Advances in Life Science and Technology
ISSN 2224-7181 (Paper) ISSN 2225-062X (Online)
Vol.77, 2020

Assessment of Endophytic Fungi Associated with Greavillea Robusta Tree Planted as Urban Green Infrastructure in Hawassa Town: Its Implication to Understand the Current Health Condition of Urban trees

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Abstract
Endophytic fungi represent a genetically diverse group of microorganisms associated with healthy tissues of terrestrial plants. They are believed to be mostly beneficial to their host plants and produce novel antimicrobial compounds or may be latent pathogens that become active at specific stage of development or under a set of environmental conditions. The aim of the current study was to assess the diversity of culturable endophytic fungi in the leaf of diseased and healthy looking Grevillea robusta from different streets having different traffic flow. Leaf samples of G.robusta were collected from those trees growing in the road side, parks and home gardens at Hawassa town. Identification of the isolates to the genus level was performed on the basis of cultural characteristics and spore morphology. Accordingly, a total of 387 endophytic fungal isolates were recovered from 192 leaf fragments across five different streets. The highest isolation rate of endophytic fungi was recorded on a street from South Star Hotel to Bus station (IR=3.81) where as the smallest isolation rate was recorded on a street from Bus station to Atote (IR=0.71). The highest relative frequency of endophytic fungi was recorded for Pestalotiopsis spp 2 (RF=25.58%) where as the smallest was recorded was recorded for Unidentified spp 5 (RF=0.51%). Additionally, the highest diversity of endophytic fungi was recorded on the road from South Star Hotel to Bus station (H'=3.01) where as the smallest diversity of endophytic fungi was recorded on a street from Atote to Teachers Teaching College (H'=1.79).
Keywords: - Disease, Urban trees, Diversity, Grevillea robusta
DOI: 10.7176/ALST/77-04
Publication date: January 31st 2020

1. INTRODUCTION

Urban forests are made up of trees and other vegetation within the built environment. Urban trees are conglomeration of native tree species that existed prior to the development of the city and exotic species which might be introduced by residents or other means. Thus, urban forests often have a tree diversity that is higher than surrounding native landscapes (Nowak *et al*., 2008). Trees around residential area or private compound, streets, in urban parking lots, beside school compounds, and throughout city parks are part of an urban forest (sources).

Urban trees are essential parts of many cities' beautification, sustainability, and healthy community goals and initiatives. Urban forests and trees are an integral part of our community's ecosystems and help provide people with numerous benefits including: increasing property values, improving water quality and minimizing erosion by slowing the flow of precipitation during storm events, providing valuable habitat for wildlife, providing enjoyment for city residents, contributing to climate change mitigation and adaptation by sequestering carbon, minimizing/moderating daily temperature extremes, and reducing energy expenditures on cooling, providing shade and improving local air quality by removing air pollutants and producing oxygen (Dwyer *et al*. 1992; Kuoand Sullivan 2001; Wolf 2003; Nowak and Dwyer 2007).

Increased tree diversity can minimize the overall impact or destruction by a species-specific insect or disease, but the increase in the number of exotic plants can also pose a risk to native plants if some of the exotic species are invasive plants that can potentially out-compete and displace native species (source). Urban trees highly influenced by people and other factors, such as vehicles, buildings, pavement, utility lines, underground pipes, animals, and other plants. The care and management of many urban trees can be complicated by natural and social factors including: insects and diseases; wildfire; natural catastrophic events (such as ice storms and wind storms, including hurricanes); invasive plants; climate change; development; air pollution; lack of adequate management; and other social factors. As urban expansion continues, such challenges are likely to increase and new ones might emerge (Dwyer *et al*., 1992).

Plant diseases are a major concern to forest managers throughout the lodge pole pine types. In many areas, diseases constitute the primary management problem. Since urban tree are grow in the face of different challenges and constraints, they can be faced to different diseases and pests. In urban areas this can be aggravated by the presence of compacted and poor soil, city streets, and drive ways and underground utility services can constrain their roots. A shortage of water and nutrients, common in urban areas, can kill them. Road salt, pollution and

pesticides used to treat lawns can contaminate their air and water. Other trees and buildings can block their sunlight and vandalism, vehicle accidents, lawn mowers, weed trimmers, snow plows and poor pruning can damage their trunks and branches. These can cause potentially killing trees and reducing the health, value and sustainability of the urban forest (Illinois University 1998).

The most common urban tree diseases are bacterial Leaf Scorch, fungal diseases (anthracnose, blumeriella, needle cast, tubakia) and Phytophthora root rot and canker diseases (Illinois University 1998). Theses pathogens affect various parts of the plant; root, stem, leaf and internal organs of the plant. As a result it leads the loss of urban tree canopy cover and health, and to shifts or loss of species that would diminish the quality of the urban environment and numerous ecosystem services derived from trees and forests. These potential changes could increase environmental management and human health costs, as well as decrease the quality of life of urban residents (Illinois University 1998).

Statistically sound data on the urban forest health and diversity are required to properly assess the magnitude of these benefits. To optimize forest benefits, information on costs associated with vegetation management should also be assessed. It is critical to assess the urban tree health and diversity for proper urban tree/forest planning to help sustain or enhance environmental quality and human health and well-being in cities.

Hawassa town being one of the fastest growing urban centers in Ethiopia and its economic growth and developmental activities such as building, road construction, private residence expansion and many other anthropogenic activities have been on steadily increasing. Consequently, the vegetation of the area has been under enormous human impact and may be on the decline. Moreover, the health condition of the existing urban tree found in different green infrastructures (GIs) of the city has not been assessed. Thus, it is imperative to assess the impact on tree species diversity and forest health of the city. Unfortunately, little is known about the urban tree disease. Results from this study will be used to advance the understanding of the urban tree disease and health, diversity, improves urban forest policies, planning and management, provide data for potential inclusion of trees within environmental regulations, and determine how trees affect the environment and consequently enhance human health and environmental quality in urban areas.

There are number of tree species diversity in Hawassa town planted as Urban Green Infrastructure such as *Grevillea robusta*, *Callistemon* species,"zenbaba", *Rosa chinensis, Azadirachta indica, Pinus patula, Hevea brasiliensis* (rubber tree), *Millettia ferruginea*, *Schinus molle* and *Cordia africanai.* In all the sampling sites *Grevillea robusta* is the most dominant tree species planted on the road side, home garden, and parks.

This study emphasizes on two different concepts i.e., implementation of a well thought out risk reduction strategy improves the overall health of the urban forest, which results in a safer urban environment. This goal is universal, regardless of national boundaries and documentation and implementation of tree risk management policies forms the foundation for a government agency's defense, if litigation ever occurred.

The objective of this research work was to isolate and identify the diversity of endophytic fungi associated with *Grevillea robusta* trees and to assess the severity and susceptibility of the tree in the city to plant pathogens in the study area. In addition, this research work was intended to know the major disturbances which cause adverse impacts on the urban trees health condition and to recommend an appropriate preventive mechanism of disease to improve the health condition of the urban forests.

2. MATERIALS AND METHODS

2.1 Study Area

The study was conducted in Hawassa town in different streets with different traffic flow. Hawassa is located in the southern part of the country on the shore of Lake Hawassa in the Great Rift Valley; 273 km south of Addis Ababa. Its geographical location is 703' latitude North and 380 28' longitudes East.

Hawassa town accommodate about eleven modern city buses, eighty minibuses, more than two thousand five hundred bajaj taxi and a varying number of old and new public and private cars visiting the city (http://www.hawassaonline.com/subpage.php?id=70).

2.2 Site selection and Study Design

To achieve the intended objectives, first preliminary survey was made to understand the overall condition of the urban forests of the city especially on *Grevillea robusta* and to select the appropriate study sites. Moreover, the pilot study plays a great role during the identification of diseased and healthy trees of the study area. The sampling sites were categorized in to five different streets characterized by having high and different traffic flow. These are the road from **Saint Gabriel Church** to **Piazza', South Star Hotel** to **Buss station, Bus station** to **Atote, Atote** to **Hawassa teachers teaching college (TTC),** main road from **Shashemene-Hawassa- Dilla.** In order to obtain more representative data the samples were collected from street median, walkway squares and private garden. Samples were collected purposively using systematically random sampling from diseased looking *Grevillea robusta* tree. When describing the study site in terms of car flow and road side plantation, the dominant tree species in the road side of all the straights was found to be *Grevillea robusta*. In all the sites the health condition of

Grevillea robusta was different i.e. in some place there was a sign of cholorosis and canker where as in other area the tree trunk is covered with lichen and mosses, which serve as an indicator in the level of environmental pollution. On the other hand the flow of the car in all the straights was apparently different i.e. relatively the highest car flow is found in the straight form South Star Hotel to Buss station and followed by Saint Gabriel Church to Piazza'.

2.3 Data Collection Methods

2.3.1. Field Assessment

Physical observation was made gently going through each sampling sites by looking each parts of the *G. robusta* tree planted as urban GIs. During the pilot study the tree were observed for defoliation of leaf, wilting and rising of gums on the sampled trees and which serve as in identifying a diseased. For the diseased trees, sample was taken from leaf of the plant, because it shows an apparent disease symptom as compared to other plant organs. The collected samples were brought to the laboratory by using sterilized polyethylene bag for isolation of the fungal endophytes and the possible plant pathogens.

2.3.2. Sample Inoculation and Culturing

The sample which was collected during sample collection was brought to the laboratory placed aseptically with an intermittent wetting till sample processing. A total of two trees per site and four (4) leaves per trees were taken from bottom, middle and top of the tree crown from each of the six sites. A single leaf was fragmented in to four pieces of each measuring 10mm. The leaf fragments were disinfected with 70% ethanol, placed in 33% hydrogen per oxide as for one minute and the fragments were washed five times serially with distilled sterilized water in order to remove the effect of ethanol and hydrogen per oxide. Finally, the fragments were blotted onto a sterilized tissue paper in an aseptic environment.

To isolate those endophytic fungi which inhabit the leaf of diseased *G. robusta,* sterilized four leaf pieces or fragments were inoculated on a 2% malt extract agar (OXOID) and the endophytes were allowed to grow on the plate in a temperature ranging from 25-27^{0}C for 5-7 days. After seven days of inoculation, the fungal endophytes and the possible pathogens were grown on the plate as a mixed culture. The mixed cultures were the purified separately onto malt extract agar and then the plates were placed inside an incubator with a temperature ranging from25-27^{0}C for 5-7 days.

2.3.3. Identification of the Pathogens

Identification of the endophytic fungi was made based upon cultural and microscopic (conidial) characteristics of the isolates. The cultural characteristics includes the color of the upper and reverse sides of the cultures, mycelial color formation, colony diameter, shape of colony margin, mycelial growth patterns such as aerial hyphae, appressed or submerged hyphae, formation of aerial hair-like tufts of hyphae were used to characterize the fungal endophytes.

Based on culture characteristics and spore morphology, the isolated endophytic fungi were categorized into morphotaxa identified to the genus level, while those which could be separated into distinct groups based on culture characteristics but could not be identified to any of the known genera were recognized as unidentified taxa. Lactophenol cotton blue staining solution was used for staining of non-pigmented fungal spore for microscopic examination purpose. Conidial morphological characteristics including shape and color of the spores, separation, presence or absence of specialized appendages on the spores were used to characterize the fungal structure.

In addition, different standard identification manuals were used to provisionally to identify the fungal isolates to the genus level. Whenever more than one morphological group occurs within a genus, they were designated as sp.1, 2, 3, etc, where sp., refers to species.

2.4. Data analysis and presentations

Data obtained from the research work was presented using tables as percentages. The isolation rate (IR), is a quotient calculated by dividing the number of isolates obtained from needle segments by the total number of needle segments incubated. This allows for the measurement of fungal species richness in a needle (Lv *et al.,* 2006). IR was calculated according to the equation below:-

$$IR = \frac{\text{Number of isolates obtained from plant segment}}{\text{Total number of segments incubated}}$$

The relative frequency (RF, expressed as a percentage) was calculated as the total number of isolate from a single taxa divided by the total number of isolates from taxa obtained from all tissue incubated (Lv *et al,* 2006). It was used to determine the most frequently isolated taxa among the rest of taxa.

$$RF = \frac{\text{Number of isolates of a taxon}}{\text{Total number of isolates of all taxa obtained from all incubated tissues}} \times 100$$

Species diversity

The Shannon diversity index (*H'*) was used to characterize species diversity in a community. Shannon's index accounts for both abundance and evenness of the species present. Shannon index of diversity was calculated to all factors which were assumed to have effect on the diversity of endophytic fungi. Here it was used to determine which location of the study area has higher endophytic fungi diversity. It is calculated as follows:-

$$H' = -\sum_{i=1}^{S}(p_i \ln p_i)$$

Species similarity

Sorensen Similarity index (*QS*): is a statistic used for comparing the similarity of two samples. It is calculated as follows:-

$$QS = \frac{2C}{A+B}$$

2.5 Methods of Data Analysis

Once the data is collected, relevant analysis tools notably descriptive statistics, inferential statistics and qualitative analysis will be used to generate the envisaged information with respect to the research objectives.

The endophytic fungal diversity which was collected from the trees was calculated using Shannon diversity index, which will be calculated as (Pielou, 1975):-

$$H' = -\sum_{i=1}^{S}(p_i \ln p_i)$$

3. RESULT AND DISCUSSION

A total of 387 endophytic fungal isolates were recovered from 192 leaf fragments collected from all sampling points having different traffic flow. The highest rate of endophytic fungal isolation is recorded on a street from South Star Hotel to Bus station which is found to be 3.81 (Table 1) this might be due to the poor health condition of the road side plantation caused by the pollution raised mass flow of cars on the straight. However, the lowest isolation rate of endophytic fungi was recorded on a street from Bus Station to Atote which was found to be 0.71 (Table 1). The lowest isolation rate on the road from Bus station to Atote might be due to the low traffic flow as compared to South Star Hotel to Bus station and Saint Gebriel to Piazza. The highest isolation rate of endophytic fungi in the two roads is highly dominated by prominent plant pathogens causing leaf spot, leaf chlorosis, canker and dieback disease in *G. robusta* and other trees species. When we observe the nature of the endophytes recovered the highest relative frequency (44.7 %) (Table 2) of the endophytes isolates was taken by *Pestalotiopisis* spp, which is a plant pathogen that predominantly affects leaf blade and petiole and followed by *Botryospheria* spp, which accounts with relative frequency of 35.05% and known in causing stem canker disease in plants.

Table 1: Isolation rate of endophytic fungal isolates from all sites

S. No	Name of Morphotaxa	St. Gabriel to Piazza	South Star Hotel-Bus Station	Buss station to Atote	Atote to TTC	Road to Shashemene to Dilla
1	*Pestalotiopsis spp1*	9	5	1	13	2
2	*Pestalotiopsis spp2*	33	13	7	32	12
3	*Pestalotiopsis spp3*	11	8	1	24	0
4	*Xyleria spp1*	0	2	3	0	0
5	*Xyleria spp2*	1	3	0	3	0
6	*Botryospheria spp1*	9	13	0	12	3
7	*Botryospheria spp2*	2	7	0	0	4
8	*Botryospheria spp3*	3	10	0	1	2
9	*Botryospheria spp4*	1	9	0	1	3
10	*Botryospheria spp5*	2	10	0	3	5
11	*Aspergillus spp1*	0	2	4	0	0
12	*Penicillium spp1*	0	4	2	0	3
13	*Fusarium spp1*	6	2	1	2	3
14	*Fusarium spp2*	2	3	0	1	0
15	*Fusarium spp3*	3	0	1	0	0
16	*Fusarium spp4*	5	0	0	0	0
17	*Phoma spp1*	1	5	1	0	0
18	*Phoma spp2*	1	2	1	0	0
19	*Phoma spp3*	2	3	0	0	0
20	*Phoma spp4*	1	1	0	0	0
21	*Unidentified spp1*	0	3	0	2	3
22	*Unidentified spp2*	2	2	1	0	1
23	*Unidentified spp3*	1	9	0	3	0
24	*Unidentified spp4*	1	2	0	0	0
25	*Unidentified spp5*	1	1	0	0	0
26	*Unidentified spp6*	5	3	0	1	1
	Total	102	122	23	98	42
	Isolation rate	3.1875	3.8125	0.71875	3.0625	1.3125

There is an indication that the tree is now facing stress from the polluted environmental condition caused by a polluted gas emitted from cars. Among this, as compared to those tress found in a relatively in the rural area, none of the trees found in the city are colonized by lichen species (indicators for environmental condition). Additionally, the two endophytic fungi with the highest relative frequency, which are *Pestalotiopsis spp* and *Botryosphaeria spp* are known in causing leaf spot, leaf chlorosis, canker and dieback disease when the plant suffers environmental stress (Njuguma, 2011).

Table 2: Relative frequency of endophytic fungal isolates from all sites

S.No	Name of Morphotaxa	Number of isolates	Relative frequency (%)
1	*Pestalotiopsis spp1*	30	7.75
2	*Pestalotiopsis spp2*	99	25.58
3	*Pestalotiopsis spp3*	44	11.37
4	*Xyleria spp1*	5	1.29
5	*Xyleria spp2*	7	1.8
6	*Botryospheria spp1*	38	9.82
7	*Botryospheria spp2*	10	4.67
8	*Botryospheria spp3*	18	6.07
9	*Botryospheria spp4*	14	5.14
10	*Botryospheria spp5*	20	9.35
11	*Aspergillus spp1*	6	2.8
12	*Penicillium spp1*	9	2.33
13	*Fusarium spp1*	14	3.61
14	*Fusarium spp2*	6	1.55
15	*Fusarium spp3*	2	0.516
16	*Fusarium spp4*	5	1.29
17	*Phoma spp1*	7	1.8
18	*Phoma spp2*	4	1
19	*Phoma spp3*	5	1.29
20	*Phoma spp4*	2	0.51
21	*Unidentified spp1*	8	2
22	*Unidentified spp2*	6	1.55
23	*Unidentified spp3*	13	3.3
24	*Unidentified spp4*	3	0.7
25	*Unidentified spp5*	2	0.51
26	*Unidentified spp6*	10	2.58
	Total	**387**	

Pestalotiposis spp2 is the dominant endophytic fungal isolates in terms of relative frequency and it accounts 25.58% of the endophytic fungal isolates followed by *Pestalotiopsis* spp3 which accounts 11.37% of the isolates. The previous fungal isolate is also isolated from all sampling sites. The endophytic fungal isolates with the smallest relative frequency is *Phoma* spp4 and unidentified spp5, each accounts for 0.51% of the total isolate.

The data shown in the figure below shows that the highest diversity of endophytic fungi is recorded on a street from South Star Hotel to Buss station, which is 3.01 where as the smallest diversity of endophytic fungi is recorded on a street from Atote to **T**eachers **T**eaching **C**ollege, which is 1.79 however the data is not statistically significant (Fig. 1).

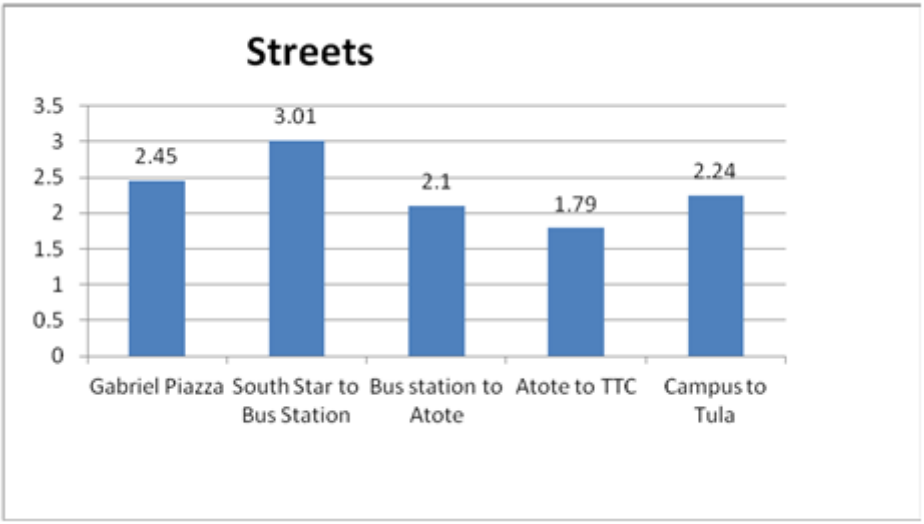

Figure 1: Graph showing diversity endophytic fungi from different sites with different traffic flow

Appendix 1: Diversity of endophytic fungi isolated from *Grevillea robusta* across the five streets

S.No	Endophytic fungal isolates	St. Gabriel to Piazza	pi*lnpi	South Star Hotel-Bus Station	pi*lnpi	Buss station to Atote	pi*lnpi	Atote to TTC	pi*lnpi	Road to Shashemene to Dilla	pi*lnpi
1	*Pestalotiopsis spp1*	9	-0.21421308	5	0.130925538	1	0.136325835	13	-0.267961588	2	0.1449773
2	*Pestalotiopsis spp2*	33	-0.365091699	13	0.238589606	7	0.362047325	32	-0.365463372	12	0.3579323
3	*Pestalotiopsis spp3*	11	-0.240175029	8	0.178660951	1	0.136325835	24	-0.344550281	0	0
4	*Xyleria spp1*	0	0	2	0.067391375	3	0.265680251	0	0	0	0
5	*Xyleria spp2*	1	-0.045342871	3	0.091116609	0	0	3	-0.106725159	0	0
6	*Botryospheria spp1*	9	-0.21421308	13	0.238589606	0	0	12	-0.257150306	3	0.1885041
7	*Botryospheria spp2*	2	-0.07709462	7	0.163989969	0	0	0	0	4	0.2239405
8	*Botryospheria spp3*	3	-0.103716486	10	0.205035734	0	0	1	-0.046785382	2	0.1449773
9	*Botryospheria spp4*	1	-0.045342871	9	0.192304657	0	0	1	-0.046785382	3	0.1885041
10	*Botryospheria spp5*	2	-0.07709462	10	0.205035734	0	0	3	0	5	0.2533609
11	*Aspergillus spp1*	0	0	2	0.067391375	4	0.30420867	0	0	0	0
12	*Penicillium spp1*	0	0	4	0.112056613	2	0.212378003	0	0	3	0.1885041
13	*Fusarium spp1*	6	-0.166659608	2	0.067391375	1	0.136325835	2	-0.079424904	3	0.1885041
14	*Fusarium spp2*	2	-0.07709462	3	0.091116609	0	0	1	-0.046785382	0	0
15	*Fusarium spp3*	3	-0.103716486	0	0	1	0.136325835	0	0	0	0
16	*Fusarium spp4*	5	-0.147820338	0	0	0	0	0	0	0	0
17	*Phoma spp1*	1	-0.045342871	5	0.130925538	1	0.136325835	0	0	0	0
18	*Phoma spp2*	1	-0.045342871	2	0.067391375	1	0.136325835	0	0	0	0
19	*Phoma spp3*	2	-0.07709462	3	0.091116609	0	0	0	0	0	0
20	*Phoma spp4*	1	-0.045342871	1	0.046051702	0	0	0	0	0	0
21	*Unidentified spp1*	0	0	3	0.105196737	0	0	2	-0.079424904	3	0.1885041
22	*Unidentified spp2*	2	-0.07709462	2	0.07824046	1	0.136325835	0	0	1	0.0889921
23	*Unidentified spp3*	1	-0.045342871	9	0.216715105	0	0	3	-0.106725159	0	0
24	*Unidentified spp4*	1	-0.045342871	2	0.07824046	0	0	0	0	0	0
25	*Unidentified spp5*	1	-0.045342871	1	0.046051702	0	0	0	0	0	0
26	*Unidentified spp6*	5	-0.147820338	3	0.105196737	0	0	1	-0.046785382	1	0.0889921
	Total	102		122		23		98		42	
	H'		**2.451642211**		**3.014722175**		**2.098595098**		**-1.794567202**		**2.245693**

Appendix 2: Isolation rate of endophytic fungal isolates from all sites

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3	*Pestalotiopsis spp3*	11	8	1	24	0
4	*Xyleria spp1*	0	2	3	0	0
5	*Xyleria spp2*	1	3	0	3	0
6	*Botryospheria spp1*	9	13	0	12	3
7	*Botryospheria spp2*	2	7	0	0	4
8	*Botryospheria spp3*	3	10	0	1	2
9	*Botryospheria spp4*	1	9	0	1	3
10	*Botryospheria spp5*	2	10	0	3	5
11	*Aspergillus spp1*	0	2	4	0	0
12	*Penicillium spp1*	0	4	2	0	3
13	*Fusarium spp1*	6	2	1	2	3
14	*Fusarium spp2*	2	3	0	1	0
15	*Fusarium spp3*	3	0	1	0	0
16	*Fusarium spp4*	5	0	0	0	0
17	*Phoma spp1*	1	5	1	0	0
18	*Phoma spp2*	1	2	1	0	0
19	*Phoma spp3*	2	3	0	0	0
20	*Phoma spp4*	1	1	0	0	0
21	*Unidentified spp1*	0	3	0	2	3
22	*Unidentified spp2*	2	2	1	0	1
23	*Unidentified spp3*	1	9	0	3	0
24	*Unidentified spp4*	1	2	0	0	0
25	*Unidentified spp5*	1	1	0	0	0
26	*Unidentified spp6*	5	3	0	1	1
	Total	102	122	23	98	42
	Isolation rate	3.1875	3.8125	0.71875	3.0625	1.3125

Authors' contributions

The first and corresponding author (Mr. Getachew Birhanu) plays the leading role in coordinating the research work whereas the second (Dr. Abdella Gure) and the third (Mr. Tikabo Gebreyesus) author participated in the research work equally with the first author.

Acknowledgment

We, the authors, want to thank the Hawassa University for financing the Research work. We exclusively want to thank Wondo Genet College of Forestry and Natural Resource, Hawassa University for funding the research work and host the laboratory activity.

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