

# Morphological Characterization of Fungal Disease on Tapped *Boswellia papyrifera* Trees in Metema and Humera Districts, Northern Ethiopia

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## አህፅርአት

የአጣን ዛፍ (*Boswellia papyrifera*) በአማራ፣ በትግራይ እና በቤንሻንጉል ጉምዝ ክልሎች በብዛት የሚገኝ የዛፍ ዓይነት ሲሆን ይህ ዛፍ የአጣን ምርትን ለማምረት ያገለግላል። አጣንን ለማምረት ዛፉን በየጊዜው በተደጋጋሚ የማድማትና ቀስ በቀስ የደማውን ቦታ ስፋትና ቁመቱ እንዲጨምር የማደረግና በተጨማሪ በሌላ በአዲስ ቦታ ላይ በተመሳሳይ ሁኔታ እንዲደማ ማድረግ ያስፈልጋል። በመተማ እና በሁመራ አካባቢ ዛፍን በተለያዩ ጊዜ እና ቦታ በተደጋጋሚ እንዲደማ ማድረግ በዛፉ ደህንነት ላይ የሚያስከትለው ጉዳት ማጥናትን ዓላማው ያደረገ ምርምር ተካሂዶ ነበር። በተከታታይ እና በተደጋጋሚ የማድማት ሥራ በተካሄደባቸው በብዙ ዛፎች ላይ የአብጠት (Galls) ምልክት ታይቶባቸዋል። እዚህ ቦታ ላይ ቅርፊቱ ልጦ በማገሳት ግንደ(እንጨቱ) ሲታይ እንጨቱ ቀለሙን ቀይሮ የመቁሰል ምልክት ታይቶቦታል። ከዚህ እንደ መቁሰል ካለው እንጨት ጫፍ ላይ ናሙና በመውሰድ በተሰማሚ ማሳደጊያ ላይ እንዲያደግ በማድረግ የችግሩ መንስኤ ምን እንደሆነ ለማወቅ ጥናት ተካሂዷል። በተደረገው ጥናትም የበሽታ ምልክት ካለበት ቦታ የተወሰደው ናሙና አድገት ሲታይ በመጀመሪያ ነጭ ቀለም እያደገ ሲሄድ ግን ቀለሙ ጥቁር የሆነ ፈንገስ አድጎ ታይቷል። የዚህ ፈንገስ መሪቢያ ዘር የሆነው (spore) ላይ በተደረገው ጥናት ደግሞ ሁለቱ ጫፎቹ ሞላል ያሉ ሆነ አቫይድ (ovoid) መሳይ ቅርጽ ያለው ሲሆን ወገቡ ላይ መሀል ለመሀል ለሁለት ሚክሩለው መስመር አለው። ይህ ፈንገስ ሲያደግ ያሳየው የቀለሙ ባህሪ ሁኔታና የመሪቢያ አካሉ (spore) ቅርጽ፣ ቁመቱና ስፋቱ ባህሪ ላሲዮዲፕሎዲያ ቲዮብሮሜ ከተባለው ፈንገስ ጋር አንድ ዓይነት/ተቀራራቢ ሆኖ ተገኝቷል። በመሆኑም በአጣን ዛፍ ላይ ማድባት በተደረገበት ቦታ አብጠት ካለበት ቦታ የተገኘው በሽታ ላሲዮዲፕሎዲያ ቲዮብሮሜ የተባለ ግንድን የሚያቆሰል ፈንገስ አማካይነት የሚመጣ በሽታ እንደሆነ ለማወቅ ተችሏል። በላቦራቶሪ በትንሽ ሳህን (Petri dish) ላይ ያደገውን ይህን ፈንገስ የተመረጡ ጤናማ ዛፎችን በመውጋት በዛፉ ላይ በሽታ ያስከትል እንደሆነ ለማወቅ በተደረገው ጥናት በፈንገሱ የተወጋው ዛፍ ተመሳሳይ የበሽታ ምልክት ታይቷል። በመሆኑም ይህ ፈንገስ በአጣን ዛፍ ላይ በሽታን የሚያስከትል መሆኑን ለማወቅ ተችሏል።

## Abstract

*Boswellia papyrifera* is a tree species which is found in Amhara, Tigray, and Benshangul Gumuze region and used for the production of frankincense. Frequent tapping at different rounds and at different position of the tree is made to produce frankincense. A study aiming at evaluating the health status of tapped *Boswellia papyrifera* was carried out on samples obtained from Metema and Humera. Galls were commonly observed on most tapped *Boswellia* trees and a black wood discoloration was found on the wood beneath these galls. Isolation made from the discolored part of the wood resulted in growth of fungal colonies with a white to gray fluffy aerial mycelia which later changed into blackish mycelial growth as it matures. The spore of the fungus has hyaline, ellipsoid to ovoid shaped and it has one septa. The spore has a rounded base and truncate apex. The color, the shape of the colony and the characteristics of the conidia is found to be identical with description of *Lasiodiplodia theobromae*. Hence, based on the morphological characteristics of the colony and the spore, the fungus isolated from symptomatic trees, is tentatively identified as *Lasiodiplodia theobromae*. Molecular characterization is essential to confirm the identity of the fungus. The pathogenicity test resulted into development of lesion depicting that the fungi isolated from *Boswellia papyrifera* can cause disease on the tree.

**Keywords:** *Boswellia*, Incense production, *Lasiodiplodia*, Pathogen, Tapping

## Introduction

Dry forests, forests found in dry areas, are characterized by long dry period and low water availability (Murphy and Lugo 1986). These dry forests constitute a unique biodiversity and it harbors diverse valuable resources of economic importance (Timmermann and Hoffmann 1985). The Acacia-Comiphora woodlands are among the dry forest vegetation types found in Africa. These forests constitute diverse species of *Acacia*, *Boswellia* and *Comiphora* tree species and these tree species are known to yield commercial gums and resins (Abiyu *et al.* 2010). *Boswellia papyrifera* (Del.) Hochest belongs to Burseraceae family. This specie is widely distributed in Africa and it is found in Ethiopia, Nigeria, Cameroon, Central African Republic, Chad, Sudan, Uganda and Eretria (Friis, 1996; Lovett and White 1983; Friis 1991; Vollesen 1989).

In Ethiopia, *Boswellia papyrifera* is found in the Acacia-Comiphora wood lands of Amhara and Tigray, (Vollesen 1989). It also occurs in Oromia, Benshangul and SNNP Regions. It grows on degraded sites with very shallow soils, steep rocky slopes, lava flows or sandy river valleys with in an altitudinal range of 950-1800 masl (Fichtl and Admasu 1994). This tree species is among the economically important trees and it is widely used for incense production (Lemenih *et al.*, 2003). In Ethiopia, for example, more than 80% of the exported gum and resin products come from *Boswellia papyrifera* (Eshete 2002, Tadesse *et al.*, 2007).

As in many gum and resin producing trees, gum and resin production is induced as a response to wounding made on the bole of the tree (Langenheim 2003). Similarly, the process of gum and resin production activity in Ethiopia involves tapping, where a piece of the bark is removed from the bole of the tree. Tapping is a technique used for harvesting of frankincense, has been practiced since ancient times (Groom 1981). Tapping is a cyclic practice repeatedly applied every 15 to 20 days after the first tapping. During these subsequent tapping the wound made on the stem is refreshed and widened by removing more bark at the upper edge of the previous tapping spot. Similarly, the numbers of the tapping spot on a tree various between 6-16 spots depending on the size of the tree (Abeje *et al.*, 2011, Gebrehiwote 2003).

The frequent and intensive wounding could affect the normal growth and reproduction of this tree species. For example, a high incidence of insect attack and high proportion of aborted seeds, seeds without embryo have been observed in seeds from heavily taped trees and old trees (Ogbagize et al 2006, Rijkers et al. 2006, Nigussie 2008). However, there is no information on the prevalence of pathogenic infection on these tapped trees. In several circumstances where trees are subjected to continuous wounding, the wounding provide avenue for microbial infection and the plants become highly prone to disease infestation. This study is therefore, conducted to investigate pathogens associated with tapped *Boswellia* trees.

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## Materials and Methods

### The study sites

The study was conducted in Metema and Humera areas situated in northwest and northern part of Ethiopia. These two sites are among the sites where vast areas of natural *Boswellia* stand are found and these sites are known for the large quantity incense production. Metema, one of the study sites is situated in North Gonder, Amhara Regional State, Ethiopia. Its altitudinal range varies between 600-1200 masl. The mean annual rainfall of Metema ranged between 870-1390mm. The diurnal minimum and maximum temperature is 19.6°C and 37.5°C respectively (Eshete *et al.*, 2011). Humera, the other study site, is situated in western parts of Tigray. The altitude of this site ranges between 560 -1849 masl. The mean annual rain fall of Humera is about 581.2mm and the temperature is in ranges between 20°C to 41°C (Sertse 2003).

### Sample Collection and Isolation

Prior to sample collection, attempts were made to identify clear external symptoms and sign of infection and infestation on part of the tree. Samples were then collected from the symptomatic part of *Boswellia* trees and taken from tapped trees. The pieces of wood samples were collected from different symptomatic trees and kept separately in paper bags.

Isolation was made from sample collected from different parts of the tree with disease symptom. Two different isolation techniques were used. This involves, direct transfer of sterilized symptomatic wood pieces (2-3mm) on Malt Extract agar. Incubating the pieces of wood (5mm) with symptoms of infection on moist chamber is the other isolation technique used. A 9cm diameter Petri dish containing moisten tissue paper was incubated at room temperature and allowed to grow for a week is used. Malt extract agar (MEA, Oxid Ltd. Basingstoke, Hants England) containing 30g Malt extract, 15g Agar and 5g peptone, is the growth media used for fungal isolation. The MEA used for the isolation was treated with streptomycin to minimize bacterial contamination. Small plug of resultant mycelial growing on symptomatic wood on MEA were then transferred onto MEA growth medium. Similarly, the fruiting structures grown on the piece of woods were picked up with needle and transferred onto MEA. These isolates were incubated at room temperature. Following this, segments of the resultant mycelial growth were again transferred onto new media (MEA) to get pure culture to be used for further study. All fungal isolates were kept in fridge where the temperature is adjusted to about 5°C. A Piece of fungal mycelial from edge of clean colony was transferred on to Water Agare (WA) containing three to four sterilized pine needle to initiate spore production (Alemu *et al* 2004; Ismael *et al.* 2012)..

## Morphological characterization of the fungi

The color of the colony growth on MEA, both at the top and bottom of the Petri dish was used to detect the variation between different cultures. For this purpose, the color of all isolates obtained from all sample trees were evaluated and compared.

In addition, spore morphology, color, size, septation and shape of spores were also used to characterize the fungal isolates. Representative sample isolates obtained from the different study sites were selected and used for spore characterization. The spores used for this purpose were obtained from the pycnidia produced on moist chamber, spores produced on the colony growing on MEA and the spore produced on Water Agar (WA) that contained sterilized pine needle. It was incubated for 10 days at room temperature. The spores obtained from different sources were placed on slide and the shape and size of conidia were evaluated under microscope (ASK-ANIA, RML 5, Germany). The lens magnification range was set on 40x magnification and the eyepiece was set at 10x magnification capacity. The lengths and width of the spores were measured with ruler mounted on the eyepiece of the microscope (Alemu et al 2004; Ismael et al. 2012).

Fungal classification key was used to determine the identity of the fungi isolated from *B. papyrifera* trees and the colony and spore characteristics of these isolates were compared with the characteristics of its close relative (Alemu et al 2004; Ismael et al. 2012).

### Pathogenicity test

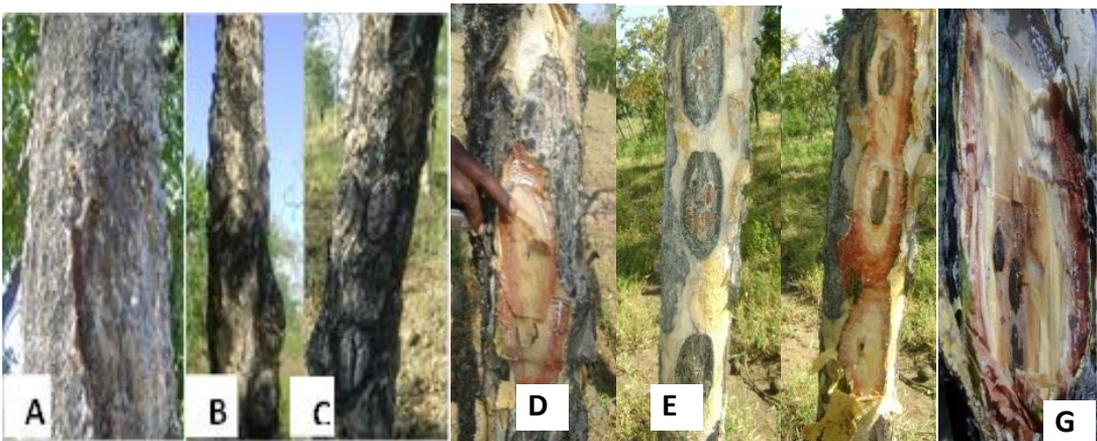
Inoculation test was conducted on branches of big and healthy looking *Boswellia* trees to evaluate if the fungal isolate obtained from symptomatic trees is pathogenic on *B. papyrifera* trees. For this purpose five randomly selected isolates, two isolates (Agam Wuha W 4 and Gubay 5) obtained from Metema and three isolates (Ayay T1, Medeb MT2 and Humera HC) obtained from Humera were used in the inoculation test. The isolates used for inoculation were grown for one week on 9cm Petri dish containing MEA. In this inoculation test, trees inoculated with a pure growth media that only contained MEA was used as control. The inoculation was carried out on healthy 10 arbitrarily selected branches of *Boswellia* trees.

A 5 mm bark borer was used to prepare the pieces of mycelial plug to be inoculated on the branches of the selected trees. The same bark borer was used to make the hole by removing the barks from the branches. Sterilized scapula was used to pick up the mycelial plugs and insert it into each of the hole opened on each branch. After inoculation with the mycelial plugs, the holes were covered with Para film to prevent desiccation and contamination. The inoculation test was made around Agam Wuha, Metema. After three month the lesion development on inoculated branches was evaluated and the length of the lesion was measured after three months of inoculation (Alemu et al 2004; Ismael et al. 2012).

## Results and Discussion

This study demonstrated that all tapped *Boswellia papyrifera* trees are subjected to infection from pathogenic fungi. The occurrence of symptoms of infection is associated with wound made during tapping. Most tapped *Boswellia* trees have been subjected to frequent and intensive tapping (Abeje *et al.*, 2011, Gebrehiwot 2003). This traditional tapping resulted into wounding of trees and exposes the trees to attacks from insects, fungi bacteria and virus (Trapp and Croteau 2001, Rijkers *et al.* 2006, Nigussie 2008, Abiyu *et al.* 2010). It has been reported that tapping for incenses has a negative impact on the survival and growth rate as well as the reproduction of the tree (Rijkers *et al.* 2006). Disease symptoms including canker formation, exudation of gum, wilting, dieback, vascular browning and death of tree has been observed on *Boswellia* trees (Alemu *et al.*, 2014).

Most *Boswellia* trees in the study areas have been repeatedly tapped at different direction and positions for incense production. Therefore, it is common to find a swelling on almost all taped trees at taping spot, which gradually developed into gall like structures (Fig. 1B, C, D). The number and size of the galls formed on *Boswellia* trees varied from tree to tree and the size of the tree. When the galls are removed by chopping in to the wood, beneath the galls, it was common to find blackish or chocolate like discoloration on the stem. This signifies that the galls and wood discoloration beneath it are good symptoms of infection. According to Short and Castner (2005), galls occur on a wide variety of plants and it can be caused by fungi, bacteria, nematodes or mites.



**Figure 1.** Disease symptoms (A) Excessive whitish resin drops all over the stem, (B) Stem malformation at spot of tapping, (C) Formation of swelling that develop in to gall (D) Color of wood of normal wood (E) Taping scar (F) Lesions developed at different tapping positions (G) Extent of lesion on the sap wood

### Growth characteristics of the isolates

The isolation made from each symptomatic sample tree using different isolation methods has resulted into a colony that has identical growth characteristics. At initial stage, the colony has whitish and sparsely distributed mycelial growth. However, as it grew older

the color of the mycelia changes into black and it densely covered the whole surface of the Petri dish. The color of the colony can be described as grey to black color at the top and blackish at the reverse side of the Petri dish (Figure 2A).

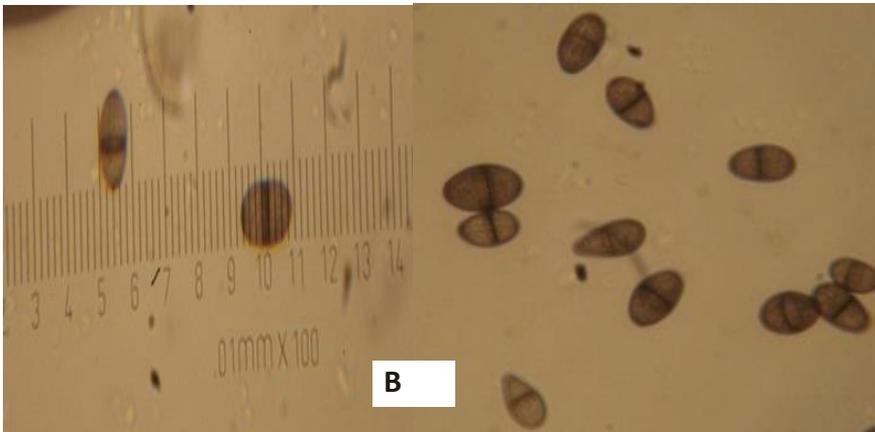
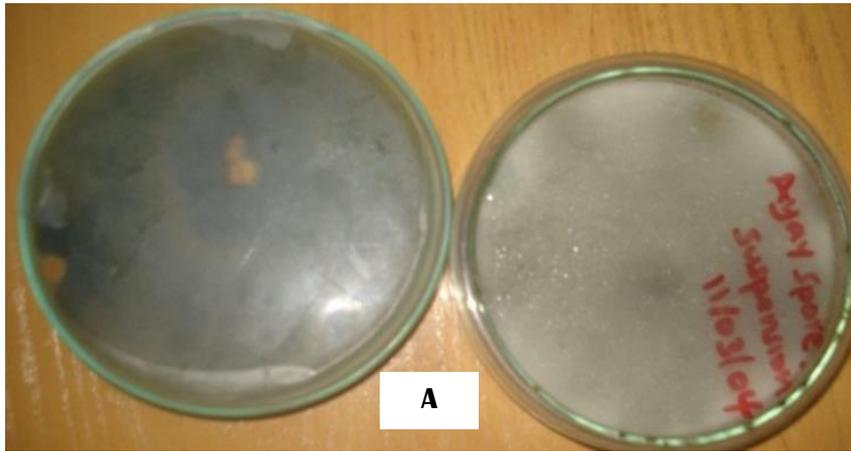


Figure 2. (A) Mycelial growth on Petri dish and (B) Spore of the fungi

**Table 1.** The average dimension of the conidia

Sample Source	Sample ID*	Length (L)**	Width (W)**	Ratio (L/W)
<i>Humera</i>	Humera A	7	5	1.40
	Humera TB	7.6	4.8	1.58
	Medeb 2	7.9	5	1.58
	Ayay T4A	8	4.9	1.63
	Ayay T3	8.1	5	1.62
	Ayay T5	8.4	5.1	1.65
<i>Metema</i>	Lt T3B	7.5	4.7	1.60
	Lt T2	8.8	5.2	1.69
	Gubay 4	7.8	5.4	1.44
	Gubay 8	10.3	5.8	1.78

\* The samples were obtained from different trees.

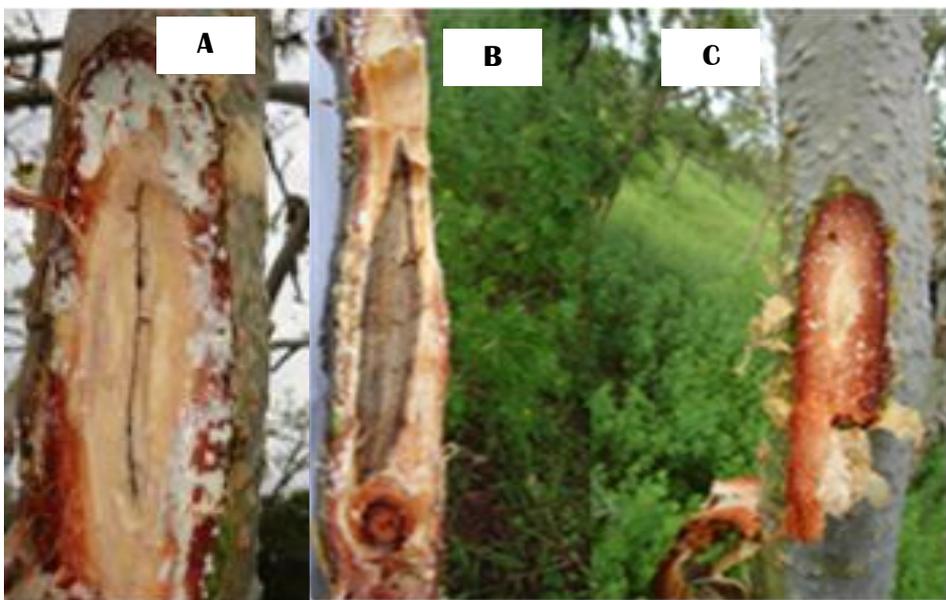
\*\* Mean length and width of 10 randomly selected spores

Spores were obtained from pieces of wood incubated in moist chamber, from mycelial plug inoculated on pure Water Agar (WA) and mycelial plug inoculated on WA containing sterilized pine needle. The spores obtained from all these sources exhibited similar characteristics. These spores are identical in shape and spore size (Figure 3). The spores have oval shape and it comprises of spore with septa that is thick walled and the conidia have granules on its surface (Alemu *et al* 2014). The average dimension of the spore is given in table 1. The length of the conidia varied from  $7\mu$  to  $10.3\mu$  and its width is between  $4.7\mu$  and  $5.8\mu$  and the length to width ratio is in a range of  $1.4\mu$  to  $1.78\mu$ . The characteristics of the isolates including the morphology of the colony and morphology of the spore resemble to the description of *Lasiodiplodia theobromae* (Slippers & Wingfield 2007, Rubini *et al* 2005, Mohali *et al* 2005).

*L. theobromae* is a cosmopolitan fungus that occurs on a wide range of monocotyledonous, dicotyledonous and gymnosperm hosts (von Arx & Müller 1954, Barr 1987). Several of the *Lasiodiplodia* species are commonly found in tropical and subtropical regions. These group of fungi are associated with various symptoms of plant disease such as shoot blights, stem cankers, fruit rots, root rots, dieback and gummosis (von Arx 1987) and they are also known as endophytes (Slippers & Wingfield 2007, Rubini *et al* 2005, Mohali *et al.*, 2005). Based on the results of morphological characterization, it appears that the fungus found associated with symptoms of stem canker of tapped *Boswellia* tree seemed to be similar with that of *Lasiodiplodia theobromae*.

### Pathogenicity test

The inoculation test revealed that the fungus had caused infection on inoculated *Boswellia* trees. The average lesion length differed between 14.3cm and 15.7cm. No lesion was found on the branch inoculated with the control. This might imply that *Lasiodiplodia theobromae* is pathogenic to this tree species (Table 2).



**Figure 3.** (A) Lesion developed on inoculated trees, (B) Lesion developed on the cambium, (C) stem inoculated with the control.

Table 2. Lesion development to inoculated *Boswellia* trees

Tree No	Tree DBH	Mean Branch Diameter	Length of Lesion (cm)					
			Control	Agame Wuha 4	Gubay 5	Ayay 1	Medeb 2	Humera C
1	22.6	11.7	0	18	15	21	12	22.5
2	28.9	10.25	0	17	13	16	15.4	14.4
3	15	6.9	0	15.7	14.7	14.3	14.2	14.5
4	17.4	9.6	0	21	15	14	19	15.5
5	20.5	9.9	0	10	8	12	5	12
6	24.6	12.05	0	15.5	15	14	15	13
7	15.2	8	0	11.6	18	18	17	14.5
8	24.6	10.95	0 <sup>l</sup>	16 <sup>l</sup>	9	10	12.5	17.5
9	15.5	7.4	0	14	14.5	14.5	19.5	12
10	15.9	8.05	1.5	18	24	9.2	17.5	9
Sum			1.5	140.8	146.2	143	147.1	144.9
Mean			0.15	14.08	14.62	14.3	14.71	14.49
Stdv				3.64	4.72	3.73	4.49	3.86

### I = Insect tunnel had developed at the point of inoculation of the control

This fungal species is a cosmopolitan fungus that occurs on a wide range of monocotyledonous, dicotyledonous and gymnosperm hosts (von Arx & Müller 1954, Barr 1987). Several of the *Lasiodiplodia* species are commonly found in tropical and subtropical regions. These group of fungi are found associated with various symptoms of plant disease such as shoot blights, stem cankers, fruit rots, root rots, dieback and gummosis (von Arx 1987) and they are also known as endophytes (Slippers & Wingfield 2007, Rubini et al 2005, Mohali et al 2005).

Based on this study, it could be inferred that *Lasiodiplodia* is a cause of stem canker on the *Boswellia* trees. Hence, it is essential to develop sustainable production system where trees are allowed longer resting period to ensure the tree recover or heal the wounding trauma from previous years wounding. Eshete *et al.*, (2011) indicated that trees tapped from the smallest diameter class gave lower amount of frankincense. Hence, determining critical minimum diameter of the tree to be tapped to minimize the damage on small diameter class could reduce mortality of adult trees and sustain the production of frankincense. It is also important to look for other alternative incense production options and consider protecting wounds from direct exposure to insect and fungal infection

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