were found on par with each other and superior over endophytes from leaf bits. At interaction zone variation in growth pattern of fungus

with thickening of the hyphal strands (SRSE-01 and SLSE-02), anastomosis (SRSE-01, SLBE-02 and SLSE-05) and chlamydospores formation was observed. SRSE-01 was significantly superior over other isolates with high number of chlamydospores (222.67/ microscopic field) followed by SLSE-04 (135.33/ microscopic field) and SLSE-05 (117.67/ microscopic field) which were on par with each other. Lysis of the fungal mycelium was not observed in any of the interactions.

In detached leaf technique all the isolates were superior over control in per cent spotted area, isolates SRSE-01 was found superior with minimum diseased area of 0.38 cm<sup>2</sup> and with least per cent spotted area of 2.29% and was on par with SLSE-05 isolate (2.67%). No significant difference was observed among the isolates with respect to disease index. Based on the superior characters of SRSE-01, SLSE-04, SLSE-05 isolates in dual culture and detached leaf techniques, were advanced for other studies when found compatible with each other.

All the three isolates tested positive for biochemical characters such as utilization of nitrogen sources, production of proteases, amylase and catalase whereas they failed to produce siderophore and IAA. SRSE-01 and SLSE-05 were found to solubilize phosphate indicating their ability in phosphate solubilization. Zone of solubilization around their colonies was more pronounced in SLSE-05 isolate compared to SRSE-01 isolate.

Phenols content was found significantly high in the treatment where all the endophytes were simultaneously inoculated (2.06 mg g<sup>-1</sup>) compared to other treatments where endophytes are associated, however it was not superior over phenol from fungicide treated plants (2.29 mg g<sup>-1</sup>). Similarly, under green house conditions combination of all the three bacterial endophytes resulted in significantly high inhibition of disease (40.70%) at 64 DAS with minimum AUDPC value of 516.35 as against the control (739.53).

## Chapter – I

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### *Introduction*

## Chapter I

# INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench), a C4 plant of the family Poaceae, was originated in northeast Africa and is the fifth most important cereal crop grown after wheat, rice, maize and barley in the world. It is termed as 'poor man's crop' being used as staple food crop particularly those in the semi-arid tropics and a multipurpose crop raised for fodder, energy and starch production. It is cultivated under tropical, subtropical and temperate regions of the world and is a highly reliable crop suitable for hot and dry environments that performs well in marginal lands with low fertilizers and inputs. As it confronts prolonged drought in arid climate and due to its high water use efficiency, it is referred to as "camel crop". With the present changing climatic conditions it is a "climate change-ready crop" that provides food security and income for millions of poor farmers (Dogget, 1988).

Sorghum cereal is gluten free, 100 g of it contain 74.63 g carbohydrates, 6.3 g dietary fibre, 3.30 g fat, 11.30 g protein (exploreit.icrisat.org), minerals (Mg, Fe, Cu, Ca, P and K) and vitamins (niacin, riboflavin and thiamin) and is considered as 'power house of nutrients' (USDA, 2018). Smolensky *et al.* (2018) reported the presence of anti cancer agents such as antioxidants, anthocyanins and polyphenols in its barn.

The major sorghum producing countries in the world are United States of America, Nigeria, Ethiopia, Mexico and India. During 2018-19 it occupied an area of 42.14 M ha with an annual production of 59.34 M T and productivity of 1408 kg ha<sup>-1</sup> in the world. 16% of the global sorghum production is contributed by Indian sub continent from states of Andhra Pradesh, Tamilnadu, Karnataka, Madhya Pradesh, Maharashtra and Gujarat with the production of 480 M T from an area of 4.96 M ha and the productivity was 967 kg ha<sup>-1</sup> (FAO, 2018-19).

Sorghum yield potential is affected by biotic and abiotic stresses. It is attacked by a wide range of foliar, stem and panicle diseases (King, 1972). Foliar diseases like leaf blight, anthracnose, downy mildew and rust cause significant loss due to reduction in the photosynthetic area of the affected leaves. Leaf blight caused by *E. turcicum* is one of the most important foliar disease (Sharma and Jain, 1975) occurring in severe form in major sorghum cultivated areas in Guntur district.

When infection occurs at pre-flowering stage in susceptible cultivars, grain yield losses of up to 50% may occur (Frederiksen, 1980). It also causes yield losses from 27.6 to 90.7% in maize crop (Chenulu and Hora, 1962). Low fertilizer, narrow spacing, monocropping further intensifies the incidence with varied severity depending on the existing pathotypes (Ogolla *et al.*, 2019).

*E. turcicum* is a hemibiotrophic foliar pathogen of sorghum, teosinte, maize, *Paspalum*, *Triticum*, *Hordeum*, *Avena*, *Oryza* and *Saccharum* (Frederiksen, 1980; Bunker and Mathur, 2006). The pathogen was reported to be seed borne and survives both in time by producing chlamydospores and space by persisting in crop debris (Chidambaram *et al.*, 1973; Shree and Luke, 1983; Ahmed and Reddy, 1993). In the presence of warm climate and high relative humidity (RH) the pathogen tends to multiply in polycyclic manner on leaves of susceptible cultivar producing peculiar localised lesions that turn to elongated cigar shaped lesions. Low fertilizer, narrow spacing, monocropping intensifies the incidence with varied severity depending on the existing pathotypes (Ogolla *et al.*, 2019).

Naturally grown plants foster a wide range of microorganisms within their tissues that have unique ability to survive inside plants with little or no microbial competition, protect the plants by antagonizing phytopathogens, thus making them potential candidates for biological control (Misaghi and Donndelinger, 1990). They can be isolated from surface disinfested plant tissue that do not visibly harm the plant and are referred to as endophytes (Hallmann *et al.*, 1997). These endophytic bacteria probably have evolved in intimate relationships with their host plants through coevolutionary process and may influence various physiological processes of plants in ways that have not yet been elucidated. Moreover, endophytic bacteria are also been researched for their beneficial activities such as phosphate solubilisation activity, siderophore production, nitrogen fixation, production of plant growth hormones, extracellular enzymes and promotion of plant growth (Compant *et al.*, 2005).

Chemical control of the Turcicum leaf blight using carboxin (Khedekar *et al.*, 2012), tebuconazole (Manu *et al.*, 2017), mancozeb and propiconazole (Wani *et al.*, 2017) was reported. However, keeping in view of returns from the crop, use of chemicals for managing diseases burdens the farmers, in addition to this, increased health consciousness of the consumers about potential hazard due to usage of

chemicals in farming opened the door for natural and ecofriendly produced produce. Therefore, present work was formulated to manage the sorghum leaf blight using potential antagonistic endophytes that occur naturally in the plant system with the following objectives

**Objectives:**

1. Isolation of endophytes from sorghum roots and leaves
2. Screening endophytes for their antagonistic activity *in vitro*
3. Characterization of potential endophytes
4. Management of sorghum turcicum leaf blight using potential endophytes

## Chapter – II

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## *Review of Literature*

## Chapter II

# REVIEW OF LITERATURE

All plants are inhabited internally by diverse microbial communities. Certain bacteria and fungi enter plants as endophytes, establish a mutualistic association and play crucial roles in plant development, growth and fitness (Hardoim *et al.*, 2015). However, certain other microorganisms result in deleterious effect on plants. Therefore, prior inoculation with suitable endophyte could reduce impact of harmful fungal, bacterial, virus diseases and in some instances insects and nematodes (Kerry, 2000).

Leaf blight of Sorghum caused by *Exserohilum turcicum* is a disease of global importance, prevalent in all sorghum growing countries and its exceptional adaptability to diverse agro-ecological conditions is reported to damage in several countries (Kumar, 2018). Sorghum being referred to as poor man's crop receives very less attention for the management of diseases. Therefore use of natural means to protect the crop is of high need.

Emphasis on endophytic antagonists as a means of biological control has been given importance due to its environmental friendly approach (de Fretes *et al.*, 2018). Therefore, exploitation of endophyte–plant interactions can provide insight into and can play a significant role in low-input sustainable agriculture not only for Sorghum but also for both food and non food crops. Under this background literature pertaining to “Management of Sorghum Turcicum leaf blight using bacterial endophytes” was briefly reviewed and presented here under in this chapter.

## 2.1 BACTERIAL ENDOPHYTES

Endophytes are an endosymbiotic group of microorganisms that colonize inter and/or intracellular locations of plants without causing apparent disease (Pimentel *et al.*, 2011; Singh and Dubey, 2015). Evolutionarily, endophytes appear to be intermediate between saprophytic bacteria and plant pathogens, having protective shelter and nutrient supplies from their host. Each plant species that exist on the earth is a host to one or more endophytes (Strobel *et al.*, 2004). Endophytes are sheltered

from environmental stresses and microbial competition by the host plant, and they seem to be ubiquitous, having been isolated from various plant tissues (McInroy and Kloepper, 1995; Kobayashi and Palumbo, 2000; Posada and Vega, 2005). Endophytes are also known to occur within lichens and algae (Flewelling *et al.*, 2013).

Endophytic bacteria originate from the epiphytic bacterial communities by gaining entrance into plants through natural openings, wounds and also use hydrolytic enzymes to establish a mutualistic association (Perotti, 1926; Hallmann *et al.*, 1997; Azevedo *et al.*, 2000). Fungal and bacterial endophytes may comprise communities in plants that increase a plant's capacity to survive and thrive in its habitat (Sura-de Jong *et al.*, 2015).

Plants strictly limit the growth of endophytes. By maintaining stable symbiosis, Endophytes produce several compounds that promote plant growth and sustain well (Dudeja *et al.*, 2012). Endophytes help plants to adapt better to the environment (Lee *et al.*, 2004; Das and Varma, 2009) through its increased nutrient (phosphorus and nitrogen) uptake, protection from being attacked by fungi, insect and mammals by producing secondary metabolites (Namasivayam *et al.*, 2014) and also increases the expression of defense-related genes in plants. Thus making plants more resistant to many potential pathogens besides providing potential sources for novel natural products for their use in agriculture and nutrition (Fadiji and Babalola, 2020).

In spite of many reports of beneficial effects of endophytes, it has come to be implicit that the relationship between endophytes and hosts may be considered a balanced antagonism with both positive and negative effects on hosts depending on the environmental conditions (Kulkarini *et al.*, 2014)

### **2.1.1 Historic Perception of Endophytes**

Endophytes were first described by German botanist Link in 1809, and termed them as “Entophytae”, further were conferred to as a distinct group of partly parasitic fungi living in plants. De Bary (1866) first coined the term “Endophyte” and Vogl reported the presence of endophytes in 1898 (Venkateswarulu *et al.*, 2014).

Perotti (1926) used the term ‘endophyte’ to describe bacteria that had been isolated from within plants other than *Rhizobium* sp. Henning and Villforth (1940) reported the presence of bacteria in the leaves, stems and roots of apparently healthy plants.

Tervet and Hollis (1948) defined endophytes as microorganisms that are able to live inside plants without causing disease symptoms. In 1991, Petrini defined endophytes as “all organisms inhabiting plant organs that at some time in their life cycle can colonize internal plant tissues without causing apparent harm to their host” (Hardoim *et al.*, 2015). Conceptually, bacterial endophytes have been defined by Kado (1992) as “bacteria that reside within living plant tissues without doing substantive harm or gaining benefit other than securing residency”. While Quispel (1992) considered endophytes as bacteria that establish an endosymbiotic nature with the plant, whereby the plant receives certain ecological benefits from symbiont, such as increased stress tolerance or plant growth promotion *etc.*

Beattie and Lindow (1995) stated that an active exchange occurs between the internal and external populations of a bacterial strain of a plant. Hallmann *et al.* (1997) described endophytes as bacteria which can be isolated from surface disinfested plant tissue or extracted from inside the plant, and which do not visibly harm the plant.

## **2.2 TURCICUM LEAF BLIGHT**

Leaf blight is an economically important and widespread disease of sorghum in highly humid areas of Asia, Africa and the Americas. When infection occurs at pre flowering stage in susceptible cultivars, grain yield losses up to 50% may occur. The disease is considered more important in dual purpose and fodder sorghums than in grain sorghum under Indian conditions. Pathogen *Exserohilum turcicum* (Pass.) KJ Leonard and EG Suggs causes leaf blight in sorghum.

Symptoms are visible from the seedling stage to the crop maturity stage. Small, reddish or tan spots develop on seedlings, the spots later enlarge and coalesce resulting in wilting of young leaves. On mature plants, long, elliptical, reddish purple or yellowish lesions develop, first on lower leaves and later progress to the upper leaves and stem as well.

### **2.2.1 *Exserohilum turcicum***

Turcicum leaf blight disease was first reported by Passerini (1876) in Perma, Italy and named the pathogen as *Helminthosporium turcicum*. Leonard and Suggs (1974) established the *E. turcicum* (Pass.) for species in which the conidial hilum was

strongly protuberant. *E. turcicum* is a heterothallic fungus characterized by production of conidia on the terminals of the conidiophores often singly or in groups of 2-6 from the stomata of the diseased leaves. Conidiophores are simple, cylindrical and olivaceous brown. (Ellis and Holiday, 1971) and (Sivanesan, 1987) The conidiophores resume growth from the lateral side of conidial attachment, often become geniculate (Rani, 2015; Alcorn, 1988).

The conidia often germinate semi axially from one or both polar cells but rarely from intermediate cells (Alcorn, 1988; Thakur *et al.*, 2007). Conidia are light gray, straight or spindle-shaped or curved with rounded ends. They are three- to eight distoseptate and have a protruding basal hilum, and presence of circular conidial scars after their abscission from conidiophores (Bunkoed *et al.*, 2014)

### **2.2.2 Symptomatology of the Disease**

The disease is characterized by long spindle shaped necrotic grayish or tan lesions on leaves measuring up to 12.5 x 2.2 cm (Ullstrup, 1966; Wani, 2015). The typical symptoms of turcicum leaf blight are visible from the seedling stage to the crop maturity stage, starts with small elliptical water soaked lesions greyish in colour initially which later turn straw or brownish colour and become as large distinctive lesions that elongate to several centimetres long and 1-3 cm wide. The spots turn greenish with age and get bigger in size, finally attaining a spindle shape with straw coloured centre and dark margin. The colour of margin varies from brown, red or purple depending on host cultivar. Spores of the fungus develop abundantly on both sides of the spot. Under humid conditions, the centre of lesion develops faint grey bloom shade due to flush of conidia and conidiophores. These lesions appear first on the lower leaves and as the season progresses, the lesion number increases and all the leaves are covered. The plants look dead and grey (Frederiksen, 1980; Reddy *et al.*, 2014). Heavily infected field present a scorched appearance (Chenulu and Hora, 1962).

### **2.2.3 Prevalence of the Turcicum Leaf Blight**

Sorghum Turcicum Leaf blight is a widespread disease that has been attributed to 45% yield losses in sorghum in India (Chenulu and Hora, 1962; Elazegui, 1971; Sharma *et al.*, 2012). If the pathogen establishes itself on a susceptible host prior to

panicle exertion, yield may be greatly reduced (Sifuentes Barrera and Frederiksen, 1994; Frederiksen and Odvody, 2000).

Leaf blight is a polycyclic disease which relies vastly on sporulation from infected lesions for new infections (Ullstrup, 1966). Warm humid weather with heavy dew favours the spread and development of disease while dry weather hinders it (Ullstrup, 1966; Indira *et al.*, 2002; Agrios, 2005). Depending on the host-pathotype combinations in a particular environment, the disease incidence varies in prevalence and severity both in time and space with proportionate yield losses (Frederiksen, 1982).

The disease is more prevalent in humid areas with moderate temperatures (Pataky and Ledencan, 2006). The reduction of photosynthetic leaf area can lead, in severe cases, to grain yield losses of 20–25 % (Smith *et al.*, 1988). The grain will appear shrunken or “pinched” due to large amounts of leaf area that may be damaged by lesions (Leslie, 2002). The blighted leaves are not suitable for fodder because of the lowered nutrition value (Reddy *et al.*, 2014).

Turcium leaf blight was reported to be a common foliar disease in sorghum with high prevalence in the areas where abundant rainfall or humidity exists (Ngugi *et al.*, 2002). Similar report was also given by Abebe and Singburadom (2006) from Ethiopia when surveyed in low altitude areas *i.e.*, districts in Gambella where the disease was found to be severe.

In India, the disease was first reported by Butler in 1907 from Bihar. The disease is widely distributed in India (Ravindranath, 1980) and reported to occur in Andhra Pradesh, Tamil Nadu, Karnataka, Rajasthan and parts of Madhya Pradesh where the sorghum is mostly being cultivated (Anahosur, 1992). It was reported from many parts of the country, *viz.*, Lalmardi, Srinagar (Kaul, 1957), Punjab (Mitra, 1931), Himachal Pradesh (Chenulu and Hora, 1962) and Kashmir valley (Payak and Renfro, 1968).

## 2.3 ISOLATION OF BACTERIAL ENDOPHYTES

### 2.3.1 Surface Disinfestation and Isolation of Endophytes

Surface sterilization is the initial and mandatory step for isolation of endophytic bacteria where all the surface microbes get killed and is accomplished by treating plant tissues with a sterilizing agent for certain duration followed by 3-5 times sterile double distilled water rinsing. The confirmation of attaining sterile plant surface without affecting the host tissue and the endophytes can be confirmed through sterility checks. Only after, complete surface sterilization is confirmed; the isolated microorganisms can be referred to as endophytes (Anjum and Chandra, 2015).

The most common disinfectants are sodium hypochlorite (Gardner *et al.*, 1982; Fisher *et al.*, 1992), ethanol (Fisher *et al.*, 1992; Dong *et al.*, 1994), hydrogen peroxide (McInroy and Kloepper 1995), mercuric chloride (Gagne *et al.*, 1987; Hollis, 1951; Sriskandarajah *et al.*, 1993), or a combination of two or more of these at concentrations suitable to the sample used.

Scientists followed different surface sterilization methods for the isolation of endophytes. Gagne *et al.* (1987) took symptomless alfalfa plants (*Medicago sativa* L.) and washed its roots with soap water, rinsed thoroughly with tap water, and used it for isolation of endophytes. Tap roots were reported to cut into one cm pieces and were surface sterilized with 0.2% HgCl<sub>2</sub> in 50% ethanol for four minutes, followed by one minute rinses in three changes of sterile water, blot dried and placed onto nutrient media supplemented with 40 µg of cycloheximide per ml and incubation at 21 °C for 14 days. Similarly, for the isolation of endophytes from soybean stems, roots and nodules Hung and Annapurna (2004) took 2-3 cm long bits. After normal washing they were soaked in distilled water and drained. Later were rinsed in 70% ethanol for 30 sec and then sterilized with 0.1% HgCl<sub>2</sub> for three min in case of roots and nodules while five min for stems. The tissue was reported washed for 10 times with sterile water followed by aseptical maceration with homogenizers and serial dilution up to 10<sup>-6</sup> dilution. 100 µl from appropriate dilutions were spread plated on two different media, *viz.*, PDA and TSA.

Ahmed *et al.* (2012) used symptomless leaves, fruits, stem cuttings and roots of ethnomedicinal plants, and washed them several times under running tap water and sequentially rinsed with 70% ethanol for 30 sec, 0.01% HgCl<sub>2</sub> for five min, 0.5%

NaOCl for 2 min and finally cleaned them with sterile distilled water thrice and blot dried. The material was cut into smaller pieces and each piece was placed on potato dextrose agar (PDA) medium and incubated at 28 °C for isolating endophytes.

Anjum and Chandra (2015) described a procedure for isolation of endophytes from *Catharanthus roseus*, *Ocimum sanctum*, *Mentha arvensis*, and *Stevia rebaudiana*. Leaves, stems, and roots of plant were washed separately under tap water for 15 min followed by rinsing thrice with sterile distilled water. They recommended that surface sterilization for stem and leaves has to be done using ethanol for one min and HgCl<sub>2</sub> for 30 sec while for the roots with ethanol and HgCl<sub>2</sub> for one min each followed by rinsing in sterile water thrice and to be incubated at 28±2 °C on NA plates by adding antifungal agents. Aliquots of water from the last rinsing to be cultured onto NA plates for observing any growth.

Gupta *et al* (2015) described that leaves and roots of *Prosopis cineraria* were cut to small pieces and macerated separately in phosphate buffer of pH 7.2 with a sterile pestle and mortar. Tissue extract were prepared for tenfold dilution in sterile saline and serial dilutions (10<sup>-5</sup>, 10<sup>-6</sup>, and 10<sup>-7</sup>) of the extract were prepared and 0.1ml of the aliquot was used for streaking on NA plates separately for both roots and leaf tissue extract. Plates were incubated at 37 °C and were observed after 48 to 72 h for bacterial colonies.

### **2.3.2 Morphological Characterization of Bacteria**

Singh *et al.* (2013) reported that among the seven endophytic bacteria isolated from sugarcane most of the isolates exhibited round colonies with both wavy and smooth margins, convex elevation and white to creamy pigmentation. Likewise, Gupta *et al.* (2015) isolated three endophytes from the roots of *Prosopis cineraria* plants which were described as circular in shape, even, smooth, flat and yellowish to orange coloured with opaque nature.

## **2.4 ROLE OF BACTERIAL ENDOPHYTES IN ANTAGONISM**

Bacterial endophytes extensively colonize ecological niches of phytopathogens and create a "barrier effect", thus lessening or preventing the deleterious effects of certain pathogenic organisms (Kloeppe *et al.*, 1999; Berg *et al.*,

2005; Hallmann and Berg, 2006). Endophytic microorganisms were reported to have the capacity to control plant pathogens (Sturz and Matheson, 1996; Duijff *et al.*, 1997; Krishnamurthy and Gnanamanickam, 1997) and insects (Azevedo *et al.*, 2000) and nematodes (Hallmann *et al.*, 1997, 1998) through de novo synthesis of novel compounds and antifungal metabolites (Strobel *et al.*, 2004).

Endophytic bacteria have been recorded to demonstrate an inhibitory effect against many plant pathogenic fungi such as *Verticillium longisporum*, *Rhizoctonia solani*, *Fusarium oxysporum* and *Phythium ultimum* in balloon flower; *R. solani* and *F. oxysporum* in cotton; *Sclerotium rolfsii* in beans; *V. dahliae*, *V. alboatrum* and *R. solani* in potato and *R. solani* in ginseng (Berg *et al.*, 2005; Cho *et al.*, 2007).

Hebbar *et al.* (1991) reported antagonistic effect of endophytic bacteria viz., *Pseudomonas* sp, *Flavobacterium* sp and *Bacillus* sp isolated from the leaves and roots of sunflower on *Alternaria helianthi*, *Sclerotium rolfsii*, *R. solani* and *Macrophomina phaseolina* under *in vitro* conditions. Similarly, Raj *et al.* (2003) reported inhibitory effect of different *Bacillus* strains i.e., *B. pumilus* INR7 and *B. pumilus* S37 on pearl millet powdery mildew pathogen. Rajkumar *et al.* (2005) reported that among the 34 isolates tested, 12 isolates showed inhibitory effect on the growth of *Phytophthora capsici* radial growth. In detached leaves assay, among the isolates tested, antagonists PS27 and PS83 showed maximum suppression of lesion development on detached leaves. Shimizu *et al.* (2009) also reported that six endophytic isolates MBCu-32, 36, 42, 45, 56 and MBPu-75 to significantly reduce the number and size of the anthracnose lesions caused by *Colletotrichum orbiculare* on the cucumber cotyledons compared to the untreated control. Similarly, He *et al.* (2009) screened 175 endophytic bacteria for their antagonistic effect against *Colletotrichum gloeosporioides* and reported 86.1% inhibition by YB128 strain. Likewise, Shi *et al.* (2010) reported an endophytic bacterium capable of controlling ten parasitic and saprophytic fungi and bacteria that commonly infect harvested papaya fruit. Amaresan *et al.* (2014) highlighted the antagonistic influence of some endophytic bacteria isolated from chilli plants (*Capsicum annuum*) against the phytopathogens *Sclerotium rolfsii*, *F. oxysporum*, *Pythium* sp. and *Colletotrichum capsici*. Doye. (2013) reported that among 52 endophytic bacteria isolated from the healthy roots of maize, bacterial strain EB22 to show significant inhibition (80%) of *E. turcicum*. Selim *et al.* (2016) isolated three potential bacterial endophytes, of which isolate H8 with sharp antifungal activity against *R. solani* and *Pythium ultimum* while

isolate H18 against *Erwinia carotovora* and *R. solani* while, the isolate H40 demonstrated remarkable inhibitory influence against *E. carotovora* and *F. solani*. Rabbee *et al.* (2019) observed that detached citrus leaves infiltrated with endophyte *B. velezensis* significantly reduced the size of lesions caused by *Xanthomonas citri* subsp. *citri*. similarly Andreolli *et al.* (2019) observed that endophyte *Pseudomonas protegens* MP12 displayed a significant reduction of lesions caused by *Botrytis cinerea* detached leaves of grapevine. Likewise Liu *et al.* (2019) reported endophytic strain NEAU-S7GS2 with significant inhibitory effect on the mycelial growth of *E. turcicum* (67.6%) when compared to monoculture plate. Similar results have been observed by Kollakkodan *et al.* (2020) who observed a significant suppression of lesion caused by *Phytophthora capsici* on detached leaves of black pepper by antagonistic endophyte *B. velezensis* PCSE10.

According to Griffiths (1974), Harish *et al.* (1998) and Strunnikova *et al.* (2007), chlamydospore formation would result due to biotic and abiotic stresses. Several research teams previously reported the induction of chlamydospore formation in fungal plant pathogens by various antagonistic bacteria (Ram, 1952). Goh *et al.* (2009) reported that *B. amyloliquefaciens* SMCD 518 biocontrol candidate clearly demonstrated the capability to trigger the chlamydospore formation in *F. graminearum* and *F. sporotrichioides*. Pedersen *et al.* (1999) found that even the treatments with bacteria culture filtrates may increase the formation of chlamyospores of plant pathogens.

#### **2.4.1 Co-Inoculation of Endophytes**

The beneficial effects that the endophytes can confer on plants are discernible through their role in biological control of diseases in various crops (Bargabus *et al.*, 2004; Kloepper *et al.*, 2004). It is likely that, most cases of naturally occurring biological control results from mixtures of antagonists, rather than a single antagonist. Recently, a greater thrust is given for the development of biological consortium since it consists of microbes with different biochemical and physiological capabilities, which permits interaction among themselves and will lead to the establishment of a stable and effective microbial community. It will further provide better management of diseases by way of synergistic effect and multiple mode of action (James and Mathew, 2017)

Doye. (2013) reported the highest percentage of disease suppression against the leaf blight pathogen *E. turcicum* in the case of plants treated with a combined inoculum of endophyte isolates when compared to the singly inoculated plants under green house conditions

Santiago *et al.* (2017) reported that the potential endophytic strains T168 and R182 isolated from potato seedlings did not show any traces of growth inhibition when cross streaked with bacterial strain R170 and Irabor and Mmbaga (2017) reported that endophyte isolates B17B and Y grew well together without inhibiting each other's growth in compatibility test. Al-Hussini *et al.* (2019) reported that four rhizobacterial isolates designated D1/3, D1/8, D1/17 and D1/18 were found compatible with each other when subjected to compatibility tests using a cross-streak assay on nutrient agar medium. James and Mathew (2017) also reported that, among the 28 endophyte isolates, five isolates VRF-1, VSF-3, CSF- 1, MyRF-1 and ASF-3 to be highly compatible when subjected to mutual compatibility test by cross streak method. Interestingly Iqbal *et al.* (2020) observed compatibility among the bacterial endophytes (ZE15, ZE19 and ZE32) and rhizobacterial isolates (ZR3 and ZR19) through cross streak method.

## **2.5 THE MECHANISMS OF PLANT GROWTH PROMOTION**

Endophytes were reported to promote plant growth by a number of mechanisms *viz.*, ammonia production (Zahran, 1999), phosphate solubilization activity (Verma *et al.*, 2001; Wakelin *et al.*, 2004), Indole Acetic Acid production (Lee *et al.*, 2004), production of siderophores (Costa and Loper, 1994), supply of essential vitamins (Pirtila *et al.*, 2004), osmotic adjustment, stomatal regulation, modification of root morphology, enhanced uptake of minerals and alteration of nitrogen accumulation and metabolism (Compant *et al.*, 2005). Recently they are being used in forest regeneration and phytoremediation of contaminated soils. In some cases, they are reported to accelerate seedling emergence and to promote plant establishment under adverse conditions (Chanway, 1997; Bent and Chanway, 1998).

### **2.5.1 Utilization of Nitrogen Sources**

Some bacterial endophytes are capable of converting stable atmospheric nitrogen gas into ammonia by using the enzyme, nitrogenase (Zahran, 1999) thus providing a portion of the nitrogen demand of the host plant. They positively influence plants by improving growth and root development that enhances plant tolerance to various environmental stresses (Ullah *et al.*, 2019).

On the other hand, production of ammonia increases the plant defence against pathogens colonization therefore, production of ammonia by endophytes is a desirable trait for plant growth promotion and soil fertility (Li *et al.*, 2016).

Saini (2015) reported that two isolates from nodules and three isolates from the root produced ammonia. Prasad and Dagar (2014) reported that six endophytic bacterial isolates G1, G2, G1, SA3, SA4 and A1 isolated from black grapes and avocado that were able to produce ammonia. Out of 33 isolates, from sweet sorghum five isolates, BA8, BA10, MA11, MA32 and MA33 were capable of nitrogen fixation (de Fretes *et al.*, 2018). Brigido *et al.* (2019) reported high proportion (69.5%) of the endophytic bacterial isolates showed the ability to produce ammonia.

## **2.5.2 Siderophore Production**

Iron is an essential trace element for plants, acts as a cofactor in enzymes to catalyse redox reactions involved in fundamental cellular processes. Iron exists mainly in its oxidized ferric ( $\text{Fe}^{3+}$ ) state which is unavailable plant at neutral and basic pH levels that generally prevail in natural habitats. Under such conditions plants get benefited from bacteria that have potential to produce siderophores which chelate iron by a ligand exchange reaction resulting in uptake of siderophore-Fe complexes (Schmidt, 1999). Siderophores are low molecular weight compounds with high  $\text{Fe}^{3+}$  chelating affinity (Sharma and Johri, 2003) responsible for the solubilization and transport of this element into bacterial cells which in turn helps the plants in iron sequestration and facilitates the plant growth (Dimkpa, 2016).

The production of siderophores by endophytes can inhibit the growth of plant pathogens as they deprive the fungal pathogens from iron because their siderophore is having less affinity to iron than bacteria (Loper and Henkels, 1999; Sharma and Johri, 2003;). Large numbers of bacterial genera such as *Pseudomonas*, *Azotobacter*, *Bacillus*, *Rhizobium*, and *Enterobacter* are known to synthesize siderophores (Wittenwiler, 2007). Siderophores also form stable complexes with other heavy metals and help to relieve the stresses imposed on plants by high levels of heavy metals and can offer protection against reactive oxygen species (ROS) created by UV radiation or antibiotic stressors (Burke *et al.*, 1990; Jin *et al.*, 2018).

Forchetti *et al.* (2007) reported that none of the endophytic strains (SF2, SF3, SF4, SF5, SF7) isolated from sunflower were able to produce siderophores on CAS agar plates. Govindasamy *et al.* (2017) isolated 280 bacterial endophytes and reported that only 13 isolates (5%) developed strong yellow-orange halo zones on Chrome Azurol Sulphonate (CAS) agar plates, an indicative of their ability to produce siderophores.

### **2.5.3 Phosphate Solubilisation**

Phosphorous is an important essential element required for the growth and productivity of plants, involved in various biochemical and physiological activities of plants (Mittal *et al.*, 2008), increases the strength of cereal straw, flower initiation, seed formation and fruit production (Sharma *et al.*, 2011). However, a large proportion of P is present in insoluble forms and is consequently not available for plant nutrition. Plants absorb phosphorous as the monobasic ( $\text{HPO}_4$ ) and the dibasic ( $\text{H}_2\text{PO}_4$ ) forms (Glass, 1989).

Microorganisms play critical role in providing soluble phosphates to the plant through transformation (Sharma *et al.*, 2011). They produce organic and inorganic acids, exo-polysaccharides and enzymes that solubilize phosphates (Singh and Reddy, 2011; Arif *et al.*, 2017; Zhu *et al.*, 2011).

de Fretes *et al.* (2018) reported four endophytic isolates with phosphate-solubilizing activity BA3, BA8, BA14 and MA24. Brigido *et al.* (2019) reported, 17.5% of the bacterial endophytes isolated from Chickpea plants to solubilise phosphorous. Similarly AlKahtani *et al.* (2020) reported nine endophytes with significant ability to solubilize inorganic phosphate with clear zone on the Pikovskaya medium.

### **2.5.4 Indole-3-Acetic Acid (IAA) Production**

Indole-3-Acetic Acid (IAA) is one of the most physiologically active auxins. It is the key hormone that regulates plant growth and many physiological processes, such as cell division and differentiation, protein synthesis, elongation of stems (Lestari *et al.*, 2015), elongation of roots, lateral roots and root hairs, nutrient uptake and increased water uptake (Datta and Basu, 2000).

Endophytic bacteria such as *Azospirillum*, *Alcaligenes faecalis*, *Klebsiella*, *Enterobacter*, *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* has shown the ability to produce auxins (Hallmann *et al.*, 1997).

The amino acid tryptophan is identified as the main precursor for IAA and thus plays a role in modulating the level of IAA biosynthesis. Endophytic bacteria synthesize auxins which interferes in the physiological processes of plants by altering the plant auxin pool (Sukmadi, 2013). Singh *et al.* (2013) reported two isolates (StBn and RtBn) with IAA production in Yeast peptone mannitol medium supplemented with tryptophan. Similarly, Mohite (2013) reported nine endophytic isolates that produced significant amounts of IAA. Hung and Annapurna (2004) reported 15 endophytic isolates capable of producing IAA in the presence of the precursor tryptophan. Latif Khan *et al.* (2016) observed Endophytic strains MPB 2.1 and LK14 with IAA producing ability that significantly enhance the development of tomato seedling. Bind and Nema (2019) with 27 endophytes isolated from pigeon pea that lacked IAA producing ability.

### **2.5.5 Production of Enzymes**

Endophytic microbes provide an additional resource of beneficial enzymes which help the host to combat diverse arrays of biotic and abiotic stresses. Extracellular enzymes are the product of microbial cell growth and perform their function outside the cell in many biological or environmental processes. Plant endophytic bacteria have been widely accepted as an economic resource of vital and novel biomolecules (Vijayalakshmi *et al.*, 2016).

Enzymes namely, amylases and proteases are some of the key enzymes produced by endophytic bacteria which are detected through agar-based methods (Sturz *et al.*, 2000; Carrim *et al.*, 2006). Amylase is an extracellular enzyme responsible for breaking down plant starches (Krishnan *et al.*, 2012). Protease producing bacteria are reported to exhibit biocontrol activity on plant pathogenic fungus through lysis of fungal cell wall (Srivastava *et al.*, 2013). Catalases are involved in decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS).

Krishnan *et al.* (2012) reported amylase activity of *Bacillus* sp. PE-5, SP-1, PE-LR-3 and PE-LR-4. Singh *et al.* (2013) reported casein hydrolysis by endophytic isolate, RtChII and catalase activity by five other endophytic isolates. Gupta (2015) reported endophytic bacteria *B. subtilis* isolated from roots with high amylolytic, and catalase activity. Vijayalakshmi *et al.* (2016) isolated five endophytic bacteria from medicinally important plants, producing amylase and protease. A total of 10 isolates were tested positive for casein hydrolysis and catalase test in an investigation by Bhagya *et al.* (2019).

Dunne *et al.* (1997) have shown the suppression of phytopathogenic fungus, *Pythium ultimum*, in the rhizosphere of sugar-cane was due to the elaboration of extracellular protease by strains of *Stenotrophomonas maltophilia* W81. *Pseudomonas fluorescens* CHA0 synthesizes an extracellular protease active against the phytopathogenic nematode *Meloidogyne incognita* (Siddiqui *et al.*, 2005). Protease is a hydrolytic enzyme which is involved in the suppression of pathogenic growth and subsequent reduction in damage to plants (Bashan and de-Bashan 2005). Hydrolytic enzymes help with their antimicrobial activity related to mechanisms promoting plant defence to phytopathogens (Bootkotr and Mongkolthanaruk 2012). Bacterial strains possessing a catalase are assumed to be highly resistant to various environmental, mechanical and chemical stress (Kumar *et al.*, 2012).

Amylase enzyme is known to promote early germination and increase the availability of starch assimilation (Gholami *et al.*, 2009). Indirect action of PGPR involve the elimination of harmful microorganisms via the production of antibiotics or (and) lytic enzymes (Protease, amylase), HCN or catalase (Patel and Pratibha, 2014).

Amylase and catalase enzymes play an important role in promotion of plant growth and biological control of plant diseases (Sousa *et al.*, 2008). Krishnan *et al.* (2012) reported amylase activity of *Bacillus* sp. PE-5, SP-1, PE-LR-3 and PE-LR-4. Gupta (2015) reported endophytic bacteria *B. subtilis* isolated from roots with high amylolytic, and catalase activity. Vijayalakshmi *et al.* (2016) isolated five endophytic bacteria from medicinally important plants, producing amylase and protease.

Singh *et al.* (2013) reported casein hydrolysis by endophytic isolate, RtChII and catalase activity by five other endophytic isolates. Bacterial strains showing catalase activity are reported as highly resistant to environmental, mechanical and

chemical stress (Geetha *et al.*, 2014). A total of 10 isolates were tested positive for casein hydrolysis and catalase test in an investigation by Bhagya *et al.* (2019).

### **2.5.6 Phenols Production**

Antioxidant phytochemicals like phenols are secondary plant metabolites constructed with one or more hydroxylated aromatic or phenolic rings. Many phenolic derivative phytochemicals are important in plants to resist oxidative and environmental stresses like UV radiation, pathogens and parasites.

Endophytic microbes intricately modulate host defense pathways upon challenge with any form of environmental stress. Formation of phenolic deposits in immediate vicinity of the pathogen invaded area results in severe damage to their hyphal structure (Benhamou and Nicole, 1999). Phenol synthesis include either enhancement of defense responses within the host by manipulating host metabolic pathway (Lattanzio *et al.*, 2006) or initiation of direct antimicrobial activities against the pathogens (Mandal *et al.*, 2010)

Endophytic microbes primarily behave as microbial elicitors aiding the host for an augmented buildup of defense against biotic stress (Singh *et al.*, 2014; Jain *et al.*, 2015). Shi *et al.* (2011) reported that level of phenolic content increased in papaya fruits treated with endophyte, MGY2 which significantly reduced the post harvest diseases incidence during the storage. Ray *et al.* (2020) reported that plants treated with endophytic bacteria exhibited a sharp elevation in the total phenol content in comparison to their infected control counterparts.

Sequeira (1983) reported that, phenol oxidation results in products that are highly toxic to pathogens. Phenols are reported as the secondary metabolites produced by plants that act as phytoalexins, antioxidants *etc.* (Blainski *et al.*, 2013). They get synthesized in plants in response to pathogen infection which are associated in the resistance mechanism (Lattanzio *et al.*, 2006).

It is an established fact that phenols are produced more rapidly in resistant plants than susceptible ones (Matta, 1967). It was also reported that non pathogenic fungi induce plants for the synthesis of more phenols than pathogenic forms (Matta, 1969).

Therefore, increase in phenols in challenge inoculated plants over uninoculated control and significant increase in phenols in plants treated with endophytes over control is a confirmation that even non pathogenic are bacterial endophytes to trigger phenols which in turn reduced the disease severity.

## **2.6 MANAGEMENT OF SORGHUM TURCICUM LEAF BLIGHT USING BACTERIAL ENDOPHYTES**

Sorghum turcicum leaf blight can be managed by various methods. Conidia and hyphae of the fungus survive in crop debris and seed, proper field sanitation (e.g., burying sorghum debris via tillage) and the use of pathogen-free seed is advisable (Frederiksen and Odvody, 2000; Leslie, 2002). In a study to examine the application of host genotype mixtures (*i.e.*, hybrid mixtures) as a disease management tool for controlling a sorghum disease, Sifuentes Barrera and Frederiksen (1994) found that incorporation of leaf blight-resistant hybrids improved yield and reduced the disease severity of the susceptible hybrids in the same mixture. Further, Ngugi *et al.* (2001) found that intra-row mixtures of non-host maize and sorghum could delay leaf blight and anthracnose infection and reduce season-wide disease progress, which has implications for small-holder intercropping strategies.

Crop rotation with non-hosts of *E. turcicum*, such as dicotyledonous crops and removal of infected grasses or volunteer sorghum and destruction of reservoir hosts in and around production fields is an effective control strategy (Ratnadass *et al.*, 2012).

Turcicum leaf blight was reported to be effectively controlled with use of chemicals such as carboxin (Khedekar *et al.*, 2012), tebuconazole (Manu *et al.*, 2017), mancozeb and propiconazole (Wani *et al.*, 2017). However, prolonged usage of these chemicals results sever damage to the environment So, eco-friendly, sustainable and cost effective alternatives are being researched. Bacterial endophytes now-a-days are being used as biocontrol agents in many other cases may be used effectively to reduce the disease.

Shiomi *et al.* (2015) reported that bacterial strains, *B. subtilis* *B. lentimorbus*, *Streptomyces* sp. and *B. agaradhaerens* of nine endophytic bacterial strains evaluated against northern corn leaf blight (*E. turcicum*), by microbiolization of seeds and aerial parts of maize under greenhouse conditions showed control of diseases from 42 to 61%. Similarly Kim *et al.* (2016) reported that spray of GYL4 bacterial suspensions

significantly reduced the area of anthracnose lesions on pepper leaves under greenhouse conditions. Dawut *et al.* (2016) reported that two endophytic bacterial strains of *B. subtilis*, XJAS-AB-13 and XJAS-AB-11 with broad antifungal activity against maize pathogen *E. turcicum* with 63.33% and 45.0% control respectively. In the same way Liu *et al.* (2018) reported that the disease indices of northern corn leaf blight in potted maize seedlings were significantly reduced by foliar spraying of endophytic bacterial strain LB-1 culture broth before or after the inoculation of *E. turcicum*, with control efficacies of 27.87% and 37.85%, respectively. Whereas Kollakkodan *et al.* (2020) reported that through pre-planting bacterization process by endophytic bacterial strains PCRE10, PCSE10 and PCLE3 the highest disease suppression of 75.05% over the pathogen control was observed in plants treated with the isolate PCRE10.

Presence of *Pseudomas* spp. in internal tissues of stem was reported to suppress sheath blight disease in rice (Krishnamurthy and Gnanamanickam, 1997). Antagonistic effect of *B. subtilis* against *Cryphonectria parasitica* causing chestnut blight was reported to reduce lesion area on stem (Wilhelm *et al.*, 1998). Kalraa *et al.* (2010) isolated four endophytic bacteria (OS-9, OS-10, OS-11, OS-12) from *Ocimum sanctum* and reported that OS-9 to have high inhibitory effect on the growth of *R. solani*, *A. solani*, *F. solani* and *C. lindemuthianum* while OS-11 antagonistic to *A. solani* alone. Mmbaga *et al.* (2018) tested 16 endophytes, isolated from *Cornus florida* stem tissue against root rot pathogens, *F. solani*, *F. oxysporum*, *Macrophomina phaseolina* and *Phytophthora* and found an isolate A22F1 to significantly reduce disease severity.

## Chapter – III

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### *Material and Methods*

## **Chapter III**

# **MATERIAL AND METHODS**

The research on “Management of Sorghum Turcicum leaf blight using bacterial endophytes” was carried out during 2019-20 at the Agricultural College, Bapatla. Laboratory experiments were done in the Department of Plant Pathology, Agricultural College, Bapatla. The material and methods used in the research are described in this chapter.

### **3.1 GLASSWARE**

Glassware made of Borosil was used throughout the present investigation. They were Petri plates, test tubes, conical flasks, beakers, pipettes and measuring cylinders were first washed with detergent followed by thorough cleaning with tap water before placing them in cleaning solution (Potassium dichromate 25 g, concentrated sulphuric acid 50 ml and water 1000 ml) for 24 h and finally were rinsed with distilled water for 3-4 times and are air dried before use.

### **3.2 CHEMICALS**

Analytical laboratory grade chemicals were used in the present investigation.

### **3.3 EQUIPMENTS**

Hot air oven and autoclave were used for sterilization of glassware and media respectively. Incubation was done in biological oxygen demand (BOD) incubator and cultures were temporary stored at 4 °C in refrigerator. Compound microscope Labomed (L X 400) was used for microscopic studies. Weighments were done on a single pan electronic balance with a sensitivity of 0.001g. Other tools that are used in the present investigation for various purposes like camel brush, inoculation needle, inoculation loop, cork borer, slides, cover slips, *etc.* were sterilized prior to usage.

### **3.4 PREPARATION OF MEDIA**

Media used was prepared in distilled water and autoclaved at 15 lbs pressure for 15 min. While preparing broth, agar-agar was omitted from the media. Compositions of various media used in the present investigation are given below

### 3.4.1 Tryptic Soy Agar (TSA) Medium

Tryptic Soy Agar medium was prepared by suspending 45 g of Tryptic soy agar, (Himedia) in 1000 ml distilled water and was heated till boiling to dissolve the medium completely and was followed by sterilization.

### 3.4.2 Nutrient Agar (NA) Medium

The following ingredients were used to the prepare the medium followed by autoclaving at 15 lbs pressure (121 °C) for 15 min prior to use.

Beef extract	3.0 g
Peptone	5.0 g
Agar agar	20 g
NaCl	5 g
Distilled water	1000 ml
pH	7.0

### 3.4.3 Potato Dextrose Agar (PDA) Medium

The following ingredients were used to the prepare the medium followed by autoclaving at 15 lbs pressure (121 °C) for 15 min prior to use.

Potato	250 g
Dextrose	20 g
Agar agar	20 g
Distilled water	1000 ml
pH	5.6 ± 0.2

### 3.4.4 Chrome Azurol-Sulphonate (CAS) Agar Medium

**i) Solution A (Fe – CAS indicator solution):** 50 ml of dye solution containing 60.5 mg CAS was prepared in distilled water and mixed with 10 ml of iron solution containing 1mM FeCl<sub>3</sub>.6H<sub>2</sub>O in 10 mM HCl. The mixture was slowly added to a solution containing 72.9 mg Hexadecyl trimethylammonium bromide (HDTMA) in 40 ml of distilled water by constant stirring. The resultant dark blue liquid was autoclaved at 15 lbs pressure for 10 min.

**ii) Solution B:** Basal medium was prepared using deferrated 1M sucrose (3 ml), deferrated 1M CaCl<sub>2</sub> (0.4 ml), deferrated 1M MgSO<sub>4</sub>.7H<sub>2</sub>O (0.8 ml), 2% K<sub>2</sub>HPO<sub>4</sub> (10 ml), 10% NaCl (2 ml), 5% Na<sub>2</sub>MoO<sub>4</sub> (0.1 ml), Pipes buffer (30.42 g) and agar (15 g) in 800 ml of distilled water. The pH of the medium was adjusted to 6.8 by the addition of 50% (w/w) NaOH and autoclaved at 15 lbs pressure for 20 min.

After cooling of solution B to 50 °C, 30 ml of 10% mannitol solution was supplemented as a carbon source and then solution A was added by mixing gently to avoid the formation of air bubbles. The molten medium was distributed in sterilized Petri plates (Schwyn and Neilands, 1987).

### **3.4.5 Luria Bertani Agar Medium**

Luria Bertani Agar, (Himedia) of 40 g was added to 1000 ml distilled water and was boiled to dissolve completely followed by autoclaving at 15 lbs pressure (121 °C) for 15 min prior to pouring into petri plates for use.

### **3.4.6 Skimmed Milk Agar Medium (SMA)**

The media was prepared using the following ingredients followed by autoclaving at 15 lbs pressure (121 °C) for 15 min prior to use.

Skim milk	15 g
Yeast extract	0.5 g
Agar agar	20 g
Distilled water	1000 ml

### **3.4.7 Starch Agar Medium**

Media was prepared and autoclaved prior to use at 15 lbs pressure (121 °C) for 15 min.

Peptone	10 g
Beef extract	5 g
Starch (soluble)	10 g
Agar agar	20 g
Distilled water	1000 ml
pH	7.0

**3.4.8 Water agar medium:** To obtain pure cultures 2% water agar (20 g in 1000 ml of distilled water) was used.

**3.4.9 Preparation of PDA slants:** Sterilized test tubes containing PDA media were kept in slanting position and solidified slants were stored in refrigerator for further use.

**3.4.10 Preparation of NA slants:** Sterilized test tubes containing NA media were kept in slanting position and solidified slants were stored in refrigerator for further use.

### **3.5 ISOLATION OF ENDOPHYTIC BACTERIA**

#### **3.5.1 Preparation of Plant Samples**

Endophytic bacteria were isolated from the root and leaf bits of green-house grown healthy sorghum seedlings of M35-1 variety at 10, 20 and 30 days after seedling emergence. The samples were rinsed thrice with tap water followed by sterile distilled water twice and were cut to small pieces with a sterile scalpel.

#### **3.5.2 Surface Disinfestation**

Leaf and root bits were sterilised separately using 70% ethanol for 5 min, later were passed through series of four washes using sterile double distilled water for two min each. It was followed by sterilization using 0.1% mercuric chloride for five min and four washes of sterile double distilled water for two min each, and were blot dried as per the modified protocol of de Fretes *et al.* (2018)

#### **3.5.3 Isolation**

Inoculation was done by placing sample bits on tryptic soya agar plates and serial dilution method

**3.5.3.1 Isolation using leaf bits:** Leaf samples were cut into small bits using flame sterilised scalpel and were placed on TSA plates and incubated at  $28\pm 2$  °C for 5-7 days.

**3.5.3.2 Isolation by serial dilution:** Leaves and roots were macerated separately in phosphate buffer solution using sterilised pestle and motor and were used for preparing serial dilutions upto  $10^{-8}$ . 0.1 ml of  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$  dilutions were spread on TSA plates using a flame sterilized spreader and were incubated at  $28 \pm 2$  °C for 2-5 days.

### **3.5.4 Sterility Check**

0.1 ml of final rinsed distilled water obtained after second sterilization was spread on TSA plate for sterility check and was incubated at  $28 \pm 2$  °C.

### **3.5.5 Selection and Purification of Different Bacterial Morphotypes**

Each plate was examined for the selection of bacterial morphotypes on the basis of morphological parameters *viz.*, colony size, shape, colour, margin and texture and pigmentation under Compound microscope Labomed (L X 400). All morphotypes were purified by quadrant streaking on NA plates and pure cultures obtained were preserved on NA slants at 4 °C as working culture

## **3.6 SCREENING FOR POTENTIAL ANTAGONISTIC BACTERIAL ENDOPHYTES *IN VITRO***

### **3.6.1 Isolation and identification of pathogen**

Sorghum leaves with typical turicum blight symptoms collected were used for isolating the pathogen. To confirm presence of the pathogen in the collected specimen, the diseased leaves were scraped and observed under microscope.

Leaf bits of four mm<sup>2</sup> with healthy and infected leaf portion were cut, surface sterilized using 1% sodium hypochlorite for a minute and rinsed in three changes of distilled water to remove the disinfectant. Leaf bits were blot dried before transferring aseptically on to PDA plates and was incubated at  $27 \pm 1$  ° C in incubator.

Three day old mycelial bits developed from diseased leaf were aseptically transferred to glass slide and observations were made to confirm their identity based on morphological characters (conidia and conidiophore). The obtained pathogen cultures were sub-cultured on PDA after confirmation.

### 3.6.2 Dual Culture Technique

Dual culture technique was used to obtain the potential antagonistic endophytes against *E. turcicum*. Mycelial disc of pathogen, *E. turcicum* was inoculated at the center of PDA plate. Test bacterial endophytes were streaked individually on both the sides of the *E. turcicum* disc at 2.5 cm distance leaving a space of 2.0 cm from periphery. Plates inoculated with *E. turcicum* alone served as check. Inoculated plates incubated at  $28 \pm 2$  °C were used to record zone of inhibition after four days of inoculation where *E. turcicum* has shown maximum growth in monoculture plate. Per cent inhibition of mycelial growth of test pathogen over control was calculated by the formula given by Vincent (1947).

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Per cent reduction in growth of test pathogen.

C = Radial growth (mm) in monocultured check.

T = Radial growth (mm) in dual cultured plates.

**3.6.2.1. Microscopic observations:** Signs of antagonism/ hyperparasitism and lysis were observed using compound microscope. Number of chlamydospores per microscopic field was counted and comparisons were made with the monoculture plate.

### 3.6.3 Leaf Detachment Technique

Fresh and healthy sorghum leaves were cut into small bits and were placed in Petri plate containing moist filter paper after surface sterilization. A leaf bit was used per Petriplate and was replicated thrice using each endophytic bacterium. The leaf bits were inoculated with 3 ml of endophytic bacterial suspension ( $1 \times 10^8$  CFU ml<sup>-1</sup>) using a hand sprayer and was incubated at 30 °C for 24 h in dark chamber (Sun *et al.*, 2013). Later, pathogen ( $10^8$  spores/ml) was inoculated @ 3 ml per leaf after 24 h. The sterile distilled water sprayed leaves served as negative controls. Data on number of spots, total spotted area was measured by graphic method from the drawings on tracing the paper at 5 DAI with endophytic bacterial endophytes and calculated the total spotted area, mean per cent spotted area. The per cent disease index was calculated based on 1 to 9 scale given by Thakur *et al.* (2007) (Table 3.1).

$$\text{Disease index (\%)} = \frac{\text{Sum of individual disease scale}}{\text{Total number of leaves scored} \times \text{Maximum disease scale value}} \times 100$$

**Table 3.1. Leaf Blight Severity Rating Scale: 1 to 9 (Thakur *et al.*, 2007)**

Severity rating	Symptom and lesions types
1	0 to <1% leaf area with mild yellow flecks
2	1-5% leaf area covered with hypersensitive small lesions
3	6-10% leaf area covered with hypersensitive small lesions
4	11-20% leaf area covered with small necrotic lesions
5	21-30% area covered with small necrotic coalescing lesions
6	31-40% area covered with large coalescing necrotic lesions
7	41-50% leaf area covered with large coalescing necrotic lesions
8	51-75% leaf area covered with large coalescing necrotic lesions
9	76-100% leaf area covered with large coalescing necrotic lesions

### **3.7 COMPATIBILITY AMONG THE ENDOPHYTIC BACTERIA**

The selected bacterial endophytes were tested for their compatibility by cross streak method. Two different bacterial isolates were streaked vertically and horizontally on NA mediated plates. The plates were incubated for 48 h at room temperature and observed for inhibition at the juncture of the streaks (James and Matthew, 2017)

### **3.8 BIOCHEMICAL CHARACTERIZATION OF ENDOPHYTIC BACTERIA**

The purified isolates of the potential antagonistic endophytic bacteria were functionally screened for biochemical characters.

#### **3.8.1 Utilization of Nitrogen Sources**

24 h old endophytic cultures were transferred to 10 ml peptone broth in each tube and incubated at  $28 \pm 2$  °C for 48-72 h. Nessler's reagent was added to each tube after incubation. The development of faint yellow to dark brown colour was a positive test for ammonia production (de Fretes *et al.*, 2018).

### **3.8.2 Siderophore Production**

The CAS (Chrome Azurol-Sulphonate) agar plates were spot inoculated with 24 h old endophytic bacteria and incubated at 30 °C for 2-3 days. Development of a deep yellow to orange colour surrounding the colony was a positive indication for siderophore production (Govindasamy *et al.*, 2017).

### **3.8.3 Phosphate Solubilisation**

Phosphate solubilizing activity of the isolates was screened on Pikovskaya Agar (Pikovskaya, 1948). Each plate was inoculated with a spot of 24 h old endophytic bacterial culture. Incubation of the plates was done for 48-96 h at 30 °C for the observation of clearing zones which is an indicator of P-solubilization (de Fretes *et al.*, 2018).

### **3.8.4 Indole-3-Acetic Acid (IAA) Production**

One day old endophytic bacterial isolates were aseptically inoculated into sterile nutrient broth, and incubated for 72 hours. The cultures were then centrifuged at 12,000 g, for 5 min, at 25 °C to obtain cell free broth. Few drops (0.5 ml) of Salkowski's reagent (0.5 M ferric chloride+35% perchloric acid ) were then added to the cell free broth and incubated for thirty min at room temperature after which observations on colour change were done (Bind and Nema, 2019).

### **3.8.5 Enzymatic Activity**

**3.8.5.1 Protease test:** To measure protease enzyme, 24 h old fresh cultures of bacterial endophytes were placed on the center of the SMA medium and incubated for 48 h. Colourless halo zones around bacterial colonies were indicative of protease production (Ullah *et al.*, 2018).

**3.8.5.2 Starch hydrolysis:** A loopful of the 24 h old endophytic bacterial culture was spotted on starch minimal medium plates. Plates were incubated at 28±2 °C for 48-72 h. Testing for amylase producing bacteria was done by flooding the surface of the starch agar plates with Gram's iodine. A clear zone around bacterial colony indicated hydrolysis of starch (Ullah *et al.*, 2018).

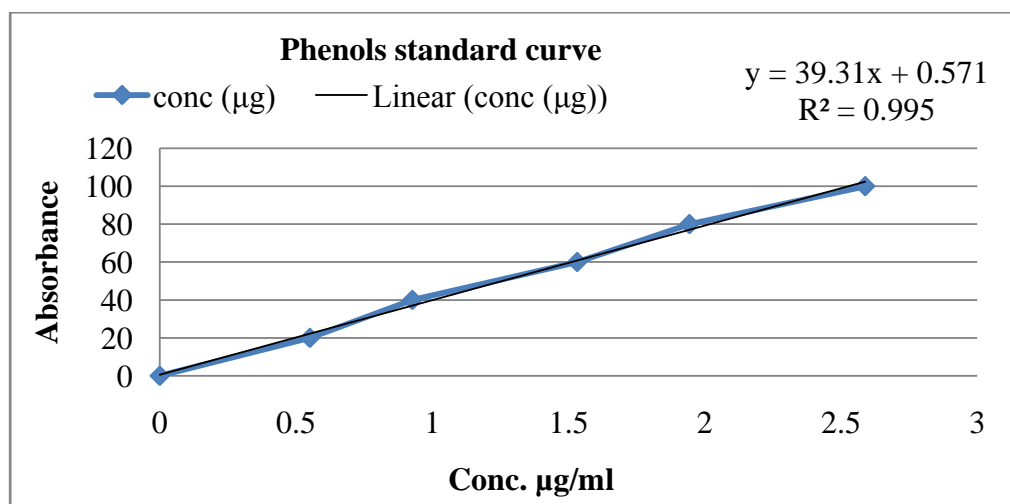
**3.8.5.3 Catalase production test:** Endophytic Bacterial cultures were grown for 24 h on NA medium slants. Then, a loopful of bacterial growth was taken on the slide and few drops of 3% H<sub>2</sub>O<sub>2</sub> were added. A positive catalase reaction was indicated by emission of effervescence due to production of oxygen from breakdown of H<sub>2</sub>O<sub>2</sub> (Ullah *et al.*, 2018).

### 3.8.6 Phenol Estimation

0.5 to 1.0 g of the sample was weighed and ground with a pestle and mortar in 10-time volume of 80% ethanol, homogenate was centrifuged at 10,000 rpm for 20 min. and the supernatant was saved and re-extraction was done with residue with 5 times the volume of 80% ethanol, centrifuged. The supernatants were pooled. Supernatant was evaporated to dryness. Residue was dissolved in a known volume of distilled water (5 ml). Aliquots of 0.2 to 2 ml were pipetted into test tubes, and volume in each tube was made to 3 ml with water.

0.5 ml of Folin-Ciocalteu reagent was added to each tube. After 3 min, 2 ml of 20% Na<sub>2</sub>CO<sub>3</sub> solution was added to each tube, and mixed thoroughly. Tubes were placed in boiling water for exactly one min cooled and absorbance was measured at 650 nm against a reagent blank.

Standard curve was prepared using different concentrations of catechol. From the standard curve the concentration of phenols was found out in the test sample and expressed as mg phenols/100 g material (Sadasivam and Manikam, 1992)



**Fig. 3.1. Standard curve of phenols**

### 3.9 *IN VIVO* MANAGEMENT OF SORGHUM TURCICUM LEAF BLIGHT

Treatment No.	Seed treatment + Foliar spray
T <sub>1</sub>	SLBE-09 (ST+FS)
T <sub>2</sub>	SLBE-10(ST+FS)
T <sub>3</sub>	SRBE-01(ST+FS)
T <sub>4</sub>	SLBE-09+SLBE-10(ST+FS)
T <sub>5</sub>	SLBE-10+SRBE-01(ST+FS)
T <sub>6</sub>	SLBE-09+SRBE-01(ST+FS)
T <sub>7</sub>	SLBE-09+SLBE-10+SRBE-01(ST+FS)
T <sub>8</sub> (positive control)	Mancozeb @0.1%(ST+FS)
T <sub>9</sub>	CONTROL

#### 3.9.1 Preparation of Endophytic Bacteria Cultures

Bacterial endophytes were cultured in nutrient broth for 48 h and were used for seed treatment and foliar spray by diluting using sterile distilled water to maintain  $10^8$  CFU ml<sup>-1</sup>

#### 3.9.2 Seed Treatment

Seeds of sorghum were surface sterilized twice using sterile dis. H<sub>2</sub>O, 70 % ethanol for 5 min followed by 4 % sodium hypochlorite for 20 min and finally rinsed four times with sterile deionized water. Surface sterilized seeds were soaked in the endophyte culture for six hours and allowed for shade drying. To maintain +ve check, seeds were treated with mancozeb @ 0.1 %.

#### 3.9.3 Sowing

Sorghum seeds were sown in green house plastic cover of size 25x25 cm filled with potting mixture of 1.5 kg (Soil: FYM: Sand in 6:2:1). The treatments were arranged in a completely randomized block design with three replications per treatment.

#### 3.9.4 Pathogen Inoculum Preparation

Fungal isolate was grown in PDB for 10 days and conidia were obtained by filtering the cultures through double-layered muslin cloth and spore concentration was adjusted to  $10^8$  CFU ml<sup>-1</sup> with the help of haemocytometer. 21-day old Sorghum

seedlings raised after endophyte seed treatment were spray-inoculated with inoculum using a hand atomizer. The spore suspension was added with 0.1% Tween-20 to ensure uniform spread of inoculum (Dhingra and Sinclair, 1985).

### 3.9.5 Foliar Spray Using Bacterial Endophytes

Two sprays were given at 15 day interval with the first spray initiated at 35 DAS. The plants were maintained with maximum relative humidity and examined every day for appearance of lesion. Disease severity of Turicum leaf blight was assessed on the day before spraying, 7, 14 days after each spraying by following the scale given by Thakur *et al.* (2007) in Table 3.1 and the PDI was calculated.

The efficacy of bacterial endophytes was tested using two sprays. First spray was initiated 15 days after pathogen inoculation that coincides with disease occurrence and followed by second spray after 15 days, the plants were maintained with maximum R.H and examined regularly for disease development. Disease severity was scored at weekly interval following scale given by Thakur *et al.* (2007) (Table 3.1) and PDI was calculated as mentioned earlier.

**3.9.6 Area under Disease Progress Curve (AUDPC):** The efficacy of different treatments will be assessed based on AUDPC values using the formula given by Wilcoxson *et al.* (1975).

$$\text{AUDPC} = \sum_{i=1}^k \frac{1}{2} (S_i + S_{i-1}) \times d$$

Where,

$S_i$  = Disease incidence at  $i^{\text{th}}$  day of evaluation

$k$  = Number of successive evaluation of the disease

$d$  = Interval between  $i$  and  $i-1$  evaluation of disease



**Plate 3.1. Pots covered with polypropylene covers to maintain humid conditions**

### **3.11 STATISTICAL ANALYSIS**

The data obtained from lab and green house experiments were analysed statistically by following the standard procedures given by Gomez and Gomez (1984).

## Chapter – IV

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## *Results and Discussion*

## Chapter IV

# RESULTS AND DISCUSSION

### 4.1 ISOLATION, PURIFICATION AND MAINTENANCE OF BACTERIAL ENDOPHYTES FROM SORGHUM

#### 4.1.1 Source of Isolation

Sorghum seeds of M35-1 variety were raised in green house at the Department of Plant Pathology, Agricultural College, Bapatla. Roots and leaves from healthy seedlings of 10, 20 and 30 days old plants were used for isolation of endophytes.

#### 4.1.2 Isolation, Purification and Maintenance of Endophytes

Isolation of endophytes from roots and leaves of sorghum was standardized by surface disinfecting them using 70% ethanol and 0.1 % mercuric chloride for five minutes each and washing with sterile distilled water four times before and after second chemical treatment which was modified from the protocol described by de Fretes *et al.* (2018). Sterility check maintained using final washings was found free from any colony growth (Plate 4.1). Variation in colony occurrence was observed with respect to whole leaf samples as well as root and leaf samples. Variation in the number of colonies was also observed at different dilutions. The colonies were regarded as endophytes after being confirmed, by obtaining colony free plates kept as sterility checks as described by Anjum and Chandra (2015). Of the obtained colonies 38 isolates were selected for their purification using streak plate method. Purified colonies were sub cultured and were maintained in NA plates and slants at 4 °C for further studies. Three isolates from root and 10 isolates from leaves were separated for antagonistic studies. Selected endophytic bacteria were designated based on the plant part from which they were isolated as given in Table 4.1.

**Table 4.1. Bacterial endophytic isolates, abbreviation and source**

S. No.	Name of the Isolate	Abbreviation	Source
1	Sorghum Root Sap Endophyte-01	SRSE-01	Roots
2	Sorghum Root Sap Endophyte-02	SRSE-02	Roots
3	Sorghum Root Sap Endophyte-03	SRSE-03	Roots
4	Sorghum Leaf Bit Endophyte-01	SLBE-01	Leaves
5	Sorghum Leaf Bit Endophyte-02	SLBE-02	Leaves
6	Sorghum Leaf Bit Endophyte-03	SLBE-03	Leaves
7	Sorghum Leaf Bit Endophyte-04	SLBE-04	Leaves
8	Sorghum Leaf Bit Endophyte-05	SLBE-05	Leaves
9	Sorghum Leaf Sap Endophyte-01	SLSE-01	Leaves
10	Sorghum Leaf Sap Endophyte-02	SLSE-02	Leaves
11	Sorghum Leaf Sap Endophyte-03	SLSE-03	Leaves
12	Sorghum Leaf Sap Endophyte-04	SLSE-04	Leaves
13	Sorghum Leaf Sap Endophyte-05	SLSE-05	Leaves

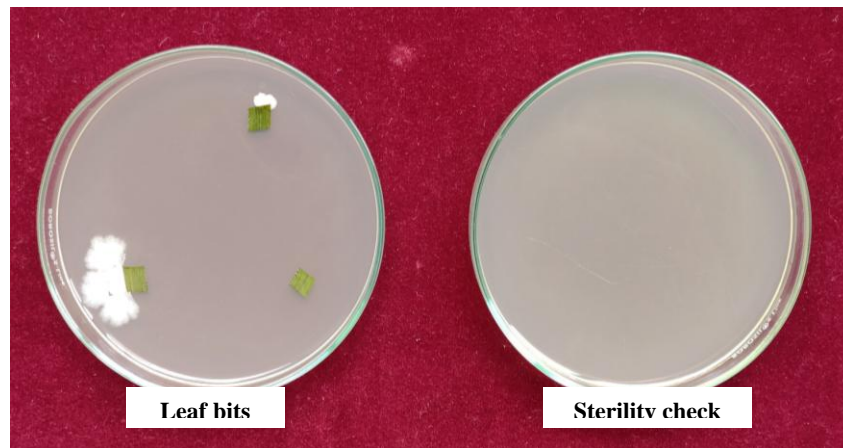
Number of colonies obtained from 10, 20 and 30 days old leaves were uniform ( $3.52 \times 10^8$ ) indicating consistent occurrence of endophytes irrespective to the age of the leaf used for their isolation. However, the number of colonies was high from roots than leaves indicating the abundance of endophytes in root than leaves with an incubation period of 2.6 days leaf endophytes and 2.0 to 2.8 days for root endophytes (Table 4.2). The incubation period for the endophytes from the sorghum leaf bits ranged from 4.3 to 4.6 days

Our results are in accordance with the work of Gupta *et al.* (2015) who reported that population density and type of endophytes was more in roots than in leaves of *Prosopis cineraria*. Similarly, Liu *et al.* (2017) stated that despite endophyte being detected in all the plant parts, roots having most intimate contact with soil may function as the first avenue for the recruitment of endophyte bacteria.

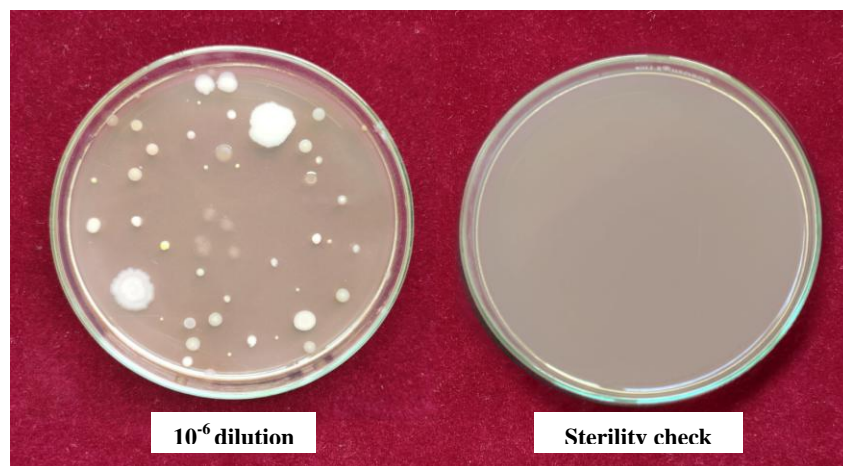
**Table 4.2 Population of bacterial endophytes in roots and leaves of sorghum**

Age of Leaf	Leaf Sap		Root sap	
	Incubation period (Days)*	No. of Colonies*	Incubation period (Days)*	No. of Colonies*
10DAS	2.6	$3.52 \times 10^8$	2.0	$4.52 \times 10^8$
20DAS	2.6	$3.54 \times 10^8$	2.6	$4.44 \times 10^8$
30DAS	2.6	$3.50 \times 10^8$	2.8	$4.48 \times 10^8$
Mean	2.6	$3.52 \times 10^8$	2.46	$4.48 \times 10^8$

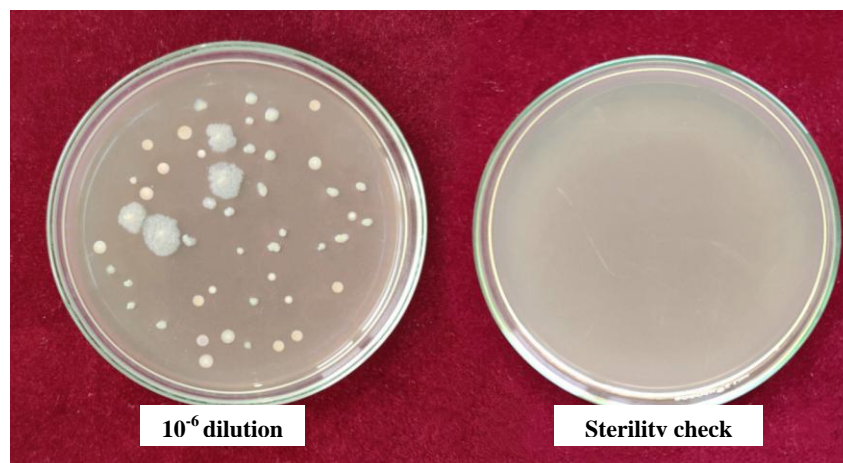
\*n= Mean of three replication



**a) Bacterial endophytes from sorghum leaf bits**



**b) Bacterial leaf bits from sorghum leaf sap**



**c) Bacterial endophytes from sorghum root sap**

**Plate 4.1 (a, b, c) Isolation of bacterial endophytes from sorghum roots and leaves**

### 4.1.3 Cultural Characterization of Endophytes

Bacterial endophytic colonies were characterized based on observations of culture plates under Compound microscope (Labomed LX 400, U.S.A.). Colony characters like shape, margin, elevation, texture, opacity and pigmentation were studied.

The bacterial endophytic colonies were morphologically characterized and described and given by Dasgupta (1994) (Table 4.2). All the endophytic isolates from the roots were round except SRSE-01 which was irregular in shape. The endophytic bacteria from leaf bits were round in shape except SLBE-02 which was irregular. Most of the isolates from the leaf sap were irregular except SLSE-01 which was round in shape. Overall, the endophytic colonies were round to irregular in shape.

Of the three isolates from roots, SRSE-03 colony was found to have even margin while in others it was undulate. Out of the 10 isolates from leaves, most of the isolates colony margins were even *i.e.*, SLBE-03, 04 and SLSE-01 while it was undulate in SLBE-01, 02 and 05, SLSE-02, 03 and 04 while it was lobate in SLSE-05. Eight of the isolates appeared raised in their elevation and five isolates SLBE-02, 03, 05 and SLSE-01, 05 had flat colonies. There was variation in texture of the endophytic isolates as all the colonies had smooth texture with opaque nature except SRSE-02 and 03 which were translucent (Plate 4.2). Colony pigmentation difference has been observed among the isolated endophytes. It varied from white to creamy colour where most of the isolates were creamy white coloured while, SRSE-02, SRSE-03, SLBE-01, SLSE-05 were white coloured and SLBE-04 was cream coloured.

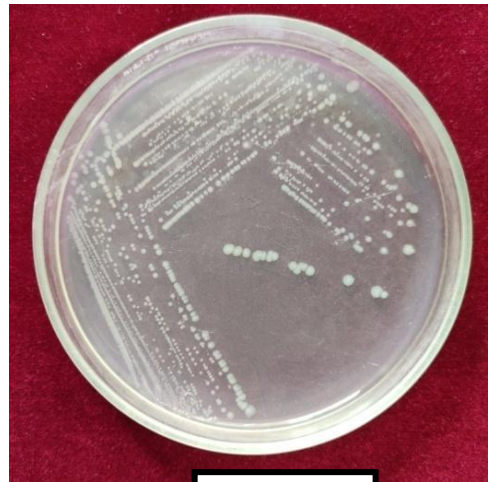
Similar results were obtained by Singh *et al.* (2013) who recovered seven endophytic bacteria from sugarcane and observed that most of the isolates exhibited round colonies with both wavy and smooth margins, convex elevation and white to creamy pigmentation. Likewise, Gupta *et al.* (2015) isolated three endophytes from the roots of *Prosopis cineraria* plants which were described as circular in shape, even, smooth, flat and yellowish to orange coloured with opaque nature.

**Table 4.3. Colony characters of bacterial endophytes**

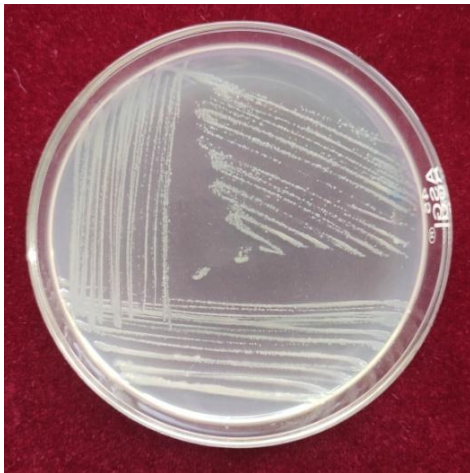
<b>S. No.</b>	<b>Isolates</b>	<b>Shape</b>	<b>Margin</b>	<b>Elevation</b>	<b>Texture</b>	<b>Opacity</b>	<b>Pigmentation</b>
1	SRSE-01	Irregular	Undulate	Raised	Smooth	Opaque	Creamy white
2	SRSE-02	Round	Undulate	Raised	Smooth	Translucent	White
3	SRSE-03	Round	Even	Raised	Smooth	Translucent	White
4	SLBE-01	Round	Undulate	Raised	Smooth	Opaque	White
5	SLBE-02	Irregular	Undulate	Flat	Smooth	Opaque	Creamy white
6	SLBE-03	Round	Even	Flat	Smooth	Opaque	Creamy white
7	SLBE-04	Round	Even	Raised	Smooth	Opaque	Creamy
8	SLBE-05	Round	Undulate	Flat	Smooth	Opaque	Creamy white
9	SLSE-01	Round	Even	Flat	Smooth	Opaque	Creamy white
10	SLSE-02	Irregular	Undulate	Raised	Smooth	Opaque	Creamy white
11	SLSE-03	Irregular	Undulate	Raised	Smooth	Opaque	Creamy white
12	SLSE-04	Irregular	Undulate	Raised	Smooth	Opaque	Creamy white
13	SLSE-05	Irregular	Lobate	Flat	Smooth	Opaque	White



**SRSE-01**



**SRSE-02**



**SRSE-03**



**SLBE-01**



**SLBE-02**



**SLBE-03**

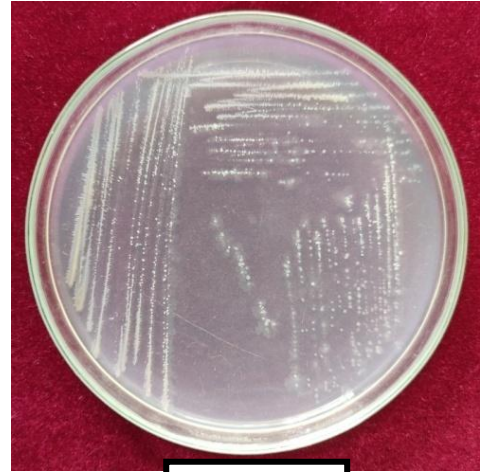
**Plate 4.2. Bacterial endophytes isolated from Sorghum roots and leaves**

**Plate 4.2 (cont.)**

Plate 4.2 (cont.)



SLBE-04



SLBE-05



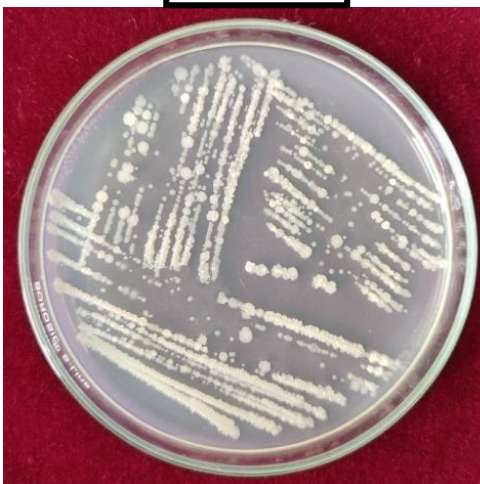
SLSE-01



SLSE-02



SLSE-03



SLSE-04

Plate 4.2 Bacterial endophytes isolated from Sorghum roots and leaves

Plate 4.2 (cont.)

**Plate 4.2 (cont.)**



**SLSE -05**

**Plate 4.2 Bacterial endophytes isolated from Sorghum roots and leaves**

## **4.2 SCREENING ENDOPHYTES FOR THEIR ANTAGONISTIC ACTIVITY IN VITRO**

### **4.2.1 Isolation of Pathogen**

Sorghum leaves showing typical symptoms of turcicum leaf blight characterized by long spindle shaped necrotic grayish or tan lesions on leaves measuring up to 12.5 x 2.2 cm (Ullstrup, 1966) were collected and used for isolation of the pathogen (Plate 4.3a). Pure culture was obtained by single spore isolation technique and stored in PDA slants at 4 °C. The conidial characters of the *E. turcicum* isolate corroborated with the standard descriptions of Leonards and Suggs (1974), Ellis and Holiday (1971) and Sivanesan (1987) and were used for further studies.

### **4.2.2 Evaluation of Antagonistic Potential of Endophytic Bacterial Isolates against *E. turcicum* Using Dual Culture Technique**

Endophytic bacteria isolated from healthy sorghum roots and leaves were screened for their antagonistic potential against the sorghum turcicum leaf blight pathogen *E. turcicum*. The inhibition potential of bacterial endophytes was tested by dual culture technique (Plate 4.3b).

Five endophytic isolates were selected from colonies obtained around cut ends of sorghum leaf bits. Likewise, 19 isolates were identified from spread plates of leaf sap and 14 from root sap at  $10^{-6}$  dilution based on variation in their cultural characters. Of these 38 isolates, five isolates from leaf bits, five isolates from leaf sap that were morphologically distinct and three from root sap were selected for testing their antagonistic potential against the *E. turcium*. All the endophytic isolates were found to inhibit pathogen when compared with monoculture plate and it ranged from 3.30 to 52.75 % (Table 4.4). Very limited inhibition was observed in SLBE-03 (3.30 %), SLBE-01 (4.95 %), SLSE-01 (6.59 %), SRSE-02 (6.59 %), SRSE-03 (6.59 %), SLSE-02 (8.79 %), SLBE-04 (23.63 %), SLBE-02 (24.18 %) and SLBE-05 (25.27 %). The isolates with clear inhibition zone formation during interaction with pathogen with more than 50 % inhibition percentage were found in SLSE-03 (50.00 %), SLSE-05 (51.10 %), SLSE-04 (51.65 %) and SRSE-01 (52.75 %) Of all the isolates, endophyte SRSE-01 was found significantly superior over other endophytes isolated from healthy leaf bits, root sap and leaf sap. Among the endophytes from leaf bits SLBE-05 was found superior in inhibiting the pathogen (25.27%). Endophytes from leaf sap,

*i.e.*, SLSE-04, 05 and 03 were found on par with each other and superior over endophytes from leaf bits in inhibiting the pathogen. Therefore, based on their inhibition percentage the three most superior isolates SLSE-04, SLSE-05 and SRSE-01 were selected for further studies.

Earlier reports as given by Doye. (2013) stated that, among 52 endophytic bacteria isolated from the healthy roots of maize, bacterial strain EB22 had showed significant inhibition (80%) on *E. turcicum*. Similarly, Liu *et al.* (2019) reported endophytic strain NEAU-S7GS2 with significant inhibitory effect on the mycelial growth of *E. turcicum* (67.6%) when compared to monoculture plate.

Based on the antagonistic effect enforced by the endophyte, growth pattern of fungus varied at the interaction zone. Most characteristic feature observed was thickening of the hyphal strands and formation of chlamyospores intercellularly and terminally at five days after inoculation. However, the isolates SLBE-04, SLBE-05 and SLSE-01 did not show any chlamyospores formation and were similar to culture in control plate (Plate 4.4) (Fig. 4.1).

Among all the isolates, SRSE-01 was found significantly superior over other isolates with high number of chlamyospores (222.67/ microscopic field) followed by SLSE-04 (135.33/ microscopic field) and SLSE-05 (117.67/ microscopic field) which were on par with each other, while SLSE-05 was on par with SLSE-03 (108.0/ microscopic field) as shown in Table 4.4.

Anastomosis of *E. turcicum* hyphae was observed on interaction with SRSE-01, SLBE-02 and SLSE-05, while clustering of hyphae resembling the formation of prosenchyma was observed in SRSE-01 and SLSE-02 interactions as shown in Plates 4.5 and 4.6. However, lysis of the fungal mycelium was not observed in any of the interactions.

Our results are in accordance with earlier workers like Griffiths (1974), Harish *et al.* (1998) and Strunnikova *et al.* (2007) who stated that chlamyospore formation would result due to biotic and abiotic stresses. In the present study, incompatible interactions with endophytes was perceived by the formation of chlamyospores which might have occurred due to antagonistic nature of endophyte due to synthesis of either enzymes or toxin or antibiotics.



**Plate 4.3a. Sorghum leaf showing typical symptoms of turicum leaf blight**

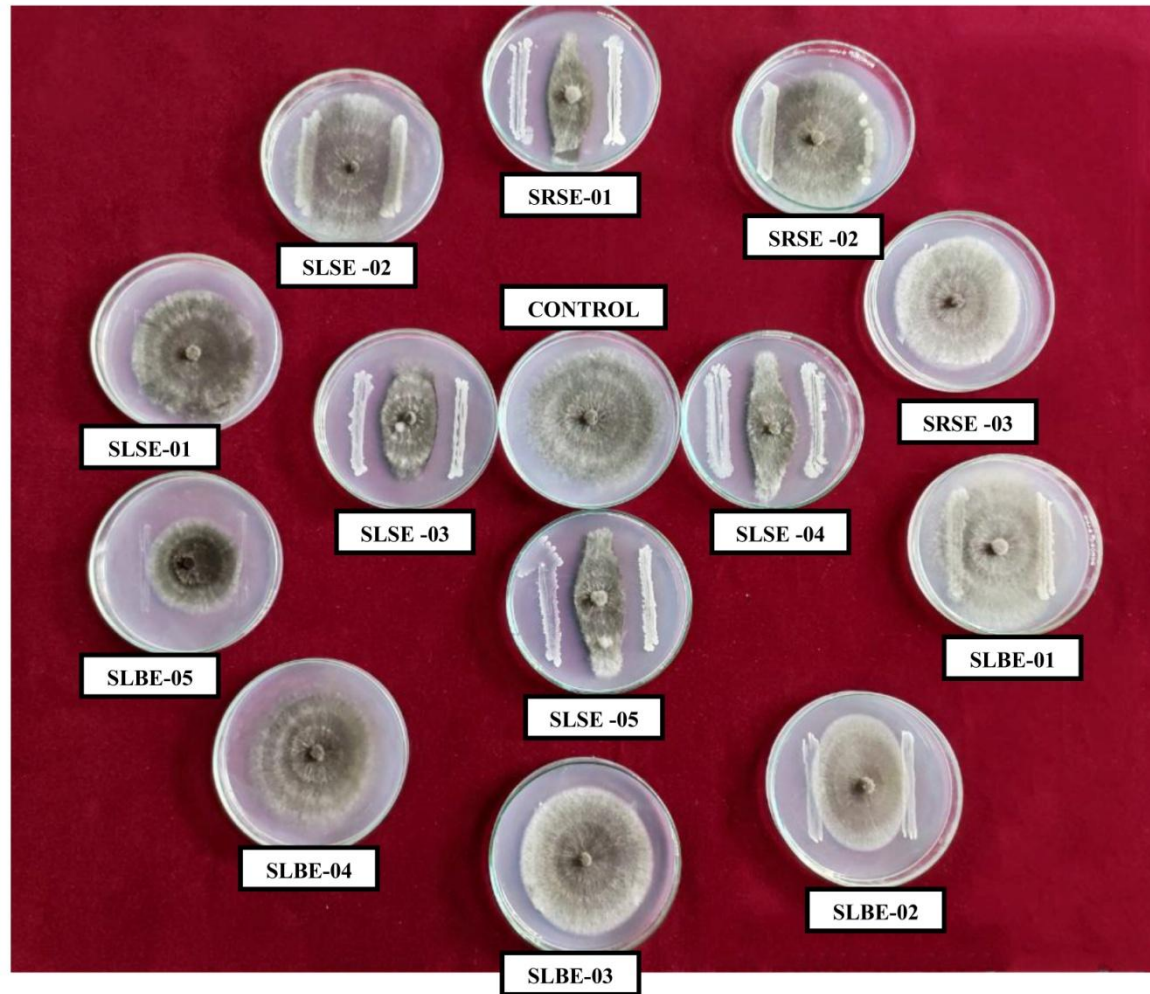


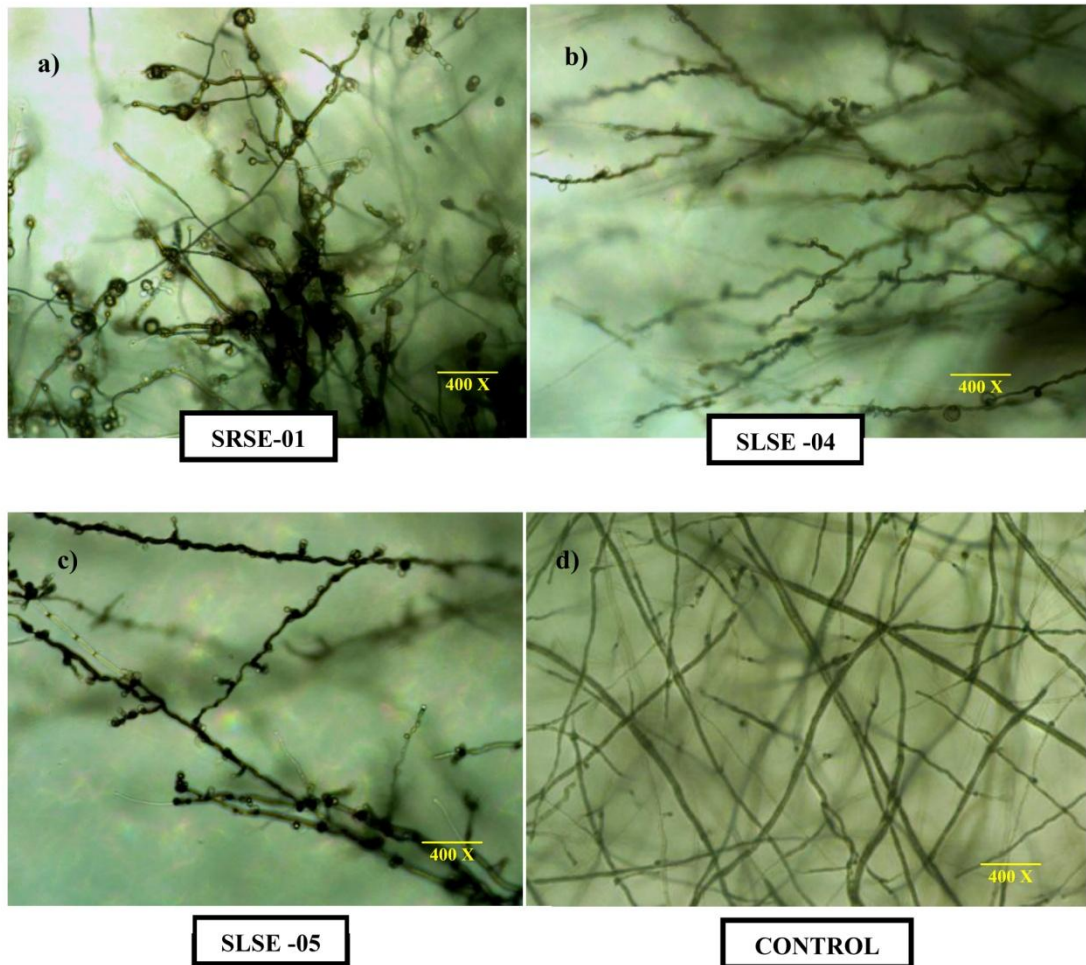
Plate 4.3b. Screening antagonistic potential bacterial endophytes against *E.turcium* *in vitro*

**Table 4.4 Antagonistic potential of bacterial endophytes against *E. turcium***

S. No.	Treatments	Radial growth (cm)	Per cent Inhibition	Zone of Inhibition (cm)	No. of Chlamydo spores per microscopic field
1	SRSE-01	2.87 <sup>a</sup>	52.75	0.6	222.67 *(2.35)
2	SRSE-02	5.67 <sup>d</sup>	6.59	-	7.67 (0.93)
3	SRSE-03	5.67 <sup>d</sup>	6.59	-	73.33 (1.87)
4	SLBE-01	5.77 <sup>de</sup>	4.95	-	36.33 (1.57)
5	SLBE-02	4.60 <sup>c</sup>	24.18	-	63.00 (1.81)
6	SLBE-03	5.87 <sup>e</sup>	3.30	-	49.33 (1.70)
7	SLBE-04	4.63 <sup>c</sup>	23.63	-	0.00 (0.00)
8	SLBE-05	4.53 <sup>c</sup>	25.27	-	0.00 (0.00)
9	SLSE-01	5.67 <sup>cd</sup>	6.59	-	0.00 (0.00)
10	SLSE-02	5.53 <sup>d</sup>	8.79	-	64.33 (1.81)
11	SLSE-03	3.03 <sup>ab</sup>	50.00	0.3	108.00 (2.04)
12	SLSE-04	2.93 <sup>ab</sup>	51.65	0.4	135.33 (2.13)
13	SLSE-05	2.97 <sup>ab</sup>	51.10	0.4	117.67 (2.07)
14	Control	6.07 <sup>f</sup>	-		0.00 (0.00)
	SEm ±	0.04			0.03
	CD (P ≤ 0.05)	0.14			0.08
	CV (%)	1.7			3.39

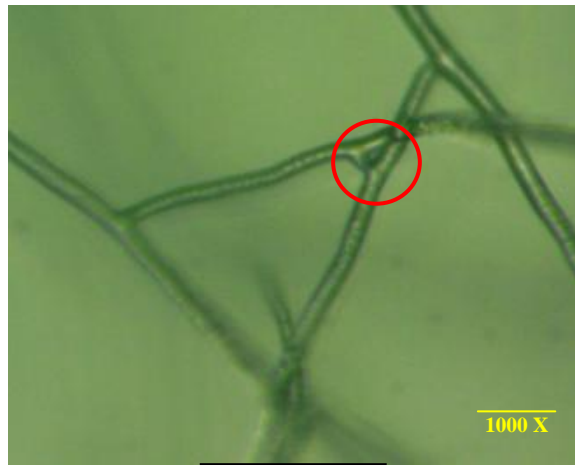
Numbers with same superscript are statistically significant

Values in parenthesis are Log transformed values



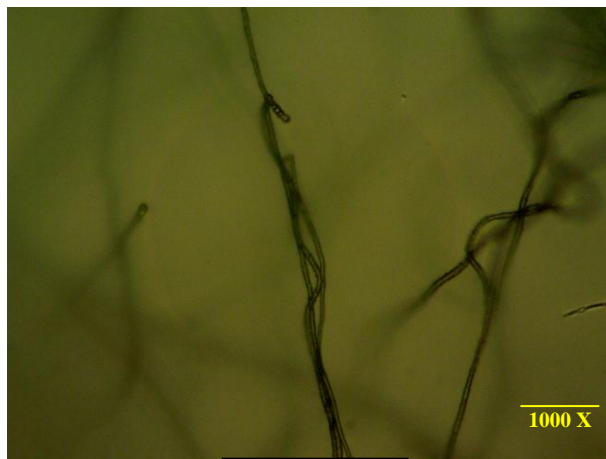
**Plate 4.4. Chlamydospores formation at interaction zone between endophytes and *E. turcium***

**(a. Interaction zone between SRSE-01 and *E. turcium*; b. Interaction zone between SLSE-04 and *E. turcium*; c. Interaction zone between SLSE-05 and *E. turcium*; d. *E. turcium* alone)**



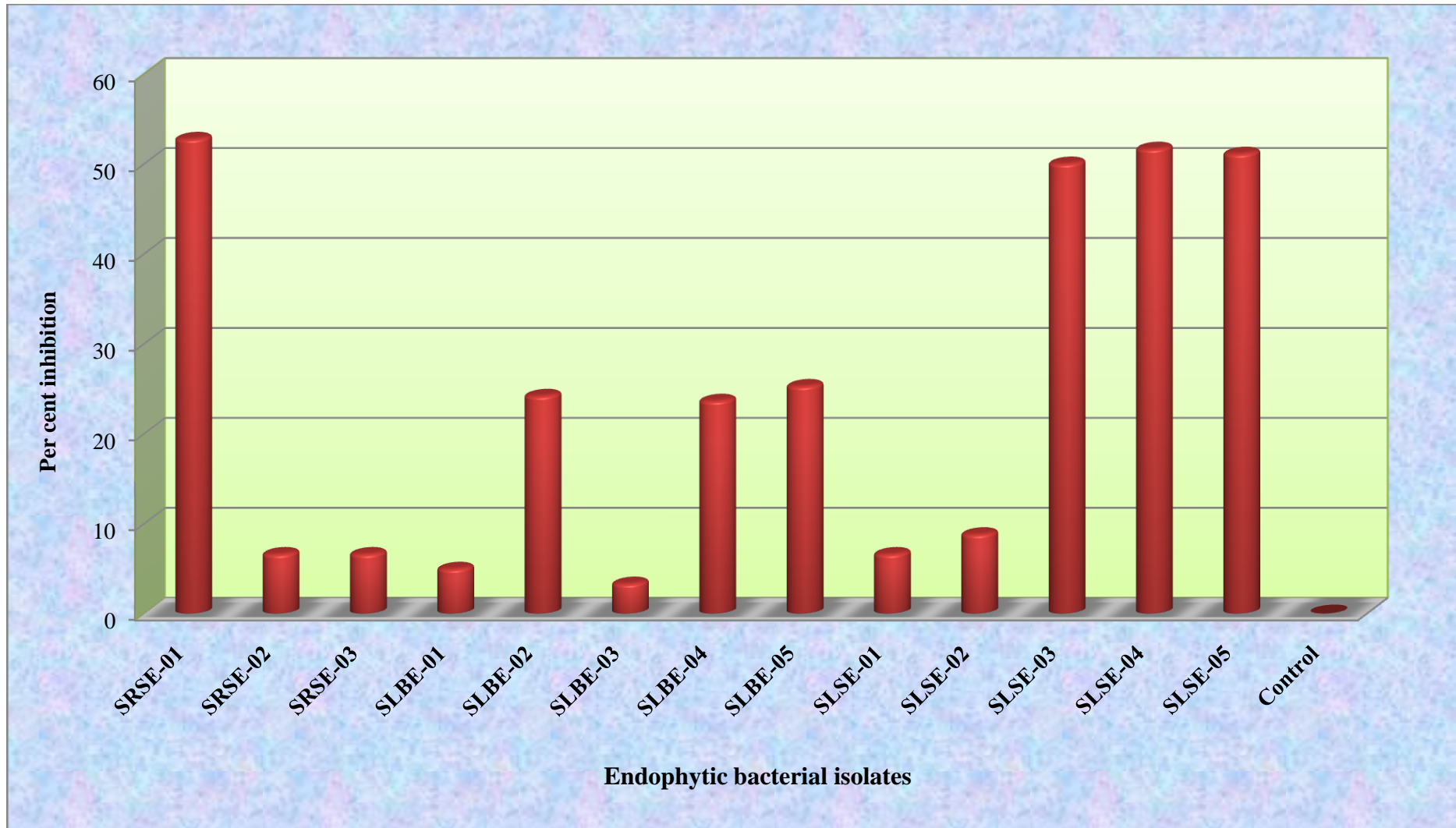
SLBE-02

**Plate 4.5** Occurrence of anastomosis of *E. turcicum* hyphae at the interaction zone with endophyte



SRSE-01

**Plate 4.6.** Hyphal clustering of *E. turcicum* on interaction with endophyte



**Fig. 4.1** Effect of endophytic bacteria on radial growth of *E. turcicum*

### 4.2.3 Evaluation of Antagonistic Potential of Endophytic Bacterial Isolates against *E. Turcium* Using Detached Leaf Technique

Fresh and healthy sorghum leaves were cut into medium sized bits and were surface sterilized prior to test antagonistic potential of the bacterial endophytes *in vitro*. Each leaf bit was coated with an endophytic bacterial isolate suspension containing  $1 \times 10^8$  CFU ml<sup>-1</sup> and was incubated at 30 °C for 24 h in dark chamber before challenge inoculation with pathogen inoculum. Leaf bits sprayed with sterile distilled water served as controls.

Samples were observed each day for the development of disease symptoms and three days after incubation observable disease symptoms were found and monitoring continued till five days of inoculation. White mycelial mass was observed at the point of inoculation (Plate 4.7).

In control, that was sprayed with distilled water followed by challenge inoculation with the pathogen had significantly more number of diseased spots and high per cent spotted area compared to leaf bits with endophyte treatment. Least number of diseased spots were observed in treatment with the endophytes SRSE-01 and SLSE-03 (a single spot) and were found superior over other isolates. Total disease spotted area was found least in leaf treated with the isolate SLSE-05 (0.37 cm<sup>2</sup>) which is on par with SRSE-01 (0.38 cm<sup>2</sup>). The isolate SLSE-03 with a single spot was found to spread and occupy a diseased area of 1.18 cm<sup>2</sup> when compared to least diseased area of about 0.37 cm<sup>2</sup> with the isolate SLSE-05. Though all the isolates were found superior over control in per cent spotted area, the lowest was recorded with SRSE-01 isolate (2.29%) which was found on par with SLSE-05 isolate (2.67%). Among bacterial endophytes least performed isolates with maximum per cent spotted area was SLBE-03 (13.99 %). Untreated check showed no disease symptoms. No significant difference was observed among the isolates with respect to disease index. Most of the leaves appeared dark green in colour except for diseased area. However, the leaves treated with the endophytes SRSE-01, SRSE-02 and SLSE-04 appeared greenish brown.

Of all the endophytic isolates SRSE-01 was found to be superior in inhibiting the pathogen development with a minute spot of 0.38 cm<sup>2</sup> and with least per cent spotted area of 2.29% (Table 4.5) (Fig. 4.2).

The present results corroborate with earlier work in different crops and across different pathogens. Raj Kumar *et al.* (2005) observed through detached leaves assay, the antagonistic isolates PS27 and PS83 with maximum suppression of *Phytophthora capsici* lesion development in bell pepper. He *et al.* (2009) also screened 175 endophytic bacteria for their antagonistic effect against *C. gloeosporioides* in *Camellia oleifera* and reported 86.1% inhibition by YB128 strain. Similarly, Shimizu *et al.* (2009) reported six endophytic isolates MBCu-32, 36, 42, 45, 56 and MBPu-75 to significantly reduce the number and size of the anthracnose lesions caused by *Colletotrichum orbiculare* on the cucumber cotyledons compared to the untreated control. Shi *et al.* (2010) reported an endophytic bacterium capable of controlling ten parasitic and saprophytic fungi and bacteria that commonly infect harvested papaya fruit. Rabbee *et al.* (2019) observed that detached citrus leaves infiltrated with endophyte *B. velezensis* significantly reduced the size of lesions caused by *Xanthomonas citri* subsp. *citri*. Similar results were reported by Andreolli *et al.* (2019) where endophyte *Pseudomonas protegens* MP12 displayed a significant reduction of lesions caused by *Botrytis cinerea* detached leaves of grapevine. Kollakkodan *et al.* (2020) observed a significant suppression of lesion caused by *Phytophthora capsici* on detached leaves of black pepper by antagonistic endophyte *B. velezensis* PCSE10.

Based on the results obtained three isolates SRSE-01, SLSE-04, 05 were selected for further studies (Table 4.5)

**Table 4.5. Effect of bacterial endophytes on Turcicum leaf spot development in sorghum**

S. No.	Treatments	Number of spots*	Total disease spotted area (cm <sup>2</sup> )*	Per cent spotted area (%)*	Disease index	Colour of the leaf
1	SRSE-01	1.00	0.38	2.29	22.22	Greenish brown
2	SRSE-02	5.00	1.11	6.44	33.33	Greenish brown
3	SRSE-03	4.00	1.29	8.62	33.33	Dark green
4	SLBE-01	5.00	1.15	6.67	33.33	Dark green
5	SLBE-02	2.00	1.17	6.55	33.33	Dark green
6	SLBE-03	4.00	1.34	13.99	44.44	Dark green
7	SLBE-04	2.00	1.35	9.62	33.33	Dark green
8	SLBE-05	3.00	1.09	6.83	33.33	Dark green
9	SLSE-01	2.67	1.22	7.66	33.33	Dark green
10	SLSE-02	4.00	1.13	7.15	33.33	Dark green
11	SLSE-03	1.00	1.18	10.21	33.33	Dark green
12	SLSE-04	4.00	0.79	4.96	22.22	Greenish brown
13	SLSE-05	3.00	0.37	2.67	22.22	Dark green
14	Control	7.00	2.26	15.02	44.44	Dark green
	SEm ±	0.0891	0.0314	0.1866	0.000891	
	CD (P ≤ 0.05)	0.26	0.09	0.54	NS	
	CV (%)	4.53	4.81	4.16		

\*n= Mean of three replication

#### 4.2.4 Compatibility studies among endophytic bacteria

The three endophytic bacteria SRSE-01, SLSE-04, 05 which proved to be antagonistic against the pathogen of interest were subjected to compatibility studies through Cross streaking method (James and Matthew, 2017). Two endophytic bacterial isolates that were streaked vertically and horizontally at each instance on NA plates that were incubated for 48 h at room temperature were observed for inhibition at the junction of the streaks. All the bacterial endophytes tested were seen to be compatible to each other without any inhibition zone seen at the intersecting point of the bacterial streaks (Plate 4.8).

Similar results were observed by Santiago *et al.* (2017) where potential endophytic strains T168 and R182 did not show any traces of growth inhibition when cross streaked with bacterial strain R170 and Irabor and Mmbaga (2017) reported that endophyte isolates B17B and Y grew well together without inhibiting each other's growth in compatibility test. Al-Hussini *et al.* (2019) reported that four rhizobacterial isolates designated D1/3, D1/8, D1/17 and D1/18 were found compatible with each other when subjected to compatibility tests using a cross-streak assay on nutrient agar medium. James and Mathew (2017) also reported that, among the 28 endophyte isolates, five isolates VRF-1, VSF-3, CSF- 1, MyRF-1 and ASF-3 to be highly compatible when subjected to mutual compatibility test by cross streak method. Interestingly Iqbal *et al.* (2020) observed compatibility among the bacterial endophytes (ZE15, ZE19 and ZE32) and rhizobacterial isolates (ZR3 and ZR19) through cross streak method.

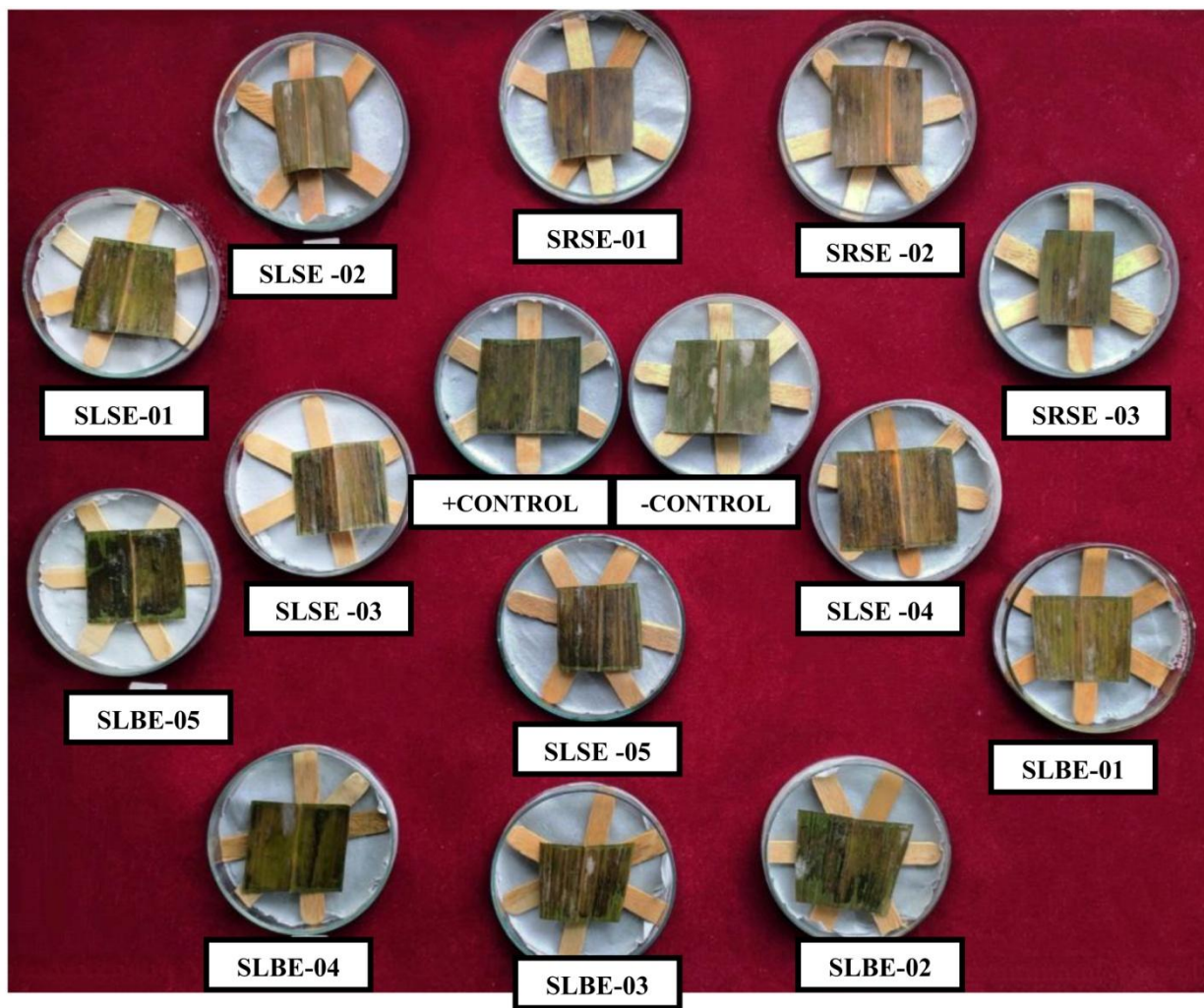
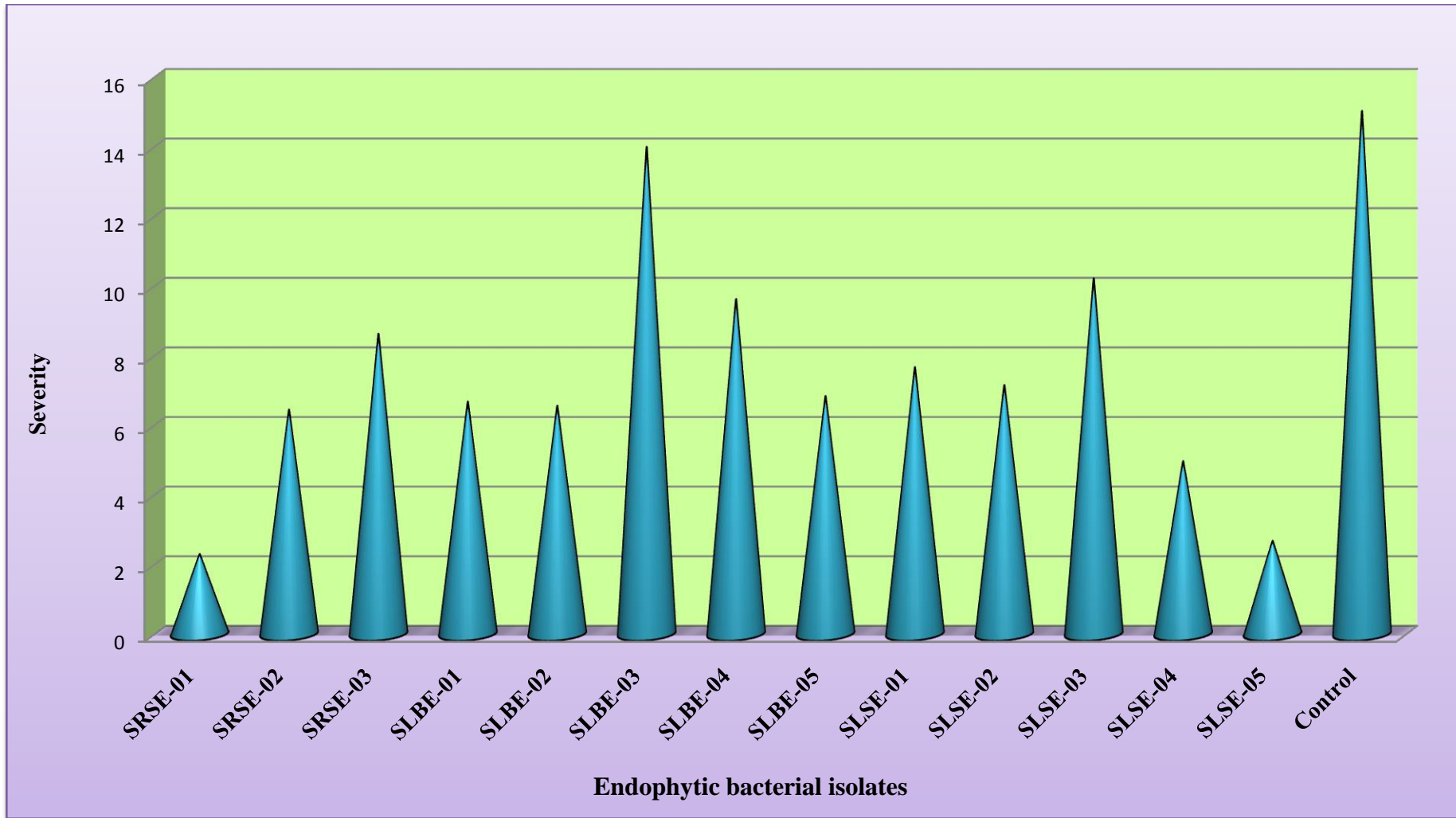
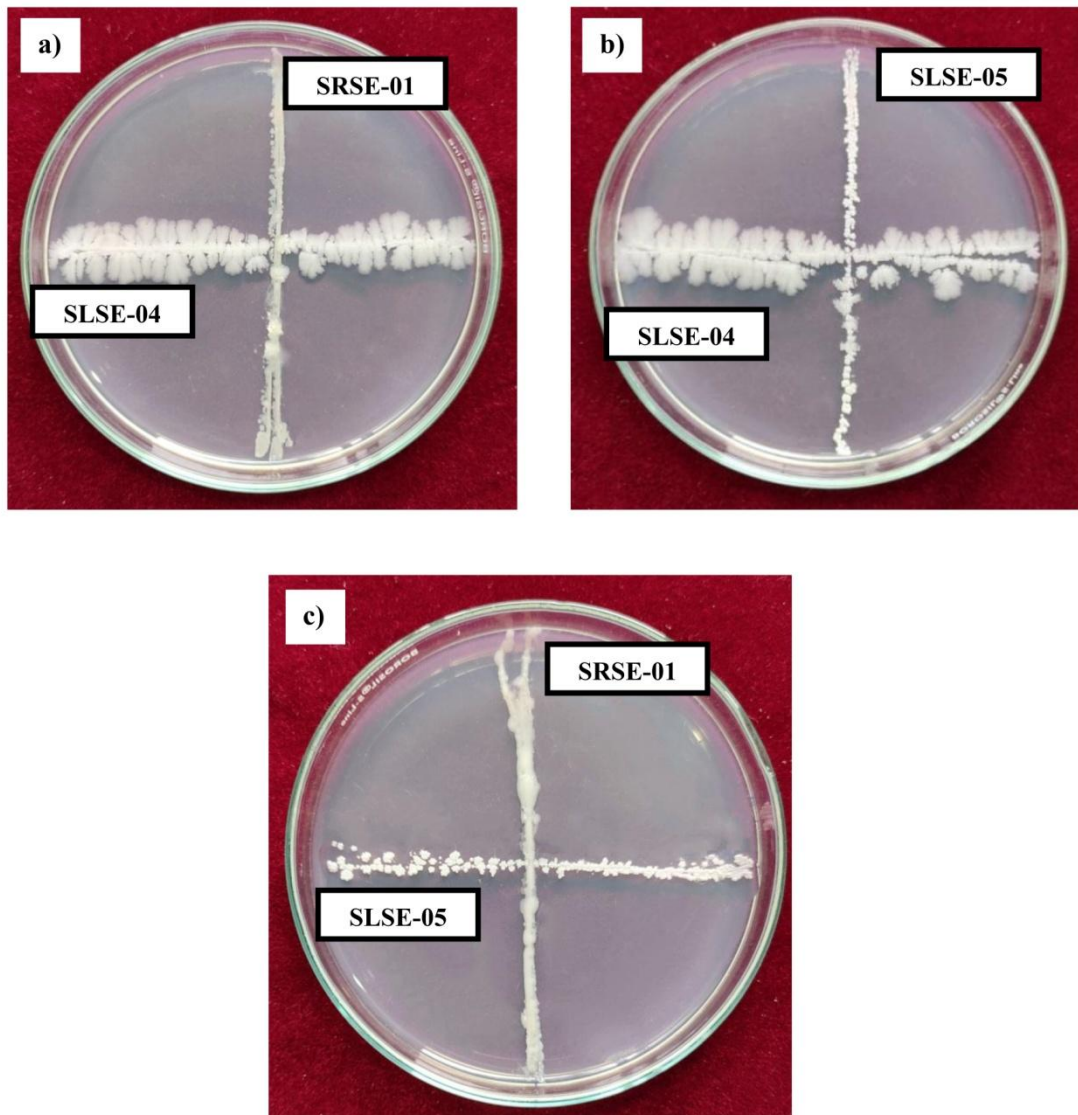


Plate 4.7. Detached leaf technique for screening antagonistic potential of bacterial endophytes against *E.turcium in vitro*



**Fig. 4.2. Effect of bacterial endophytes on Turcicum leaf spot severity**



**Plate 4.8. Compatibility studies among endophytic bacteria**  
**a. Interaction between SRSE-01 and SLSE-04;**  
**b. Interaction between SLSE-05 and SLSE-04;**  
**c. Interaction between SRSE-01 and SLSE-05)**

## **4.3 BIOCHEMICAL CHARACTERIZATION OF ENDOPHYTIC BACTERIA**

The purified isolates of the three potential antagonistic endophytic bacteria were tested for biochemical characters (Table 4.6).

### **4.3.1 Utilization of Nitrogen Sources**

All the three 24 h old endophytic bacterial cultures, when treated with Nessler's reagent, developed yellowish to dark brown colour indicating the production of ammonia due to their ability to utilize the nitrogen (Plate 4.9) and was a confirmation test for ammonia production (Cappuccino and Sherman, 1992).

The results obtained are similar with those of Prasad and Dagar (2014) who reported that six endophytic bacterial isolates G1, G2, G1, SA3, SA4 and A1 isolated from black grapes and avocado that were able to produce ammonia. Similarly, a high proportion (69.5%) of endophytic bacteria isolated by Brigido *et al.* (2019) was reported with ability to produce ammonia.

Bacteria can fix nitrogen by converting stable atmospheric nitrogen gas into a biologically useful form and positively influence plants by improving growth and root development, which increases plant tolerance to various environmental stresses (Zahran, 1999; Ullah *et al.*, 2015). Therefore all the three isolates can be utilized for the crop growth improvement in poor soils.

### **4.3.2 Siderophore Production**

The isolates failed to develop orange or yellow colour surrounding the colony which indicated inability of isolates to produce siderophore on CAS (Chrome Azurol-Sulphonate) agar plates. (Plate 4.10).

Similar observations were made by Forchetti *et al.* (2007) where none of the endophytic strains (SF2, SF3, SF4, SF5, SF7) isolated from sunflower were able to produce siderophores on CAS agar plates.

### **4.3.3 Phosphate Solubilisation**

Phosphate solubilizing activity was shown by two isolates screened on Pikovskaya Agar (Pikovskaya, 1948) with a clear zone around the colony where in SLSE-05 (3.6 cm) isolate it was more pronounced compared to SRSE-01 isolate (0.25 cm) (Plate 4.11).

Present results are according to the reports of de Fretes *et al.* (2018) who reported four endophytic isolates (BA3, BA8, BA14 and MA24) with phosphate-solubilizing activity by screening on Pikovskaya Agar media. Similarly, Brigido *et al.* (2019) reported, 17.5% of the bacterial endophytes isolated from chickpea plants to solubilise phosphorous. AlKahtani *et al.* (2020) reported nine endophytes with significant ability to solubilize inorganic phosphate with clear zone on the Pikovskaya medium.

#### **4.3.4 IAA Production**

None of the isolates tested with 0.5 ml of Salkowski's reagent, had any colour change which was an indication that the isolates failed to have any IAA producing nature (Plate 4.12).

Similar observations were reported by Bind and Nema (2019) with 27 endophytes isolated from pigeon pea that lacked IAA producing ability.

#### **4.3.5 Enzyme Activity**

**4.3.5.1 Protease activity:** Colourless halo zones around the tested bacterial colonies on SMA plates indicated protease production. Whereas the isolate SLSE-04 was found with high protease activity among the isolates (Plate 4.13).

**4.3.5.2 Amylase activity:** All the three bacterial endophytes tested positive for the amylase activity producing clear zones with orange colour around colony and each isolate varied in their amylase activity (Plate 4.14).

**4.3.5.3 Catalase activity:** Positive response to catalase activity in the form of effervescence due to production of oxygen from breakdown of H<sub>2</sub>O<sub>2</sub> was observed with all the isolates tested (Plate 4.15). Its production helps the endophytes to overcome the cellular toxicity.

Dunne *et al.* (1997) have shown the suppression of phytopathogenic fungus, *Pythium ultimum*, in the rhizosphere of sugar-cane was due to the elaboration of extracellular protease by strains of *Stenotrophomonas maltophilia* W81. *Pseudomonas fluorescens* CHA0 synthesizes an extracellular protease active against the phytopathogenic nematode *Meloidogyne incognita* (Siddiqui *et al.*, 2005). Protease is

a hydrolytic enzyme which is involved in the suppression of pathogenic growth and subsequent reduction in damage to plants (Bashan and de-Bashan 2005). Hydrolytic enzymes help with their antimicrobial activity related to mechanisms promoting plant defence to phytopathogens (Bootkotr and Mongkolthanaruk 2012). Bacterial strains possessing a catalase are assumed to be highly resistant to various environmental, mechanical and chemical stress (Kumar *et al.*, 2012).

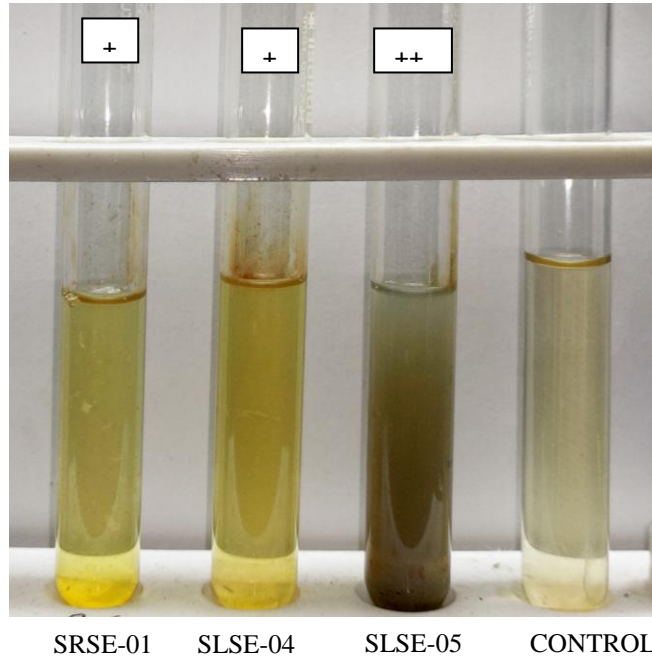
Amylase enzyme is known to promote early germination and increase the availability of starch assimilation (Gholami *et al.*, 2009). Indirect action of PGPR involve the elimination of harmful microorganisms via the production of antibiotics or (and) lytic enzymes (Protease, amylase), HCN or catalase (Patel and Pratibha, 2014).

Amylase and catalase enzymes play an important role in promotion of plant growth and biological control of plant diseases (Sousa *et al.*, 2008). Krishnan *et al.* (2012) reported amylase activity of *Bacillus* sp. PE-5, SP-1, PE-LR-3 and PE-LR-4. Gupta (2015) reported endophytic bacteria *B. subtilis* isolated from roots with high amylolytic, and catalase activity. Vijayalakshmi *et al.* (2016) isolated five endophytic bacteria from medicinally important plants, producing amylase and protease.

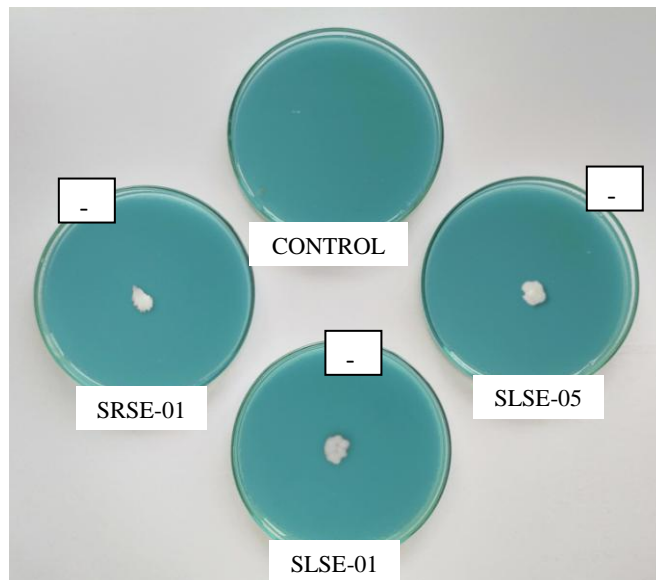
Singh *et al.* (2013) reported casein hydrolysis by endophytic isolate, RtChII and catalase activity by five other endophytic isolates. Bacterial strains showing catalase activity are reported as highly resistant to environmental, mechanical and chemical stress (Geetha *et al.*, 2014). A total of 10 isolates were tested positive for casein hydrolysis and catalase test in an investigation by Bhagya *et al.* (2019).

**Table 4.6. Biochemical characterization of bacterial endophytes**

S. No.	Name of the biochemical test	Bacterial endophytes		
		SRSE-01	SLSE-04	SLSE-05
1	Utilization of nitrogen sources	+	+	++
2	Siderophore production	-	-	-
3	Phosphate solubilization	+	-	++
4	IAA production	-	-	-
5	Protease activity	++	+++	+
6	Amylase activity	+	++	+++
7	Catalase activity	+	+	+



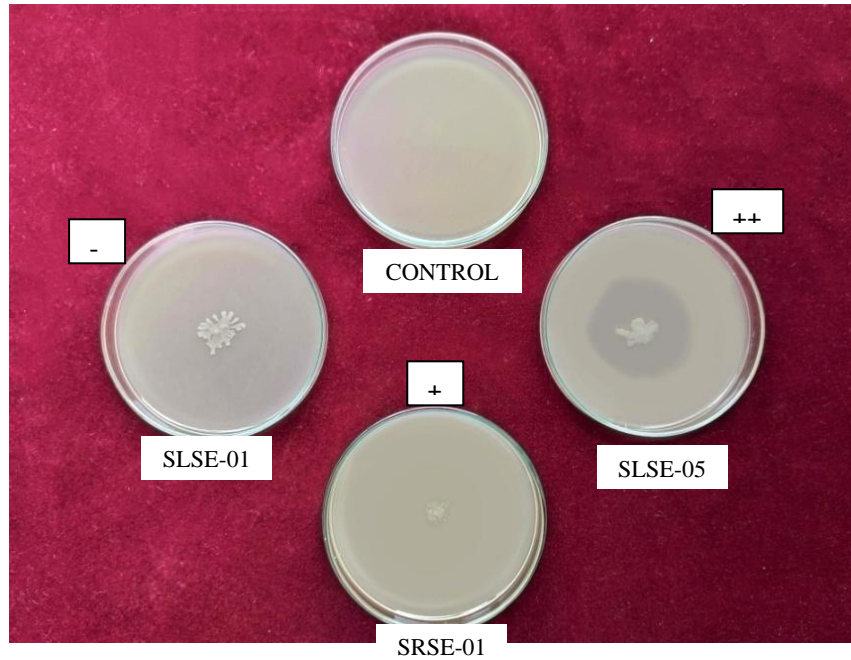
**Plate 4.9. Utilization of nitrogen by endophytic bacteria depicted by change in colour**



**Plate 4.10. Siderophore production test for endophytic bacteria**

(‘+’ reaction- yellow coloured zone around colony)

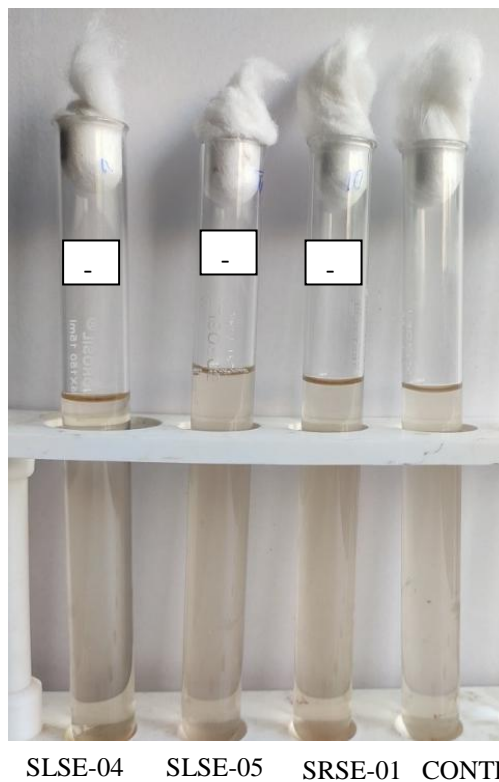
‘-’ reaction- no change in colour around colony)



**Plate 4.11. Phosphate solubilisation by endophytic bacteria**

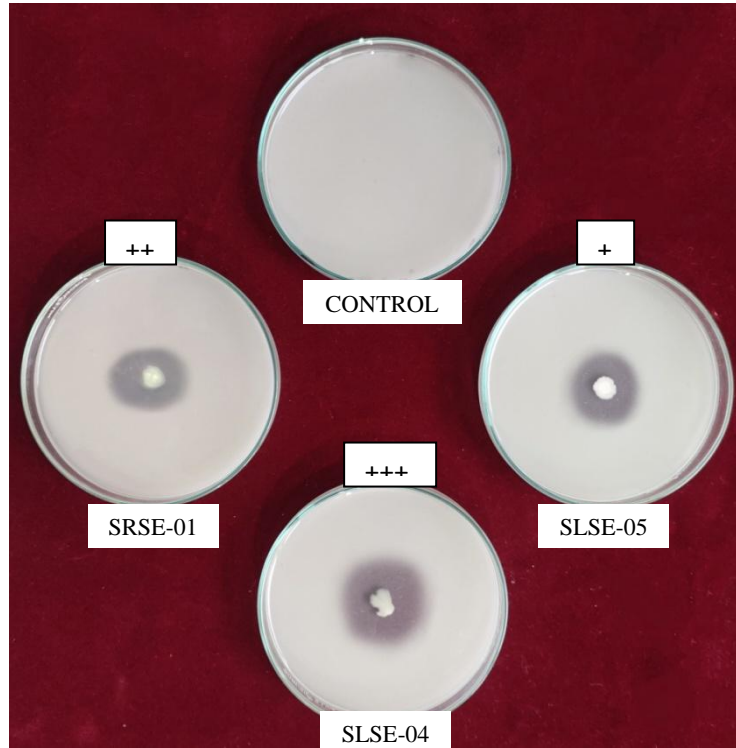
(‘+’ reaction- clear zone around colony)

‘-’ reaction- no clear zone around colony)

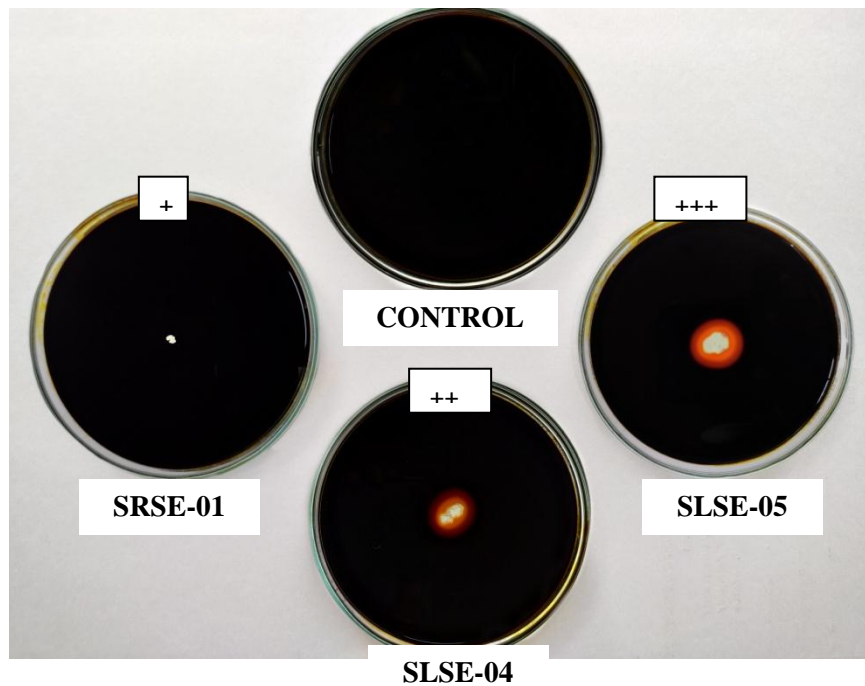


**Plate 4.12. Absence of IAA production by endophytic bacteria**

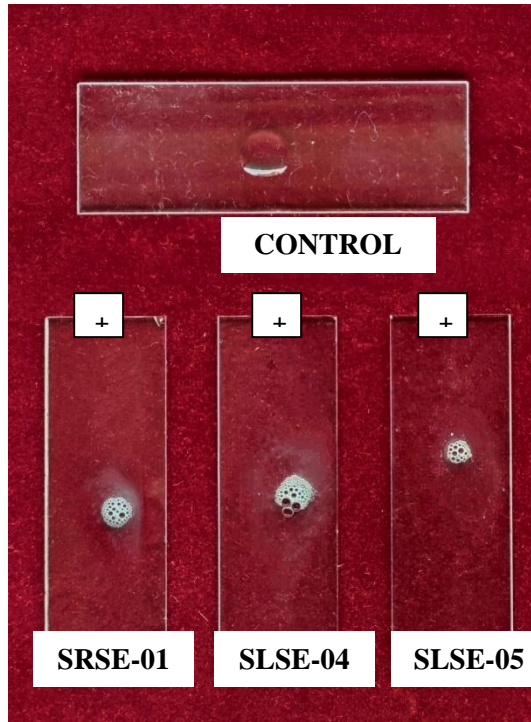
(‘+’ reaction- pink colour; ‘-’ reaction- no change)



**Plate 4.13. Protease production by endophytic bacteria  
(clear zone indicates positive reaction)**



**Plate 4.14. Amylase production by endophytic bacteria  
(‘+’ reaction- clear zone with orange colour; ‘-’ reaction- no change)**



**Plate 4.15. Catalase production by endophytic bacteria**  
(‘+’ reaction- effervescence; ‘-’ reaction- no effervescence)

#### 4.3.6 Phenol Estimation

Significant variation was observed in phenol content between endophytes treated and untreated plants. In the treatment where the pathogen was absent, phenol content was significantly low ( $0.31 \text{ mg g}^{-1}$ ) compared to challenge inoculated control ( $0.50 \text{ mg g}^{-1}$ ). Among different endophyte treated plants on challenge inoculation with pathogen there was significant increase in phenols compared to both controls, *i.e.*, with pathogen and without pathogen (Table 4.7).

Compared to other treatments, except where fungicide treated plants ( $2.29 \text{ mg g}^{-1}$ ), phenols content was found significantly high where all the endophytes were simultaneously inoculated ( $2.06 \text{ mg g}^{-1}$ ). In individual treatments, SLSE-05 was found to enhance the phenol to a greater extent of 70.81 % and was found on par with SRSE-01 isolate (69.91 %). In dual combinations, the combination of SRSE-01 and SLSE-04 was found effective with  $1.73 \text{ mg g}^{-1}$  phenol with an increase of 82.09 % over control. However, effect of the other combinations *i.e.*, SRSE-01 + SLSE-05 and SLSE-04 + SLSE-05 were found on par with each other with increase in phenol content (Fig. 4.3).

Present results are in accord with earlier findings, where Shi *et al.* (2010) reported significantly increased levels of phenols in endophyte treated papaya fruits that were used to manage post harvest diseases during storage. Similarly, Ray *et al.* (2020) reported sharp elevation in total phenol content in endophyte treated okra plants inoculated with *Sclerotium rolfsii*.

Sequeira (1983) reported that, phenol oxidation results in products that are highly toxic to pathogens. Phenols are reported as the secondary metabolites produced by plants that act as phytoalexins, antioxidants *etc.* (Blainski *et al.*, 2013). They get synthesized in plants in response to pathogen infection which are associated in the resistance mechanism (Lattanzio *et al.*, 2006).

It is an established fact that phenols are produced more rapidly in resistant plants than susceptible ones (Matta, 1967). It was also reported that non pathogenic fungi induce plants for the synthesis of more phenols than pathogenic forms (Matta, 1969).

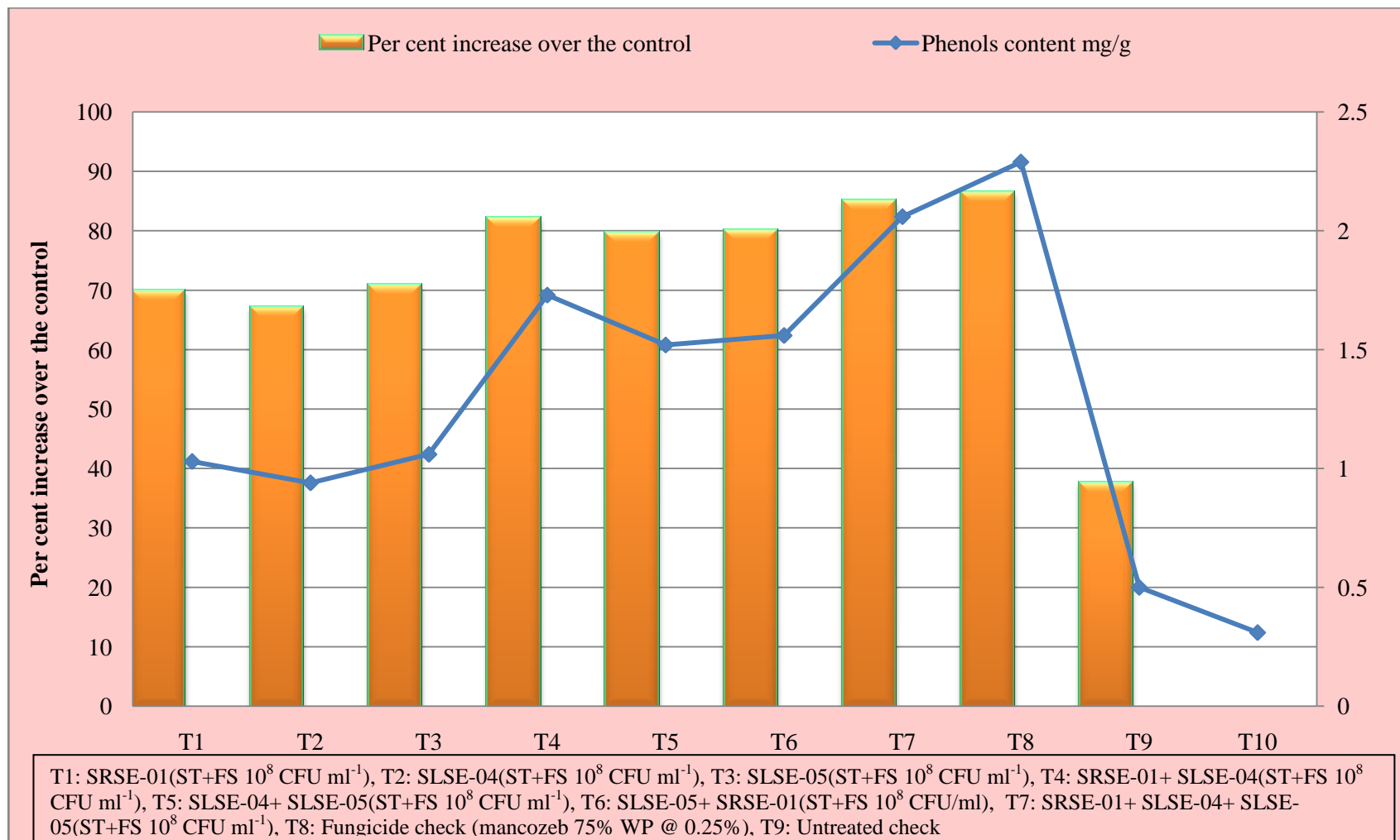
Therefore, increase in phenols in challenge inoculated plants over uninoculated control and significant increase in phenols in plants treated with endophytes over control is a confirmation that even non pathogenic are bacterial endophytes to trigger phenols which in turn reduced the disease severity.

**Table 4.7. Phenol estimation in endophyte treated sorghum plants**

S. No.	Treatment	Phenols content (mg g <sup>-1</sup> )*	Per cent increase over the control
1	T1-SRSE-01 (ST+FS 10 <sup>8</sup> CFU ml <sup>-1</sup> )	1.03 <sup>e</sup>	69.91
2	T2-SLSE-04 (ST+FS 10 <sup>8</sup> CFU ml <sup>-1</sup> )	0.94 <sup>f</sup>	67.06
3	T3-SLSE-05 (ST+FS 10 <sup>8</sup> CFU ml <sup>-1</sup> )	1.06 <sup>e</sup>	70.81
4	T4- SRSE-01+ SLSE-04 (ST+FS 10 <sup>8</sup> CFU ml <sup>-1</sup> )	1.73 <sup>c</sup>	82.09
5	T5- SLSE-04+ SLSE-05 (ST+FS 10 <sup>8</sup> CFU ml <sup>-1</sup> )	1.52 <sup>d</sup>	79.64
6	T6- SLSE-05+ SRSE-01 (ST+FS 10 <sup>8</sup> CFU ml <sup>-1</sup> )	1.56 <sup>d</sup>	80.12
7	T7- SRSE-01+ SLSE-04+ SLSE-05 (ST+FS 10 <sup>8</sup> CFU ml <sup>-1</sup> )	2.06 <sup>b</sup>	84.97
8	T8 Mancozeb 75% WP @ 0.25% (Fungicide check)	2.29 <sup>a</sup>	86.44
9	T9 Control (ST+FS CFU ml <sup>-1</sup> )	0.50 <sup>g</sup>	37.50
10	Control	0.31 <sup>h</sup>	-
	SEm ±	0.02	
	CD (P ≤ 0.05)	0.06	
	CV (%)	2.54	

Numbers with same superscript are statistically significant

\*n= Mean of three replication



**Fig. 4.3 Phenol content in endophyte treated sorghum plants**

#### 4.4 MANAGEMENT OF SORGHUM TURCICUM LEAF BLIGHT USING POTENTIAL ENDOPHYTES UNDER GREEN HOUSE CONDITIONS

There was a non significant difference in disease severity among treatments up to 15 days after inoculation with pathogen and before endophyte spray schedule indicating uniform disease occurrence in all the treatments.

After 7 days of first spray, the PDI in endophyte treated plants ranged from 16.15 to 17.46 % as against 19.27 % in control. Similarly, after 14 days of first spray it ranged from 17.65 to 20.19 % as against 24.80 % in control.

It was observed that after two sprays, *i.e.*, after first and second sprays, irrespective to the number of sprays and number of days after endophyte treatment, significant difference between endophyte treated plants and control plants in PDI was observed. All the treatments were found significantly superior (21.19-25.36%) over control (35.74 %) but not over fungicide check (19.62%).

Combination of all the three bacterial endophytes resulted in significantly high inhibition of disease (40.70%) among the treatment where endophytes were involved. However, with the endophytes in dual combination, SLSE-04 and SLSE-05 was found superior over other combinations with only 22.94 % PDI as against 23.42 to 25.36 % PDI in other combinations. When individual bacterial isolates were compared significantly low PDI was observed with SLSE-04 isolate (25.00 %) which was found on par with SLSE-05 (25.36 %) (Table 4.8).

##### 4.4.1 Impact of Different Treatments on AUDPC:

The Area under disease progress curve was depicted in Fig. 4.4 where, minimum AUPDC was observed when all three isolates SRSE-01+SLSE-04+SLSE-05 were used together (ST+FS  $10^8$  CFU ml<sup>-1</sup>) (516.35) as against the control (739.53) and among other treatments involving endophytes, the amount of diseased area varied and the maximum area was recorded in the treatment SLSE-05 (ST+FS,  $1 \times 10^8$  CFU ml<sup>-1</sup>) (598.93) which indicated its slow activity (Fig. 4.4)

Shiomi *et al.* (2015) reported that bacterial strains *B. subtilis* 0G, *B. lentimorbus*, *Streptomyces* sp. and *B. agaradhaerens* to efficiently control the incidence of northern corn leaf blight (*E. turcicum*), by microbiolization of seeds and

aerial parts of maize under greenhouse conditions. Dawut *et al.* (2016) reported that two endophytic bacterial strains of *B. subtilis*, XJAS-AB-13 and XJAS-AB-11 with broad antifungal activity against maize pathogen *E. turcicum* showing control of 63.33% and 45.0% respectively.

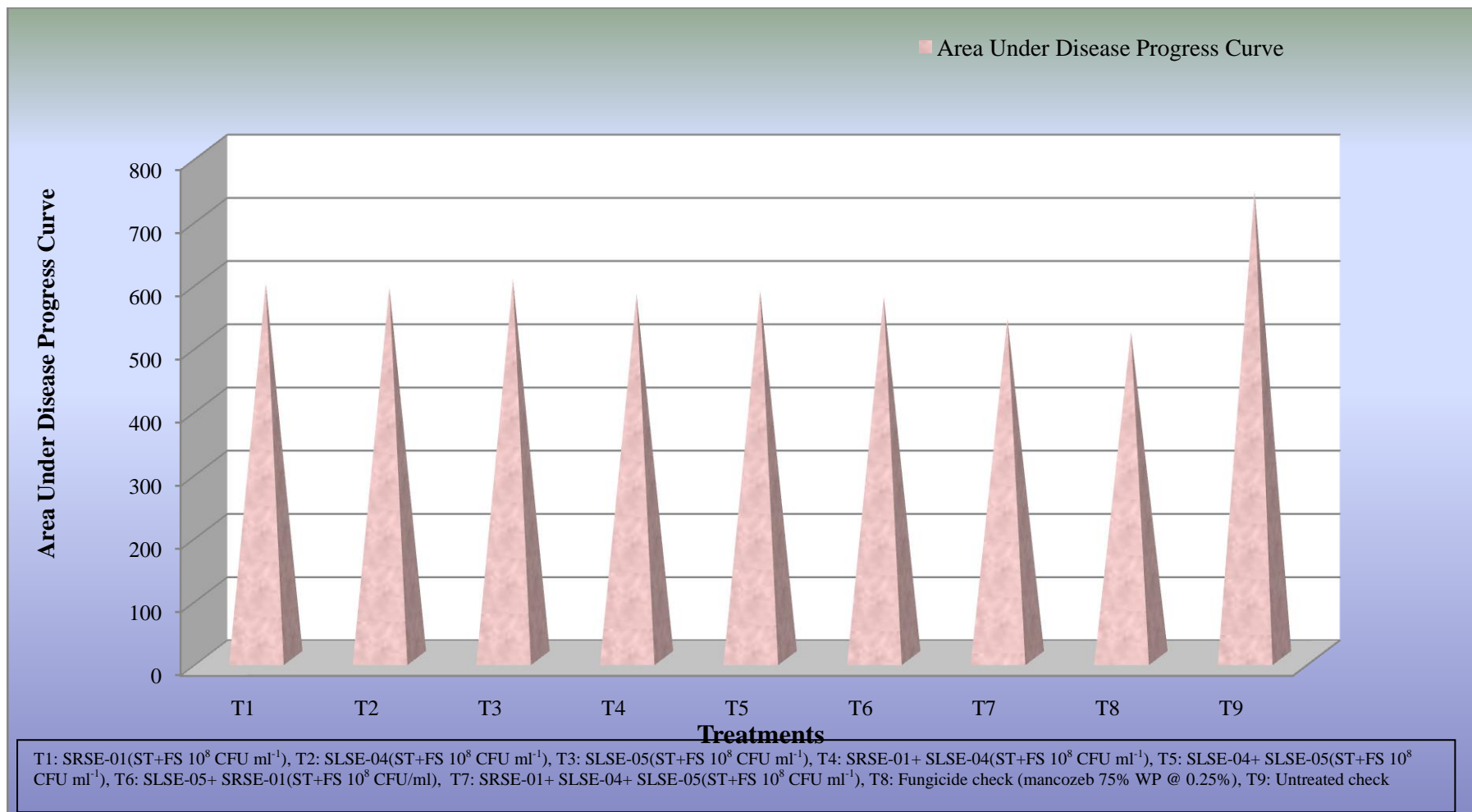
Presence of *Pseudomas* spp. in internal tissues of stem was reported to suppress sheath blight disease in rice (Krishnamurthy and Gnanamanickam, 1997). Antagonistic effect of *B. subtilis* against *Cryphonectria parasitica* causing chestnut blight was reported to reduce lesion area on stem (Wilhelm *et al.*, 1998). Kalraa *et al.* (2010) isolated four endophytic bacteria (OS-9, OS-10, OS-11, OS-12) from *Ocimum sanctum* and reported that OS-9 to have high inhibitory effect on the growth of *R. solani*, *A. solani*, *F. solani* and *C. lindemuthianum* while OS-11 antagonistic to *A. solani* alone. Mmbaga *et al.* (2018) tested 16 endophytes, isolated from *Cornus florida* stem tissue against root rot pathogens, *F. solani*, *F. oxysporum*, *Macrophomina* spp.

Based on the research carried out on the endophytic bacterial isolates from sorghum, SRSE-01, SLSE-04 and SLSE-05 were regarded to be the best isolates of all which are being submitted for identification and getting the accession number.

**Table 4.8. Management of sorghum Turcicum leaf blight using potential bacterial endophytes under green house conditions**

Tr. No.	Treatments (ST+FS)	PDI (%)					% Inhibition over control		Area Under Disease Progress Curve
		Day before first spray (34 DAS)	First spray		Second spray		After 1 <sup>st</sup> spray (49 DAS)	After 2 <sup>nd</sup> spray (64 DAS)	
			7 days (42 DAS)	14 days (49 DAS)	7 days (57 DAS)	14 days (64 DAS)			
T1	SRSE-01(ST+FS 10 <sup>8</sup> CFU ml <sup>-1</sup> )	15.12 (22.87)	17.01 (24.35) <sup>cd</sup>	20.02 (26.57) <sup>d</sup>	22.08 (28.02) <sup>e</sup>	25.59 (30.38) <sup>f</sup>	19.26	28.40	593.40
T2	SLSE-04(ST+FS 10 <sup>8</sup> CFU ml <sup>-1</sup> )	15.42 (23.11)	16.90 (24.26) <sup>cd</sup>	19.84 (26.44) <sup>d</sup>	21.60 (27.68) <sup>d</sup>	25.00 (29.99) <sup>e</sup>	20.00	30.05	586.68
T3	SLSE-05(ST+FS 10 <sup>8</sup> CFU ml <sup>-1</sup> )	15.13 (22.88)	17.46 (24.69) <sup>e</sup>	20.19 (26.69) <sup>d</sup>	22.31 (28.17) <sup>f</sup>	25.36 (30.23) <sup>ef</sup>	18.60	29.04	598.93
T4	SRSE-01+ SLSE-04(ST+FS 10 <sup>8</sup> CFU ml <sup>-1</sup> )	15.30 (23.01)	16.80 (24.18) <sup>c</sup>	19.28 (26.04) <sup>c</sup>	21.62 (27.70) <sup>d</sup>	23.42 (28.93) <sup>d</sup>	22.24	34.46	575.95
T5	SLSE-04+ SLSE-05(ST+FS 10 <sup>8</sup> CFU ml <sup>-1</sup> )	15.45 (23.13)	17.04 (24.37) <sup>d</sup>	19.29 (26.04) <sup>c</sup>	22.20 (28.10) <sup>ef</sup>	22.94 (28.60) <sup>c</sup>	22.23	35.82	580.97
T6	SLSE-05+ SRSE-01(ST+FS 10 <sup>8</sup> CFU/ml)	15.27 (23.00)	16.88 (24.25) <sup>cd</sup>	19.37 (26.10) <sup>c</sup>	20.81 (27.13) <sup>c</sup>	23.70 (29.12) <sup>d</sup>	21.90	33.69	572.04
T7	SRSE-01+ SLSE-04+ SLSE-05(ST+FS 10 <sup>8</sup> CFU ml <sup>-1</sup> )	15.10 (22.85)	16.15 (23.69) <sup>b</sup>	17.65 (24.83) <sup>b</sup>	20.03 (26.58) <sup>b</sup>	21.19 (27.40) <sup>b</sup>	28.84	40.70	537.37
T8	Fungicide check (mancozeb 75% WP @ 0.25%)	15.30 (23.01)	15.85 (23.45) <sup>a</sup>	16.14 (23.68) <sup>a</sup>	19.81 (26.42) <sup>a</sup>	19.62 (26.28) <sup>a</sup>	34.92	45.11	516.35
T9	Untreated check	15.37 (23.07)	19.27 (26.03) <sup>f</sup>	24.80 (29.85) <sup>e</sup>	29.36 (32.79) <sup>g</sup>	35.74 (36.70) <sup>g</sup>	0	0	739.53
	SEm ±	0.12	0.05	0.1	0.04	0.08			
	CD (P ≤ 0.05)	NS	0.17	0.30	0.13	0.26			
	CV (%)	0.88	0.39	0.66	0.26	0.50			

\*Figures in parenthesis are arc sine transformed values n= mean of 3 replications



**Fig. 4.4. Effect of bacterial endophytes on Turcicum leaf blight AUDPC**



**Plate 4.16. Effect of endophytes in managing sorghum Turcicum leaf blight under green house conditions**

## Chapter – V

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### *Summary and Conclusions*

## Chapter V

# SUMMARY AND CONCLUSIONS

The results of present research on “Management of sorghum *Turcicum* leaf blight using bacterial endophytes” are summarized here with.

Protocol was standardized for the isolation of endophytes from roots and leaves of sorghum where, 70% ethyl alcohol and 0.1 % mercuric chloride were used for treating leaf samples for five minutes each and washing was done with sterile distilled water for four times before and after second chemical treatments. Thirty eight colonies were selected for their purification, of which, three isolates from root and 10 isolates from leaves were separated and designated as SRSE-01, SRSE-02, SRSE-03, SLBE-01, SLBE-02, SLBE-03, SLBE-04, SLBE-05, SLSE-01, SLSE-02, SLSE-03, SLSE-04 and SLSE-05 based on the plant part from which they were isolated.

Number of colonies obtained from 10, 20 and 30 day old leaves were uniform ( $3.52 \times 10^8$ ) indicating consistent occurrence of endophytes irrespective of the age of the leaf used for their isolation. However, the number of colonies was high from roots than leaves with an incubation period of 2.6 days for leaf endophytes and 2.0 to 2.8 days for root endophytes while it was extended in endophytes from the leaf bits and ranged from 4.3 to 4.6 days.

Morphologically all the endophytes from the roots were round except SRSE-01 which was irregular in shape. Similarly, isolates from leaf bits were round except SLBE-02 which was irregular. Whereas, most of the isolates from the leaf sap were irregular except SLSE-01 which was round in shape.

Culture characters of the three isolates from roots revealed that only SRSE-03 was with even colony margin while in others it was undulate. Similarly, among the colonies from leaf bits two had even margins (SLBE-03, 04 and SLSE-01) while maximum isolates had undulated margins (SLBE-01, 02 and 05, SLSE-02, 03 and 04) and it was lobate in a single isolate SLSE-05. Eight of the isolates appeared raised in their elevation and five isolates SLBE-02, 03, 05 and SLSE-01, 05 had flat colonies. There was variation in texture of the endophytic isolates where most of the colonies had smooth texture with opaque nature except SRSE-02 and 03 which were

translucent. Colony pigmentation differed from white to creamy colour, where most of the isolates were creamy white while, SRSE-02, SRSE-03, SLBE-01 and SLSE-05 isolates had white colour and, SLBE-04 was cream coloured.

Dual culture studies revealed that endophyte SRSE-01 had maximum antagonistic potential with radial growth of sorghum *E. turcicum* with 52.75 % inhibition and was found significantly superior over other endophytes isolated from healthy leaf bits, root sap and leaf sap. SLSE-04 (51.65%), SLSE-05 (51.10%) and SLSE-03 (50.00%) isolates of leaf sap were found on par with each other and superior over endophytes from leaf bits where SLBE-05 (25.27%) was found superior in inhibiting the pathogen.

Observations after five days of inoculation at interaction zone revealed variation in growth pattern of the test fungus with thickening of the hyphal strands (SRSE-01 and SLSE-02), occurrence of anastomosis (SRSE-01, SLBE-02 and SLSE-05), chlamydospores formation intercalarily and terminally. Among all the isolates, SRSE-01 was found significantly superior over other isolates with higher number of chlamydospores (222.67/ microscopic field) followed by SLSE-04 (135.33/ microscopic field) and SLSE-05 (117.67/ microscopic field) which were on par with each other. Lysis of the fungal mycelium was not observed in any of the interactions.

All the isolates inhibited disease development on sorghum leaf when assessed using detached leaf technique. Among the endophytic isolates SRSE-01 was found to be superior in inhibiting the pathogen development with minimum diseased area and was found on par with SLSE-05 isolate (2.67%). No significant difference was observed among the isolates with respect to disease index. Based on the superior characters in dual culture and detached leaf techniques isolates SRSE-01, SLSE-04, SLSE-05 which were found compatible with each other.were advanced for other studies

All the three isolates tested positive for biochemical characters such as utilization of nitrogen sources, production of proteases, amylase and catalase whereas they failed to produce siderophore and IAA. SRSE-01 and SLSE-05 were found to solubilize phosphate indicating their ability in phosphate solubilization. Zone of solubilization around their colonies was more pronounced in SLSE-05 isolate compared to SRSE-01 isolate.

Increase in phenol content in endophyte treated plants was positive indication for their probable role in inducing disease resistance in plants. Phenols content was found significantly high in the treatment where all the endophytes were simultaneously inoculated ( $2.06 \text{ mg g}^{-1}$ ) compared to other treatments where endophytes are associated, however it was not superior over phenol from Mancozeb @ 0.25 % treated plants ( $2.29 \text{ mg g}^{-1}$ ). In individual treatments, SLSE-05 was found to enhance the phenol to a greater extent of 70.81 % and was found on par with SRSE-01 isolate (69.91 %). In dual combinations, the combination of SRSE-01 + SLSE-04 was found effective with  $1.73 \text{ mg g}^{-1}$  phenol with an increase of 82.09 % over control. However, among other combinations SRSE-01 + SLSE-05 and SLSE-04 + SLSE-05 were found on par with each other with increase in phenol content.

Combination of all the three bacterial endophytes resulted in significantly high inhibition of disease (40.70%) at 64 DAS when tested under greenhouse conditions. However, with the endophytes in dual combination, SLSE-04 and SLSE-05 was found superior over other combinations with only 22.94 % PDI as against 23.42 to 25.36 % PDI. When individual bacterial isolates were compared significantly low PDI was observed with SLSE-04 isolate (25.00 %) which was found on par with SLSE-05 (25.36 %).

Minimum AUPDC was observed when all three isolates SRSE-01+SLSE-04+SLSE-05 were used together ( $\text{ST+FS } 10^8 \text{ CFU ml}^{-1}$ ) (516.35) as against the control (739.53) and among other treatments involving endophytes, the amount of diseased area varied and the maximum area was recorded in the treatment SLSE-05 ( $\text{ST+FS, } 1 \times 10^8 \text{ CFU ml}^{-1}$ ) (598.93) which indicated its slow activity.

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**\*Original not seen**

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**Note:** The pattern of literature cited presented above in accordance with the guidelines for Thesis presentation for Acharya N G Ranga Agricultural University, Guntur.