Research Article

Enumeration and Identification of Surface Endophytes of *Solanum lycopersicum* and their Seasonal Recurrence

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ABSTRACT

Background: Endophytes have been identified in various plant species; however, only a few studies have been conducted to explore the endophytic fungi folklore in fruit-bearing plants. The present study was conducted to enumerate the foliar endophytes of *Solanum lycopersicum*. **Materials and Methods:** The leaf samples of *S. lycopersicum* were harvested in four different seasons during the year 2020-2021. Samples were surface sterilized with 0.2% $HgCl_2$, inoculated onto potato dextrose agar, amended with 0.12mg/ml chloramphenicol and incubated at 25-32°C with exposure to light and dark cycles. Identification was done by morphological typing. **Results:** Leaf samples harboured endophytes in all sampling seasons; however, season Sept-Nov which observed higher rainfall, witnessed more endophytes than the other seasons. The diversity of endophyte assemblage identified were hyphomycete and coelomycete species. *Geotrichum* sp., *Phomopsis* sp., *Trichoderma* sp., and *Humicola* sp. have proven their recurrence. *Colletotrichum* sp., being the dominant genus with higher colonization frequency, was found to be harboured in all sampling seasons. **Conclusion:** The study is the first of its kind in exploring the fungal endophytic profile in *S. lycopersicum*. Hence further studies are warranted to explore if the inherent endophytes of this plant confer any role in the metabolic physiology of the plant or host-pathogen interaction.

Keywords: Coelomycete, Colletotrichum, Endophyte, *Solanum lycopersicum*, Sporulation.

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INTRODUCTION

Fungi endophytes cause asymptomatic and mutualistic infections in the plants. They have an immense potential for novel secondary metabolite production. These novel secondary metabolites are having enormous use in clinical applications as well as in industrial fields. Apart from these applications endophytes improve the fitness of the host plants.[1] The endophytic fungi are beneficial to the plants in pathogen and pest control. The endophytes also improve plant growth and nutrient uptake and help the plant to resist abiotic stress. Hence increases the overall metabolic physiology of the plant. Production and consumption of safer food products among consumers have driven an inching demand and this is accumulated by applying novel methods of farming. For instance use of fertilizers and pesticides has been replaced with organic manure to produce organic staples. Likewise, endophytes are widely used as a pest control mechanism in the plant. Hence further insights revealing the interaction of endophytes with the plant or the host are of utmost importance. Previous studies have enumerated the potential effect of fungal endophytes in tomatoes and their horizontal acquisition within the plant. These studies were carried out to identify if the latent infection or colonization of endophytes into the host plant is horizontal or vertical transmission or if naturally acquired from the parent plant. The occurrence and distribution pattern of

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endophytic fungi in different plant parts growing in the tropics and other geographic communities have been studied somewhat well.[2] However, there are only a few studies on seasonal infection patterns. Though much of the diverse studies have focused on medicinal plants or ornamental ones limited studies have been reported on the isolation or identification of endophytes from fruit or vegetable plant. *Solanum lycopersicum* is a cultivable and indigenous plant that harvests edible berry fruit known as a tomato. The plant grows throughout the year and is considered one of the major sources of income for marginalized farmers. This plant is almost grown throughout the world round the year.[3] Hence, the present study was carried out to identify foliar endophytes of the *S. lycopersicum* plant which is grown in different seasons throughout the year.

MATERIALS AND METHODS

Sampling Technique

The leaf samples of the *S. lycopersicum* plant were collected from the fruiting plants at INTEC farm, Worabe town. The town is about 170 KM to the south of Addis Ababa, Ethiopia. The sampling was carried out from 2020 to 2021 at four different season intervals. The sampling rounds were from June to August 2020, September to November 2020, December to February 2021 and March to May 2021.

Sample Collection

Leaves weighing 100 gm weight were collected from the sampling site and put into the ziplock-based laboratory-grade plastic bags. The sample was transported the same day to the laboratory for further processing or kept in a refrigerator at 4°C until further processing.

Treatment of the Sample

The treatment of the sample, isolation, identification and induction of sporulation was done by employing the methods of Li *et al*. (2015).[4] Collected leaf samples were carefully washed with tap water continuously for 7-10 min. With the help of a sterile scalpel, washed leaf samples measuring 4-7 mm were cut from the segments using a sterile scalpel. The cut segments were surface sterilized by adopting the set protocol as follows. For one minute, the leaf sample was kept in 75% ethanol, succeeded by a solution of 0.2% $HgCl_2$ for 30 sec. After 75% ethanol and 0.2% $HgCl_2$ treatments, samples were thrice washed with sterile water with a gap of 2 min between each washing. Finally, the washed sample was blotted dry with sterile blotting paper. The samples were inoculated into the Potato Dextrose Agar (PDA) plates having chloramphenicol (0.12mg/ml) as an antibiotic to restrict the growth of bacteria. All the plates were marked with codes and incubated for 21 days or till sporulation happened. The incubation conditions were as follows. Temperature at 24 to 32°C; 12-h dark and light cycle and relative humidity maintained at 67-97%.

Subculturing and Sporulation of Isolates

The growing colonies were transferred to fresh PDA plates and subcultured. Percentage colonization frequency (CF%) of endophyte species was calculated as the number of segments colonized by a single endophyte divided by a total number of segments observed × 100 and recorded accordingly.

N: number of segments colonized by a single endophyte

M: total number of segments observed

The cultures which failed to sporulate within 2-3 months of incubation were designated as "mycelia sterile". To induce sporulation in mycelia sterile, isolates were subcultured along with sterilized host parts and incubated at 24°C with a 3-hr of UV light exposure and 21 hr of dark exposure in one method and the second one. Isolates were cultured on nutrient-deficient potato carrot agar.

Identification of Fungal Isolates

The identification of the endophytes was carried out by using the morphotyping technique along with standard mycological manuals. Briefly, wet mount preparation of the sample was prepared and stained with lactophenol cotton blue and observed under the microscope under different resolutions. Colony characteristics like colour, growth pattern, sporulation, perfusion etc were noted. The cultures were examined under a microscope after proper staining and observed for spores, hyphae, and conidia formation among other characteristics.

Statistical Analysis

The data was recorded in Excel (Microsoft Corp. USA) and subsequently analyzed using SPSS Statistic software for Mean ± Standard Deviation and descriptive statistics analysis. Colonization frequencies and proportions were calculated by taking the mean colonization rate in each sampling with consideration of 0.5 sampling error. A *p*-value of 0.05 or less was used as the cut-off level for statistical significance.

RESULTS

The leaf sample of *S. lycopersicum* harboured endophytes in all four collection seasons. Nearly 127 isolates were obtained from 100 leaf segments (Figure 1). All the isolates were successfully identified and their CF(%) were recorded (Table 1). Among the identified endophytes we

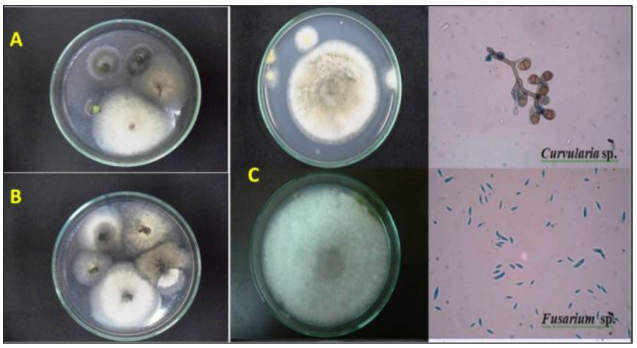

Figure 1: The diagram depicts the growth of endophytes from the edges of the plant leaf samples.

A) Species like *Fusarium*, *Trichoderma*, and *Curvelaria* can be seen growing above the margins. B) Sample collected in September to November period depicts well growth of species like *Fusarium*, *Acremonium* and *Geotrichum*. C) Morphological and microscopic visuals of *Fusarium* and *Curvularia* species.

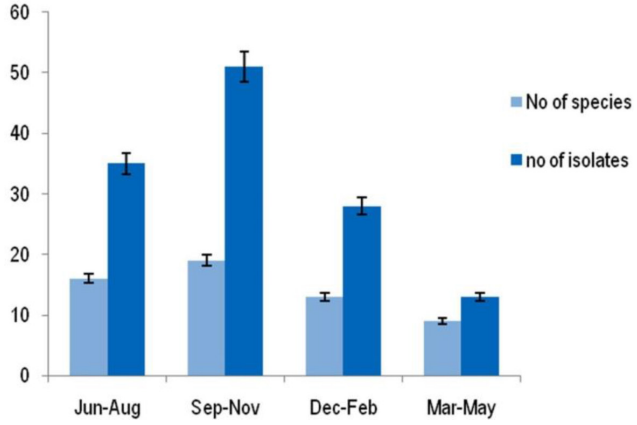

Figure 2: Bar diagram depicting several endophyte species/ isolates recovered from *S. lycopersicum* during different sampling periods.

Table 1: Percentage Colonization frequency (CF%) of endophytes isolated from the leaves of *S. lycopersicum* during various sampling periods.

Endophyte	CF%			
	June-August	September-November	December-February	March-May
Alternaria helianthi	1	2.5	NI	NI
Fusarium sp.	6	5.5	2	NI
Penicillium funiculosum	1.5	NI	NI	3.5
Phomopsis sp1	11.5	25	35.5	NI
Phomposis sp2	13.5	42.5	35	1
Acremonium sp.	NI	7.5	2	NI
Aspergillus niger	1.5	2	NI	2
Aspergillus flavus	NI	0.5	NI	NI
Cladosporium sp.	2	0.5	NI	5
Trichoderma sp.	NI	3	2.5	4.5
Myrothecium sp.	2	3.5	1	NI
Geotrichum sp.	NI	4.5	2	2.5
Colletotrichum sp1	3.5	30	17	11.5
Colletotrichum sp2	2	40.5	25	3.5
Colletotrichum sp3	6.5	52.5	19.5	2
Humicola sp.	1	3.5	0.5	NI
Mycelia sterile1	20	25.5	4.5	NI
Mycelia sterile1	14.5	29	6	NI
Mycelia sterile1	3	1.5	NI	NI
Mycelia sterile1	4.5	2	NI	NI

NI= not isolated.

could recognize 35 isolated sterile mycelia comprising 4 different species.

The foliar endophyte assemblage of *S. lycopersicum* is made up of *Colletotrichum* sp., the dominant genus with higher colonization frequency prevalently recurring in all the seasons (CF% from 2-52.5). Frequency isolation of hyphomycete and coelomycete members was recorded highest. *Phomopsis* was the second dominant genera occurring with high colonization frequencies (CF% from 1-42.5%). A higher number of endophytes were isolated much during the rainy season (September to November) rather than in other seasons (Figure 2). In many studies, leaves sampled during seasons that witness high rainfall downpour have harboured more endophytes rather than during the dry seasons. We witnessed the same trend in our study plant too. Our results are in sync with these findings. However, species like *Geotrichum, Phomopsis*, *Trichoderma* and *Humicola* recovered in all the seasons with *Colletotrichum* as the dominant genus in all the sampling seasons of the year. The study indicates that the study plant is rich in foliar endophytes that can be isolated throughout the year from the host plant. However, certain isolation optimization techniques in sampling, methodology and sampling period need to be deliberated to get the augmented output.

DISCUSSION

Much of the emphasis has been given to endophytes concerning their changing phenotypic effect on plants like changing plant growth or affecting pathogen growth in crops however, least is known about colonization frequency or pattern in host plants.[5] Hence this study was carried out in a direction to explore foliar endophytes of *S. lycopersicum*. Endophytes are assembled in the vascular tissues of the various plant parts like in leaf segments, root portions or embedded in tissues of the stem. These tissues bear endophytes either as singleton entities or in an assemblage from where most

endophytes exist as a bunch of commensal symbionts. Hence at any period or season, the endophytes can be isolated either as a single species or a bunch of species.[6] In the current study, the focus of isolation was on leaf portions owing to the rich growth of endophytes in the vascular tissues of the leaf segments. Hence endophyte isolation from the leaf sample is the best plant material for enumeration and quantification of the endophytes. Moreover using other plant parts like bark stem and root needs additional resources which were not available to the authors in the current study.

In this study, we have isolated a plethora of fungal endophytes ranging from sterile mycelia to reproductive fungi, of which *Colletotrichum* were the dominant genera. The *Colletotrichum* genera are omnipresent endophytes that have been isolated from a wide range of plant hosts.[7] Studies carried out elsewhere have also isolated different species of *Colletotrichum* from *Heisteria* and *Ouratea lucens* plants.[8] The study concludes by isolating 347 genetically distinct taxa of which 59% were single isolates and 41% the hyperdiverse. In this context, the fungal endophytes are designated as diverse colonizing agents in deep tissues whose diversity can be categorized as hypo- or hyperdiverse in nature. Likewise, mechanisms have been studied that explain the colonization diversity of cryptic endophytes within the plant tissues. These studies have found bigger endophyte population densities within vascular tissues of the leaf, which is the principal factor for the horizontal expansion of endophytes within the plant.[9] Further, in a geo-climatic context, endophytic assembly with the plant is richer in regions having sufficient rainfall or monsoon session.[1]

Fungal endophytes have vastly captured the attention of natural product chemists for the exploration of natural products. It is proven in previous studies that a web of small bioactive compounds is secreted by the endophytes, most of which have medicinal importance.[10] In this context, the current endophyte folklore of the study plant can be exploited for the natural product screening program for the identification of active metabolites.

It has been observed that the infection rate of endophytes increases with the age of the plant hence older leaves harbour more endophytes than the young ones upon isolation. But simultaneously young leaves have also contributed to much endophyte isolation which is attributed due to the conditioning precipitation that enhances foliar endophyte infection rate within the plants across all species.[11] Our findings are supported by the studies done elsewhere on the endophyte assemblage in trees, herbs and shrubs.[12-14] These studies also have demonstrated that young leaves harbour less endophyte assemblage as compared to older ones, most probably because of better exposure to infection conditions and a prolonged time for microbial growth.

Here we present the first report of endophyte assemblage in the *S. lycopersicum* plant from the present study area. Our results suggest a host of fungal endophytes colonizing within the tissues of the study plant. These endophytes can be harvested and put to use for the isolation of various metabolites having applications in medicinal or industrial arenas. Any new metabolite source could be for biotechnology or food industry use. In this context, the endophyte folklore of the study plant is significant and imperative. We suggest such studies should be carried out within other plants to disseminate the hidden endophytic biota of the plants.

Limitations of the Study

No study is without challenges and constraints. The authors believe that the use of cultivars of *S. lycopersicum* could have been a better study model to check the total endophyte assemblage. This could have generated a comparative model for the isolation of different endophytes. Secondly, the study was conducted in a resource-limited setting hence we could not characterize the secondary metabolite profile of isolated endophytes. The use of different plant samples like root or stem for endophyte isolation could also have been useful for studying the diversity of endophytes but in our study due to lack of resources we were unable to use only the leaf sample for endophyte isolation. These are some of the pitfalls associated with the study, however, the benefits associated with this study are much more than the limitation.

CONCLUSION

Based on the findings, it can be concluded that the study plant has a significant load of fungal endophytes which might infer disease resistance to the plant for various pathogens. The main benefits of endophytes to tomato plants may be attributed to the biocontrol of several insect pests and plant pathogens, as well as their ability to improve plant performance. However, more studies are warranted to prove these observations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

$HgCl_2$: Mercuric Chloride; **KM:** Kilometer; **PDA:** Potato Dextrose Agar; **CF:** Colonization Frequency; **UV:** Ultra Violet.

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