



Isolation of Bacterial Endophytes and Evaluation against *Exserohilum turcicum* (Pass.) Leonard and Suggs in Sorghum

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The present study evaluates the Isolation of bacterial endophytes and evaluation against *Exserohilum turcicum* in sorghum. Sorghum (*Sorghum bicolor* L. Moench) ranks as the world's fifth most significant cereal crop. One of the most significant foliar diseases is turcicum leaf blight, caused by *Exserohilum turcicum* (Pass.) Leonard and Suggs, is seen in severe form in the main sorghum-growing regions of the Guntur district. Chemical control is used to manage turcicum leaf blight. Given the possible risks associated with the use of chemicals in agriculture, the use of potentially antagonistic endophytes that are naturally present in the plant system has made it possible to produce natural and environmentally friendly food. During research morphologically and culturally diverse endophyte colonies were isolated. Endophyte SRSE-01, which showed 52.75% inhibition, was shown to be much better than other endophytes. Leaf sap isolates SLSE-04 (51.6%), SLSE-05 (51.1%), and SLSE-03 (50.00%) were found to be comparable to one another and better than endophytes from leaf fragments. Variation in the fungal growth pattern in the interaction zone was noted, along with thickening of the hyphal strands, anastomosis and the production of chlamydospores. In the present study, incompatible interactions with endophytes was perceived by the formation of chlamydospores which might have occurred due to antagonistic nature of endophyte due to the synthesis of either enzymes or toxins or antibiotics.

Keywords: endophyte colonies; *Sorghum bicolor*; biotic and abiotic stresses; crop debris.

1. INTRODUCTION

Bacterial endophytes are a crucial component of the phytomicrobiome, playing an essential role in agriculture and industries. Endophytes are a rich source of bioactive compounds, serving as natural antibiotics that can be effective in combating antibiotic resistance in pathogens. These bacteria interact with host plants through various processes such as quorum sensing, chemotaxis, antibiosis, and enzymatic activity (Chaudhary et al., 2022; Hnamte et al., 2024). Sorghum (*Sorghum bicolor* L. Moench), a C4 plant belonging to the family Poaceae, originated in northeast Africa. It has been recognized as 'poor man's crop' as it is being used as a staple food crop particularly those in the semi-arid tropics and serves as 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 exceptionally well in marginal lands with low fertilizers and inputs. As it confronts prolonged drought spells in arid climates 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; Bakari et al., 2023).

Despite its resilience 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; Muture et al., 2021). Foliar diseases like leaf blight, anthracnose, downy mildew and rust cause significant loss due to a reduction in the photosynthetic area of the affected leaves. Leaf blight caused by *E. turcicum* is one of the major foliar disease (Sharma and Jain, 1975) occurring in severe form in sorghum-cultivated areas in Guntur district. Infection at pre-flowering stage in susceptible cultivars can result in grain yield losses of up to 50% (Frederiksen, 1980). Low fertilizer, narrow spacing, and monocropping further intensify the incidence with varied severity depending on the prevailing pathotypes (Ogolla et al., 2019).

E. turcicum is a hemibiotrophic foliar pathogen of important cereal crops (Frederiksen, 1980; Bunker and Mathur, 2006). The pathogen was reported to be seed-borne and survives across the seasons by producing chlamydospores and in space by persisting in crop debris (Chidambaram et al., 1973; Shree and Luke, 1983; Ahmed and Reddy, 1993; Martín et al., 2022). Under the conditions of the warm climate and high relative humidity (RH) the pathogen tends to multiply in polycyclic manner on leaves of susceptible cultivars producing peculiar localized lesions that turn to elongated cigar-shaped lesions.

Traditional disease management of the Turcicum leaf blight relies on usage of chemicals such as carboxin (Khedekar et al., 2012), tebuconazole (Manu et al., 2017), mancozeb and propiconazole (Wani et al., 2017). Concerns over

chemical residues, environmental impact and increasing consumer preference for eco-friendly agricultural practices necessitate alternative sustainable disease management approaches. Plants foster a wide range of microorganisms within their tissues that have the unique ability to survive inside plants with little or no microbial competition, and 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 processes and may influence various physiological processes of plants. 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, and extracellular enzymes collectively prompting plant growth (Compant et al., 2005). The present study evaluates the Isolation of bacterial endophytes and evaluation against *Exserohilum turcicum* in sorghum.

2. METHODOLOGY

2.1 Isolation of Endophytic Bacteria

2.1.1 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.

2.1.2 Surface disinfestation

Leaf and root bits were sterilized separately using 70% ethanol for 5 min, later were passed through a 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).

2.1.3 Isolation

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

2.1.4 Isolation using leaf bits

Leaf samples were cut into small bits using flame sterilized scalpel and were placed on TSA plates and incubated at 28 ± 2 °C for 5-7 days.

2.1.5 Isolation by serial dilution

Leaves and roots were macerated separately in phosphate buffer solution using a sterilized pestle and motor and were used for preparing serial dilutions up to 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 ± 2 °C for 2-5 days.

2.1.6 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 ± 2 °C.

2.1.7 Selection and purification of different bacterial morphotypes

Each plate was examined for the selection of bacterial morphotypes based on morphological parameters viz., colony size, shape, colour, margin 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.

2.2 Screening for Potential Antagonistic Bacterial Endophytes *In vitro*

2.2.1 Isolation and identification of pathogen

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

Leaf bits of 4 mm² with healthy and infected leaf portions 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 onto PDA plates and was incubated at 27 ± 1 °C in the incubator.

Three-day-old mycelial bits developed from diseased leaves 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.

2.2.2 Dual culture technique

Dual culture technique was used to obtain the potential antagonistic endophytes against *E. turcicum*. The mycelial disc of pathogen, *E. turcicum* was inoculated at the center of PDA plate. Test bacterial endophytes were streaked individually on both 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 ± 2 °C were used to record the zone of inhibition after four days of inoculation where *E. turcicum* has shown maximum growth in the 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 monoculture check.

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

2.3 Microscopic Observations

Signs of antagonism/hyperparasitism and lysis were observed using a compound microscope. The number of chlamydospores per microscopic

field was counted and comparisons were made with the monoculture plate.

3. RESULTS AND DISCUSSION

3.1 Isolation, Purification and Maintenance of Bacterial Endophytes from Sorghum

3.1.1 Isolation, purification and maintenance of endophytes

Isolation of endophytes from roots and leaves of sorghum was standardized through surface disinfection involving sequential treatment with 70% ethanol and 0.1 % mercuric chloride for five minutes each followed by washing with sterile distilled water four times before and after the second chemical treatment which was modified from the protocol described by de Fretes et al. (2018). A sterility check maintained using final washings was found free from any colony growth. Variability in colony occurrence was observed between whole leaf samples and root/ leaf segments with further variations noted at different dilution levels. 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 the streak plate method. 3 isolates from the root and 10 isolates from leaves were separated for antagonistic studies. Endophytic bacteria were designated based on their respective plant part of origin.

Table 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

The colony count remained consistent across different ages (10,20 and 30 days) with an average of 3.52×10^8 CFU, suggesting that leaf age did not significantly influence endophyte occurrence. However, the number of colonies was higher 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. The incubation period for the endophytes from the sorghum leaf bits ranged from 4.3 to 4.6 days

These findings align 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 endophytes being detected in all the plant parts, roots having the most intimate contact with soil may function as the first avenue for the recruitment of endophyte bacteria.

3.2 Screening Endophytes for their Antagonistic Activity *In vitro*

3.2.1 Isolation of pathogen

Sorghum leaves showing typical symptoms were used for isolation of the pathogen. The 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).

3.2.2 Evaluation of antagonistic potential of endophytic bacterial isolates against *E. turcicum* using dual culture technique

Three endophytic isolates from the root and ten endophytic isolates from leaves were screened for antagonistic potential against *E. turcicum*. All endophytic isolates exhibited inhibitory activity against the pathogen compared to the monoculture plate, with inhibition percentages ranging from 3.30 to 52.75 %. 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 %). In contrast, isolates demonstrating more than 50% inhibition included 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 to 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.



Fig. 1. Screening antagonistic potential bacterial endophytes against *E. turcicum* *In vitro*

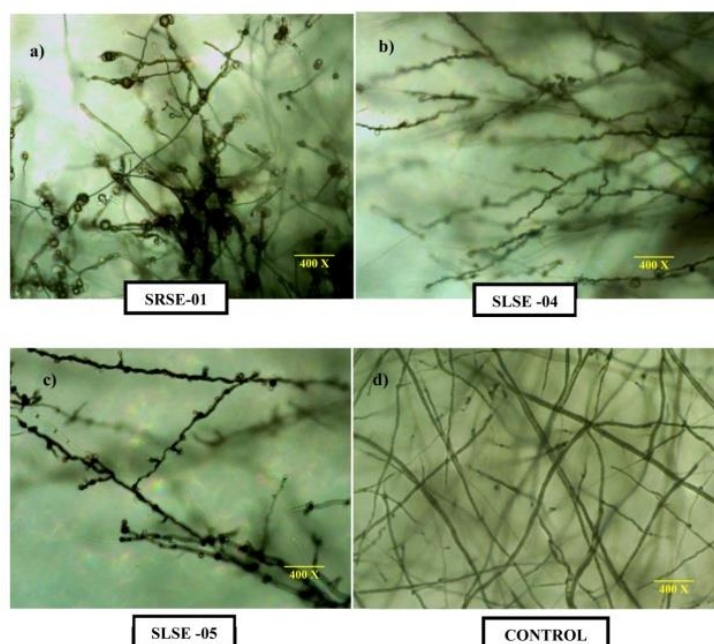


Fig. 2. Chlamydospores formation at interaction zone between endophytes and *E. turcicum* (a. Interaction zone between SRSE-01 and *E. turcicum*; b. Interaction zone between SLSE-04 and *E. turcicum*; c. Interaction zone between SLSE-05 and *E. turcicum*; d. *E. turcicum* alone)

Table 2 Antagonistic potential of bacterial endophytes against *E. turcicum*

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

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, the growth pattern of fungus varied at the interaction zone. The most characteristic feature observed was hyphal thickening and formation of chlamydospores intercellularly and terminally observed 5 days' post inoculation. However, the isolates SLBE-04, SLBE-05 and SLSE-01 did not show any chlamydospores formation resembling the culture in control plate.

Among all the isolates, SRSE-01 exhibited the highest chlamydospore production (222.67 per microscopic field) followed by SLSE-04 (135.33/per microscopic field) and SLSE-05 (117.67 per microscopic field) which were statistically on par with each other, while SLSE-05 was on par with SLSE-03 (108.0 per microscopic field)

Anastomosis of *E. turcicum* hyphae was observed in interactions with SRSE-01, SLBE-02 and SLSE-05, while clustering of hyphae resembling the prosenchyma formation was observed in SRSE-01 and SLSE-02 interactions. However, no evidence of fungal mycelial lysis was detected in any interaction.

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

4. CONCLUSION

Present 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.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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