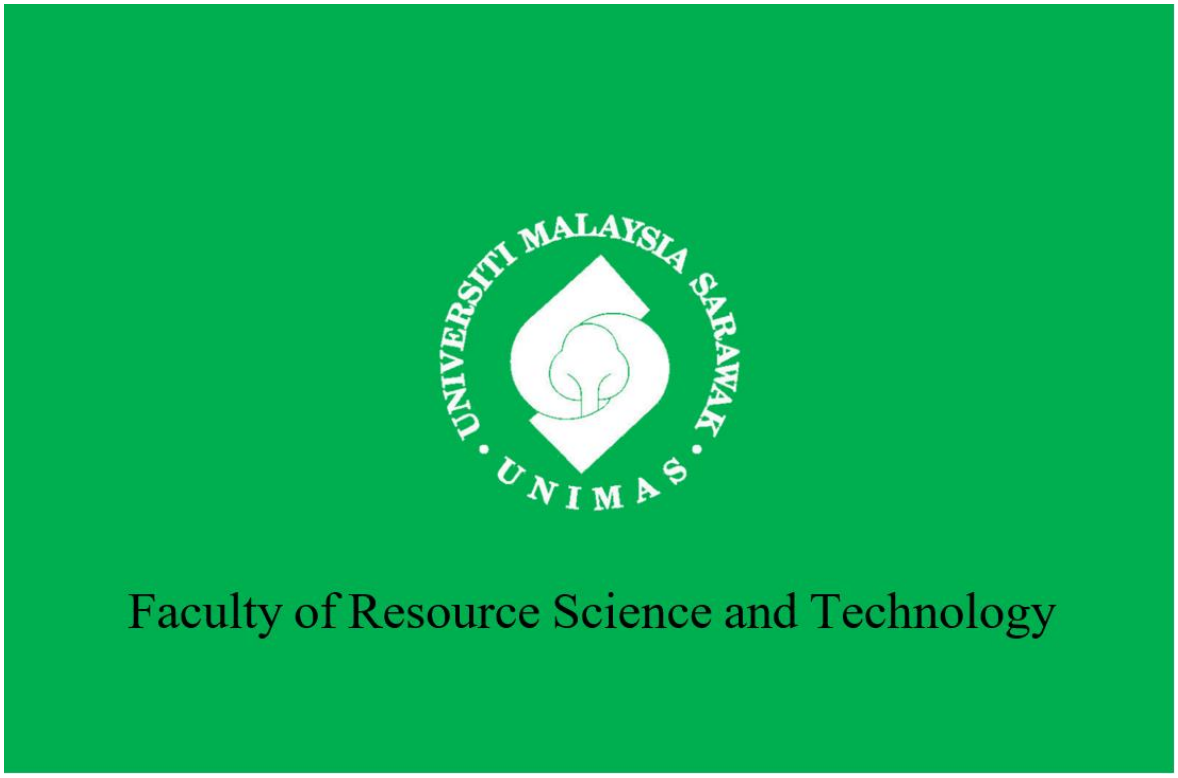

Isolation and Characterization Plant Pathogenic Fungi of Citrus Plant

Ain Nur Fitri Binti Mohammad Mahathir (68961)

Bachelor of Science with Honours
(Resource Biotechnology)
Year 2022

Isolation and Characterization Plant Pathogenic Fungi of Citrus Plant

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A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours
(Resource Biotechnology)

SUPERVISOR: DR. NGIENG NGUI SING

Programme of Resource Biotechnology
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UNIVERSITI MALAYSIA SARAWAK
2022

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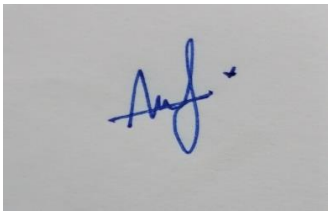

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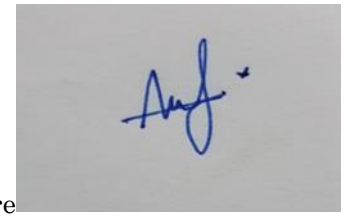
Student's signature  Supervisor's signature: simon

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ISOLATION AND CHARACTERIZATION PLANT PATHOGENIC FUNGI OF CITRUS PLANT

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ABSTRACT

Citrus plants are one of the essential crops that have a high demand in industrial commerce, especially in the Mediterranean climatic region and East Asia, including Malaysia. It has the pros and cons of profit-making citrus products as it has limited commercial field in some countries due to the less efficiency of conventional plant stock production methods. Malaysia is one of the countries affected as it has less production of citrus plants and causing our country to import more citrus products from other regions which costs twice compared to self-production market price. This is because one of the constraints faced by us is diseases that infect citrus plants that are caused by pathogenic fungus. The goal of this research is to isolate and characterize pathogenic fungus from *Citrus aurantifolia* and *Citrus limon*. Pathogenic fungi were isolated from infected plant tissue by culturing it on agar media. The pathogenicity of the isolated pure culture was tested, and the expected result which are several different pathogenic fungi of Citrus were isolated and characterize upon successful completion of this study. As a result, various types of plant pathogens are found after being categorized by experimental techniques in the laboratory.

Keywords:Citrus plant, Pathogenic fungus, Plant pathogen, Isolation and characterization

ABSTRAK

Tanaman sitrus merupakan salah satu tanaman penting yang mempunyai permintaan tinggi dalam perdagangan perindustrian terutamanya di kawasan iklim Mediterranean dan Asia Timur termasuk Malaysia. Ia mempunyai kebaikan dan keburukan produk sitrus yang mengaut keuntungan kerana ia mempunyai bidang komersil yang terhad di sesetengah negara kerana kaedah pengeluaran stok tumbuhan konvensional yang kurang cekap. Malaysia merupakan antara negara yang terjejas kerana kurang pengeluaran tanaman sitrus dan menyebabkan negara kita mengimport lebih banyak produk sitrus dari wilayah lain yang kosnya dua kali ganda berbanding harga pasaran pengeluaran sendiri. Ini kerana salah satu kekangan yang dihadapi oleh kita adalah penyakit yang menjangkiti tumbuhan sitrus yang disebabkan oleh kulat patogen. Matlamat penyelidikan ini adalah untuk mengasingkan dan mencirikan kulat patogen daripada Citrus aurantifolia dan Citrus limon. Kulat patogen telah diasingkan daripada tisu tumbuhan yang dijangkiti dengan mengkulturkannya pada media agar. Kepatogenan kultur tulen terpencil telah diuji, dan dijangka beberapa kulat patogenik Citrus yang berbeza telah diasingkan dan dicirikan apabila berjaya menyiapkan kajian ini. Hasilnya, pelbagai jenis patogen tumbuhan ditemui setelah dikategorikan oleh teknik eksperimen di makmal.

***Kata kunci**: Tumbuhan sitrus, Kulat patogen, Patogen tumbuhan, Pengasingan dan pencirian*

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
°C	Celcius
C. aurantifolia	*Citrus aurantifolia*
C. limon	*Citrus limon*
EDTA	Ethylenediaminetetracetic
g	Gram
ITS	Internal Transcribed Spaces
ITS-1F	Internal Transcribed Spaces Forward
ITS-1R	Internal Transcribed Spaces Reverse
kb	Kilobyte
mm	Millimeter
mL	Millimeter
NaCl	Sodium Chloride
PDA	Potato Dextrose Agar
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
rpm	Revolutions per minute
rRNA	Ribosomal RNA
Tris-HCl	Tris Hydrochloride
Tris-EDTA	Ethylenediamine Tetraacetic Acid
UV	Ultraviolet
%	Percentage
µl	Microlitre

CHAPTER 1: INTRODUCTION

1.1 Research Background

Pathogenic fungus are fungi that cause diseases in other living organism including human. In a brief, emerging fungal infections are associated with different pattern, pathogens, and case (Friedman & Schwartz, 2019). According to Doehlemann (2017), pathogenic fungi use variety strategies to conquer a plant and develop a diseases. It is also one of the most common pathogens in plants and identified as a major issue to plant-based food security (Doehlemann et al., 2017). Besides, plant pathogen lifespan is depending on the fungal host plant availability. Fungal pathogens survive in nature which usually in soil, inside or on the plant surface as they tend to grow along plant growing stages. Pathogenic fungus lives in a variety of ways. There are hemibiotrophic, biotrophic, or obligately biotrophic, while some are necrotrophic. Despite clear variations in lifestyle, fungal pathogens are known to utilise highly conserved proteins in infection mechanisms. As a result, the conserved proteins are promising targets for treating these fungal infections(Yang et al., 2017).Fungi are non-chlorophytic and eukaryote organism and true fungi consist of branching and filamentous structure. The majority of the over 100,000 fungus species are saprophytes. As stated by United States Environmental Protection Agency (USEPA), there are over 20,000 species of fungi that can threaten crops and plants by acting as parasites and cause a disease. By far the most common plant pathogenic organism is a fungus parasite. All plants are affected by one or more phytopathogenic fungus. Individual fungus species can parasitize one or more plant species (West, 2014).

1.2 Problem Statement

Citrus plant is believe originated from few regions such as Northeast India, Indonesia, and South China. Citrus plant is belonged to Rutaceae plant family which have various

species. Citrus plant is known as one of essential fruit crops in worldwide due to the high commercial value (Shokrollah hajivand et al., 2009). *Citrus limon* (lemon), *Citrus aurantifolia* (Kasturi lime), *Citrus reticulata* (honey orange) and *Citrus maxima* (pummelo) are common citrus plant in Malaysia. Meanwhile other citrus fruit likes *Citrus tangerina* (tangerine) and *Citrus* × *paradisi* (grapefruit) are imported from other country. As citrus plant has a high range of consumers, the commercial planting of citrus in Malaysia has its difficulties and benefits. Its difficulties are primarily caused by disease infections. Fungal pathogen is one of common disease occurred in citrus plant. In Malaysia, there are at least six fungal diseases, one viral disease and two bacterial diseases (Eng,2013). Therefore, a fundamental study of plant pathogenic fungi of citrus plant will give more details on its future impacts. This approach will help to ensure a solution for citrus plant crop disease issues.

1.3 Objectives

This research will focus on the discovery of pathogenic fungus on citrus plants as the main cause of diseases in *Citrus aurantifolia* and *Citrus limon*. The center will be to detect the presence of pathogenic fungi in *Citrus aurantifolia* and *Citrus limon*. Thus, the research's objectives are:

1. To isolate different type of pathogenic fungi from *Citrus aurantifolia* and *Citrus limon*
2. To perform pathogenicity testing for the isolated fungi
3. To characterize the isolated pathogenic fungi in *Citrus aurantifolia* and *Citrus limon*.

CHAPTER 2: LITERATURE REVIEW

2.1 *Citrus aurantifolia* (Lime)

Citrus aurantifolia also known as lime is one of the popular citrus fruits in Asian region as well as in Malaysia. *Citrus aurantifolia* is a part of the *Rutaceae* family, which includes over 150 genera and 1600 species found worldwide in tropical, subtropical, and temperate zones. Production of citrus fruit has a high demand but due to the existent of pest and pathogens, it slows down the fruit productivity. Besides, limes are considered as an affordable citrus fruit and often used as one of the main ingredients in certain dishes such as pickles and flavored juices. In addition, *C. aurantifolia* is well-known for its key biological functions, including as antibacterial effectiveness against a variety of diseases (Lemes et al., 2018).

Lime has different properties due to the various accessions by different country. For example, lime is highly consumed in Indonesia, Bhutan, and Malaysia. Lime is frequently thought to be a chance seedling. Lime also reproduces asexually via nucellar embryony. Another way for asexual reproduction of *Citrus aurantifolia* is vegetative propagation by humans. As a result, the heterozygous status of limes has been preserved throughout their evolution (Penjor et al., n.d.). However, *C. aurantifolia* had been considered as one of citrus species that easily infected by pathogen. For instance, citrus bacterial canker disease, which affects practically all citrus species and being one of the most feared citrus infections. It is widespread in tropical and subtropical citrus producing locations, causing severe canker lesions on all above-ground sections of the plant, leaf defoliation, early fruit loss, tree decline, and poor fruit marketability. Even though large surveys have already been undertaken to determine the likely presence of this condition. Typical disease signs were noticed for the first time in 2013 on lime during the rainy season, however it does not occur on other citrus trees which growing in the same orchards (Abubaker et al., 2016)

2.1.1 Fungal pathogen towards *Citrus aurantifolia*

Elsinoe fawcetti is one of fungus that has been found in *C. aurantifolia*. It is fungus that belongs to *Elsinoaceae* family and cause citrus scab. Citrus scab infects the fruit, leaves, and twigs of many vulnerable citrus cultivars, including lemons, grapefruit, and many tangerines and their hybrids, creating visible blemishes and affecting the fruit's acceptability for the fresh market. Citrus scab symptoms differ between cultivars and are strongly dependent on the age of the affected tissue (Chung, 2011).

Scab infections can appear on leaves as early as 4 days after infection and on fruit approximately 7 days later. The lesions appear as small spots, typically uneven and rough in appearance. As the host tissues grow, the afflicted regions become raised and develop erumpent scab pustules made up of both fungal and host tissues. The limes that have been affected have a flatter, corky look (Chung, 2011).

Figure 2.1: Citrus scab

2.2 *Citrus limon* (Lemon)

Citrus limon, sometimes known as the lemon tree, is a *Rutaceae* family plant which is common in Asia. Lemon fruit is also high in nutrients, such as flavonoids, citric acid, vitamin C, and minerals, all of which have health-promoting effects. The Citrus genus is a family of flowering plants that grew in tropical and subtropical Southeast Asia and have a distinct fruit with internal segments. Lemon production is restricted to a few nations and locations due to the plant's extreme sensitivity to cold temperatures. The crop is mainly in the Region of Murcia and certain neighbouring provinces of Alicante and Almera in Spain which is the

main lemon producing country in the Mediterranean area. Lemon production in Murcia Region is led by the autochthonous 'Fino' and 'Verna' kinds, followed by the foreign cultivars 'Eureka' from California (USA) and 'Lisbon' from Portugal. The 'Verna' lemon yields large fruits with high juice content and few seeds, whereas the 'Fino' lemon is smaller than the 'Verna' and has a higher juice extraction yield(Lorente et al., 2014).

Studies have showed that raw materials of *C. Limon* have been used in different ways for biological activity such as anticancer and antioxidant activity. Besides lemon is also famous in food industry as it is mostly used in beverages. Lemon production in Malaysia is considered low compared to other country. The largest exports suppliers of *C. limon* are Spain and Argentina. Malaysia being one of lemon supplier although not as much as Spain or Argentina, but Malaysia is a top lemon supplier for Singapore, Brunei, and Indonesia. Malaysia sold a total of 2,666 tons of lemon in 2020. Malaysia sold 2,820 tons of lemon in 2019. Malaysia lemon interest has climbed by 8.796% in 2019 compared to the previous year. Lemon exports declined by -9.9 percent between 2017 and 2019, earning the country $1.49 million in 2019(Page Analysis» Selina Wamucii, 2020). However, production of *C. limon* in Malaysia quite difficult due to the lemon diseases in lemon tree. It has been studied that most of diseases is caused by pathogens.

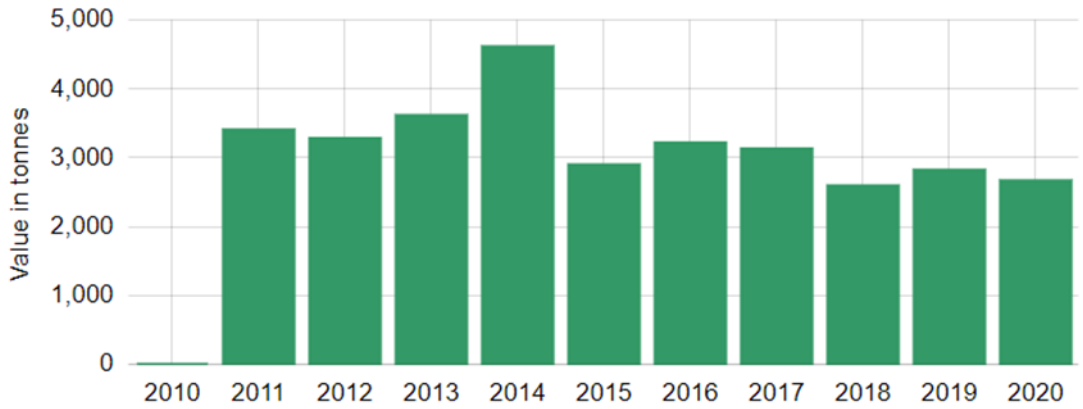


Figure 2.2: Lemon Export. Retrieved from https://www.selinawamucii.com/insights/market/malaysia/lemon/

2.2.1 Fungal pathogen towards *Citrus limon*

Melanose is one of diseases that always infected *C. limon* which cause by *Diaporthe citri*. Melanose fungus release it spores which develop in citrus tissues especially small twigs. It usually infects in dead citrus tissues within few months. *Diaporthe citri* spreads its spores through rainfall before infect lemon fruit. It takes 9 to 12 hours for the spores to germinate and penetrate the host tissues with continuous wet conditions. Melanose targets foliage, fruit, and twigs while they are quite young. These tissues grow more resistant to infection as they age. Citrus Melanose is found in various citrus-growing locations across the world and infects a wide range of citrus species. It affects immature leaves and fruits of specific citrus species or variations when the tissues develop and expand during prolonged periods of rain or humidity. This is one of the most frequent citrus fruit diseases seen across the world. It can cause significant defects on the fruit peel, but the fungus does not generally impact the pulp. External flaws, on the other hand, diminish the value of fruit destined for the fresh market. On foliage, this disease is often of modest economic relevance (Gopal et al., 2014).

A B

Figure 2.3 : Melanose on lemon tree
A: Front view of infected lemon fruit
B: Top view of infected lemon fruit

The signs of this widespread fungal infection range from tiny dots or scab-like lesions to damage patterns known as teardrop, mudcake, and star melanosis. Melanose seems to be

more widespread as trees age and the amount of dead wood in the canopy grows. The size and appearance of melanose lesions on fruit might vary depending on the maturity of the fruit at the time of infection. In addition, melanose fungus also cause a wood rot. It often happened when the trees are in a drought condition which cause a cinnamon brown discoloration at it barks. Melanose on summer growth flushes can be severe enough to produce defoliation, especially in years following freeze-induced twig die-back. On fruit, infected regions tend to develop tear-streak and water droplet patterns. Young green twigs are susceptible to infection. In most circumstances, attempting to manage melanose on foliage is both difficult and inefficient(Gopal et al., 2014).

Figure 2.4: Melanose disease on lemon fruit

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample Collection

The leaves and fruit of *Citrus aurantifolia* and *Citrus limon* were obtained from a local nursery in Kota Samarahan. Specifically, the infected looking leaves and fruits was obtained and packed in clean plastic bag then being brought to the Molecular Genetic lab of Faculty Resource Science and Technology (FRST), University Malaysia Sarawak (UNIMAS).

3.2 Isolation of pathogenic fungi *Citrus aurantifolia* and *Citrus limon* samples.

3.2.1 Isolation from infected leaves

Infected sample was rinsed with sterile water to remove any physical debris. The infected tissues, together with adjacent tiny unaffected tissue, were then sliced into small pieces around 2–5 mm squares and transfer into sterile petri dishes by using flame-sterilized forceps. The sterile petri dish was filled with 0.1% hypochlorite solution which used for surface sterilization of plant tissues. The plant part was transferred into Potato Dextrose Agar (PDA) plates and incubate for 5 to 7 days at 28°C to allows fungal growth. The resulting fungi was purified using the hyphal tips technique on Rose Bengal medium and then each isolated fungus subculture on slant medium for future studies (R. Thilagam et al., 2018).

3.2.2 Isolation of pathogenic fungi from spoilt *C. aurantifolia* and *C. limon*.

Each fruit was cut into small pieces around 3 mm in diameter with a sterile blade and the surface sterilized for 2 minutes with 1% of hypochlorite. Then the infected tissues were isolated by culturing the pieces of internal tissues.

Briefly the infected fruits will first be washed in sterile water and then swab dry by using sterilized cotton wool. The outer layer of tissues will be removed using a flame sterilized scalpel and a small portion from the central core in the advance margin of infection are removed and then put onto PDA plates and cultured for 5-7 days (R. Thilagam et al., 2018). Fungal colony that grew out was isolated and sub-cultured repeatedly until obtaining pure culture.

3.3 Pathogenicity Testing

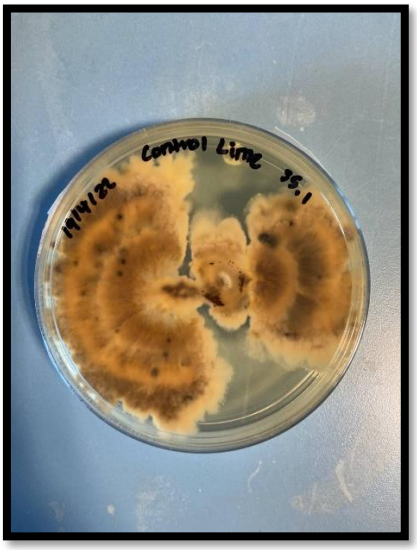
A pathogenicity or decay test was performed to determine if the isolated fungi were indeed responsible for the spoiling of *C. aurantifolia* and *C. limon* .Ethanol was used to sterilize the surface of healthy fruits. A sterilized 2mm cork borer used to cut away cylindrical plug tissues from the fruits. Agar plates with a pure fungal culture were inserted aseptically in these holes, then coated and sealed with sterile cotton wool. The technique was done for each of the fungal isolates independently. The infected samples and the control were putted in sterile petri dish and incubated for 5 days in an oven at 30□C. Each variety of fungus place of inoculation was studied and recorded. The rotting section of the lime and lemon was measured in diameter. The fungus was later recovered again from the infected fruits and compared to the original isolates (Muhammad et al, 2018).

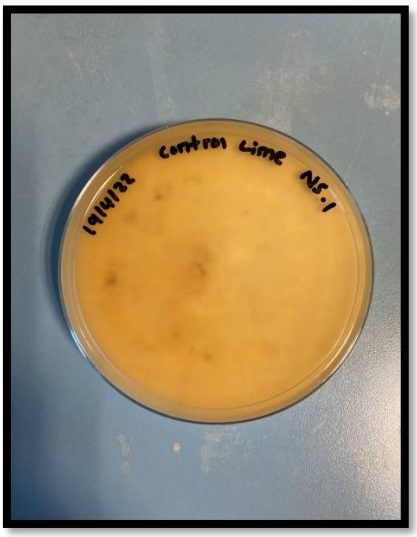
CHAPTER 4: RESULTS

4.1 Data Tabulation Pure Culture of Lime Leaves

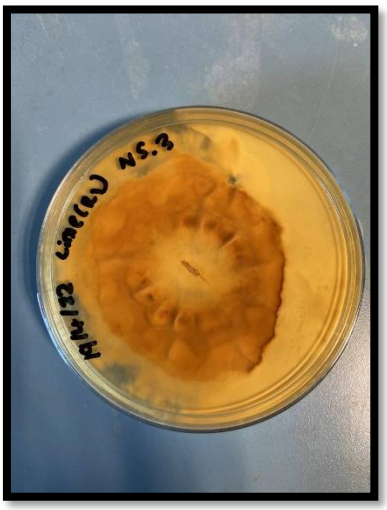
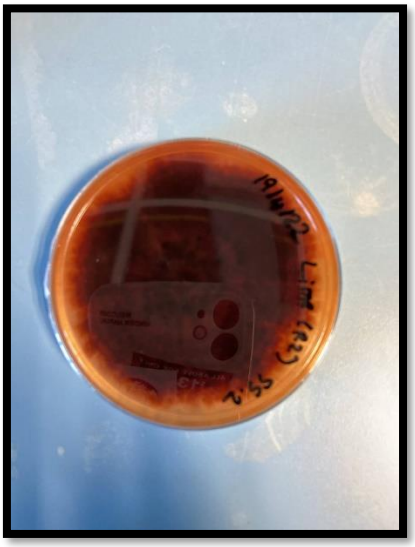
Table 1.1 showed that pure culture of lime leaves after second sub-culturing. After pure culture result was obtained, there are around 16 different pure culture of fungi. Each plate was label with its own code likes control lime SS.1. Besides, every sample was written into two codes which are SS and NS. SS code stand for surface sterilise while NS indicate for non-surface sterilise. For lime leaves pure culture it showed different characteristics in term of shape, colour and rate of growth.

Table 1.1: Lime Leaves-19/4/2022

Label	Front View	Back View	Characteristics
Control Lime SS. 1			• Colour: -White -Black spot -Brownish • Rate of Growth: Slow

Control Lime NS.1			• Colour: -White - Light brown • Rate of Growth: Fast
Control Lime NS.2			• Colour: -Milkish White -Pinkish • Rate of Growth: Slow
R1 (SS.1)			• Colour: -White -Brownish • Rate of Growth: Fast

R1 (SS.2)			• Colour: -White -Black spot -Brown Ring • Rate of Growth: Fast
R1 (NS.1)			• Colour: -Black -Spore -Brownish • Rate of Growth: Fast
R1 (NS.2)			• Colour: -White -Black • Rate of Growth: Slow

R1 (NS.3)			• Colour: -White -Yellowish -Brown ring • Rate of Growth: Slow
R2 (SS.1)			• Colour: -Grey -Black • Rate of Growth: Fast
R2 (SS.2)			• Colour: -White spot -Red brown • Rate of Growth: Slow