Investigating the Effect of Spent Coffee Ground Extract on Agriculturally Harmful Fungi and Bacteria

Group 1-35

Abstract

Microbial and fungal pests are a big problem farmers deal with today, causing diseases like crown gall disease, leading to reduced crop yields, and reduced income. Methods to manage these pests like pesticides or biocides have high costs and carry high environmental impacts. This project investigated the bactericidal, bacteriostatic and fungistatic properties of the aqueous extract of spent coffee grounds from *Coffea arabica* on agriculturally harmful fungi and bacteria, with the aim of evaluating its suitability as an alternative to current treatment methods, through well diffusion tests, colony count tests, minimum bactericidal concentration tests, and fungi growth tests. This project involved two bacteria, *Agrobacterium tumefaciens* and *Ralstonia solanacearum,* and one fungus, *Aspergillus niger.* The zone of inhibition for *A. tumefaciens*, and *R.solanacearum were* 2.3cm and 1.5cm for the test setup (p-value for both bacteria: 0.00). The number of colony forming units per 0.1μℓ for *A. tumefaciens*, and *R.solanacearum* were 162, and 45 respectively for the test setup, compared to 621 and 467 for the negative control respectively (p-value 0.002, 0.03 respectively). The minimum bactericidal concentration for *A. tumefaciens*, and *R.solanacearum* was greater than 0.5g/mℓ, and 0.25g/mℓ respectively. The diameter of fungal growth for *A.niger* was 3.2cm for the test, 2.9cm for the negative control (p-value: 0.051). This research showed that Spent Coffee Grounds Extract has bacteriostatic and bactericidal effects against *A. tumefaciens* and *R. solanacearum*, but does not have fungistatic effects against *Aspergillus niger*, suggesting that the aqueous extract of spent coffee grounds has potential to be used as a bactericide in the agriculture sector.

1. Introduction

Agriculturally harmful fungi and bacteria have caused significant economic damage to farmers. For instance, crown gall disease caused by *A. tumefaciens*, a plant pathogen, damages agriculturally-important crops (Attai *et al.*, 2018), while bacterial wilt disease, caused by the gram-negative phytopathogen *R. solanacearum*, affects a variety of crops (Cruz, Eloy, Quirino, & Carinho, 2008). *Aspergillus niger*, a common mould found in soil, causes black mould, a

disease that affects various different fruits and vegetables, like grapes, apricots, onions, and peanuts (Samson, Houbraken, Summerbell, Flannigan, & Miller, 2001).

To manage fungal and microbial pests, chemical fungicides or bactericides are used. However, their use carries a high environmental impact. Research by McArt, Urbanowicz, McCoshum, Irwin, & Adler (2017) had shown that chlorothalonil, a type of fungicide, was linked to *Nosema ceranae* infection in US bumble bees and decreased colony sizes of *Bombus impatiens*. Johnson, Dahlgren, Siegfried, and Ellis (2013) had also shown that the use of fungicides like prochloraz impairs the ability of Honey bees (*Apis mellifera*) to metabolise acaricides to kill parasitic mites, increasing chances of colony collapse disorder. The indiscriminate use of chemical pesticides has also led to pesticide resistance in many strains of bacteria and fungi, made worse by the cross resistance phenomenon where the genetic mutation that caused pesticide mutation also developed antibiotics resistance (Curutiu, Lazar, & Chifiriuc, 2017).

Biocides are also used to combat fungal and microbial pests. Strains K1026 and K84 of *Agrobacterium radiobacter* are applied on the surface of germinating seeds and the roots of shoots, to protect the plant from Crown Gall disease caused by *A. tumefaciens* (Ryder & Jones, 1991). Compared to the use of pesticides, methods of biocontrol are regarded as more environmentally friendly as they do not cause environmental pollution, and ecosystem disruption (Fujiwara *et al.*, 2011). However, biocides need to have strict environmental conditions to be effective, and are usually effective only on strains of a species that are not resistant, resulting in high overall costs (Pacanoski, 2015).

Recently, there has been growing interest and much research on the antimicrobial and antifungal properties of spent coffee grounds. Spent coffee grounds refer to the solid residue from the aqueous extraction of coffee from coffee beans. Mussatto, Machado, Martins, & Teixeira (2011) reported that over 6 million tonnes of spent coffee grounds were generated as waste annually, while Campos-Vega, Loarca-Piña, Vergara-Castañeda, & Oomah (2015) found that spent coffee grounds are currently used for low value applications such as fertiliser for mushroom growing or as a renewable source of fuel. However, such processes do not make use of the organic compounds found in spent coffee grounds, such as polyphenols, and nitrogenous

compounds like trigonelline, major caffeic and chlorogenic acids and caffeine, many of which exhibit antibacterial and antifungal properties (Mariotti-Celis *et al.*, 2017 ; Ballesteros, Teixeira, & Mussatto, 2014). Woo and Dong (2010) has found that chlorogenic acid displayed antifungal properties towards the pathogenic fungus *Candida albicans* by disrupting the structure of the cell membrane, while Sledz *et al.* (2015) showed that caffeine inhibited the growth of both gram-positive and gram-negative pathogenic bacteria. Almeida *et al.* (2012) had also shown that trigonelline displayed *in vitro* protection against the growth of *Streptococcus mutans*, a gram-positive bacteria .

Objectives

The objective of this study was to investigate the antibacterial properties of spent coffee grounds, a non-toxic and abundant resource, on the agriculturally harmful bacteria and fungi *A. tumefaciens*, *R. solanacearum*, and *Aspergillus niger* with the intention of finding a better alternative to current prevention and treatment methods.

Hypotheses

It was hypothesised that the aqueous extract of spent coffee grounds (*Coffea arabica*) had bactericidal and bacteriostatic properties against *R. solanacearum* and *A. tumefaciens*, and fungistatic properties against *Aspergillus niger.*

2. Materials and Methods

2.1 Preparation of Spent Coffee Ground Extract

Spent coffee grounds (*Coffea arabica*) were obtained from Starbucks Coronation Plaza (587 Bukit Timah, #01-03 Coronation Shopping Plaza, Singapore 269707). 800 grams of spent coffee grounds was mixed gradually with 400mℓ of deionised water in a beaker and mixed for 2 hours with a magnetic stirrer. The resulting mixture was then centrifuged at 7500 RPM for 10 minutes, and the supernatant (2g/mℓ Spent Coffee Ground Extract) was collected.

2.2 Preparation of Special Culture Media

To make Potato Dextrose agar plates (prepared with Spent Coffee Ground Extract), 19.5 grams of Potato Dextrose agar powder was mixed with 500mℓ of 2g/mℓ Spent Coffee Ground

Extract and autoclaved at 121°C for 15 min for sterilisation. 20mℓ of the autoclaved agar solution was then poured into each petri dish under a laminar flow hood and allowed to solidify. Unused agar plates were stored in a fridge at 4°C.

2.3 Preparation Of Bacterial Culture

A. tumefaciens was inoculated with a sterile inoculating loop into 9 mℓ of LB broth, while *R. solanacearum* was inoculated with a sterile inoculating loop into 9 mℓ of sucrose-peptone broth. The bacterial solutions were then incubated for 24 hours with shaking in an orbital shaker.

2.4 Well Diffusion Test

The bacterial culture was spread evenly on a Mueller-Hinton agar plate with a sterile cotton swab. The agar plate was then divided into 3 sections (test, positive control, and negative control). Wells of diameter 1 cm were drilled in the center of each section with a sterile pipette tip and filled with either 80µℓ of 2g/mℓ Spent Coffee Ground Extract for the test section, sterile water for the negative control section, or 10% bleach for the positive control section. The agar plates were then sealed with parafilm and incubated at 30°C for 24 hours. After incubation, the diameter of the zone of inhibition was measured with a meter rule. Five replicates were conducted and the experiment was repeated three times.

2.5 Colony Count Test (Spread Plate Method)

9mℓ of LB broth, and 0.5mℓ of bacterial culture were added into a centrifuge tube. 0.5mℓ of 2g/mℓ Spent Coffee Ground Extract, for the test setup, or 0.5mℓ of sterile water, for the negative control setup, were then added into the centrifuge tubes. The centrifuge tubes were then incubated with shaking at 30°C for 24 hours in an orbital shaker. After incubation, the setups were diluted with 0.85% saline to the appropriate dilution factor. 100µℓ of diluted setup was then pipetted onto the surface of a Mueller-Hinton agar plate, and spread evenly with an L-Shaped cell spreader. The agar plates were then sealed tightly with parafilm and incubated at 30°C for 24 hours. After incubation, the number of Colony-Forming Units were counted. Five replicates were conducted and the experiment was repeated three times.

2.6 Minimum Bactericidal Concentration Test (Broth Microdilution Method)

500μℓ of LB broth and 500μℓ of 100% 2g/mℓ Spent Coffee Ground Extract were added into a microcentrifuge tube. 5 Serial 2-fold dilutions were then conducted using Luria-Bertani broth to get different concentrations of Spent Coffee Ground Extract. 50μℓ of the respective bacterial culture was then added into each microcentrifuge tube. A microcentrifuge tube with 550μℓ of LB broth served as the broth control and a microcentrifuge tube with 500μℓ of LB broth and 50μℓ of the respective bacteria culture served as the bacteria control. The microcentrifuge tubes were then incubated at 30°C for 24 hours in an incubator. After incubation, a small volume (<10μℓ) from each microcentrifuge tube was spread on a Mueller-Hinton agar plate with a sterile inoculating loop. The agar plates were then sealed tightly with parafilm and incubated at 30°C for 24 hours in an incubator to allow the bacteria, if any, to grow.

After incubation, the Mueller-Hinton agar plates were observed to see if bacterial growth was present. The lowest concentration of the Spent Coffee Ground Extract with no bacterial growth on the Mueller Hinton agar plate was recorded as the minimum bactericidal concentration. Five replicates were conducted, and the experiment was repeated three times.

2.7 Fungi Growth Test

Aspergillus niger was inoculated in 20 mℓ of Potato Dextrose broth and incubated at 30°C for 24 hours with shaking in an orbital shaker. After incubation, the solution was centrifuged at 7000 RPM for 10 minutes, and the supernatant (*Aspergillus niger* overnight culture) was collected. 50μℓ of the *Aspergillus niger* overnight culture was pipetted into the center of a Potato Dextrose agar plate for the test setup, or Potato Dextrose agar plate (prepared with Spent Coffee Ground Extract) for the control setup. The agar plates were then sealed tightly with parafilm and incubated in an incubator at 30°C for 72 hours. After incubation, the diameter of fungal growth was measured with a meter rule. Five replicates were conducted and the experiment was repeated three times.

2.8 Chemical Analysis of Spent Coffee Ground Extract to determine caffeine concentration

High performance liquid chromatography was conducted using silica with C18 chains attached in the stationary phase and 20% acetonitrile as the mobile phase on the Spent Coffee Ground Extract to determine the caffeine concentration of the extract.

2.9 Statistical Analysis

The Kruskal Wallis K-test was carried out on the results of the Well Diffusion Test, while Mann-Whitney U-Test was carried on the results of the Colony Count Test and Fungi Growth Test to determine if the results of the experiment were significant (p-value <0.05).

3. Results & Discussion

3.1 Well Diffusion Test

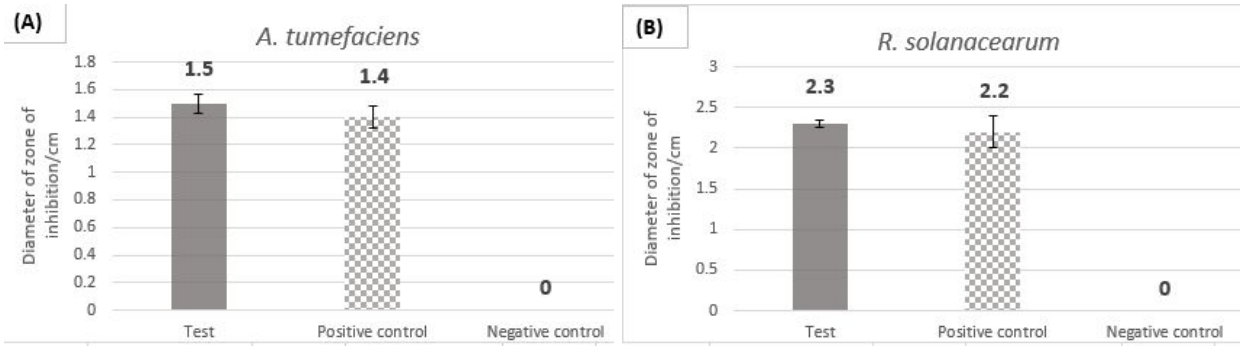

Fig. 3.1 : Diameter of zone of inhibitions recorded from Well Diffusion Test for *A.tumefaciens* **(A)**, P=0.00, and *R.solanacearum* **(B),** P=0.00. 80µℓ of extract or test solutions were pipetted in wells of the agar plate. A total of 15 replicates were conducted.

As seen in Fig 3.1, the zone of inhibitions recorded for the test setup ( ) was similar to the positive control setup ( ), while no zone of inhibition was observed for the negative control setup. The clear zone of inhibition for the test setup indicated that the Spent Coffee Ground Extract inhibited the growth of bacteria, displaying bacteriostatic properties. The results were significant as the p-values were below 0.05. The results supported previous research from Sousa, Gabriel, Cerqueira, Manso, and Vinha (2015) that showed that the aqueous extract of spent coffee grounds inhibited the growth of bacteria. It was concluded that Spent Coffee Ground Extract has bacteriostatic properties against *A. tumefaciens* and *R. solanacearum*.

3.2 Colony Count Test

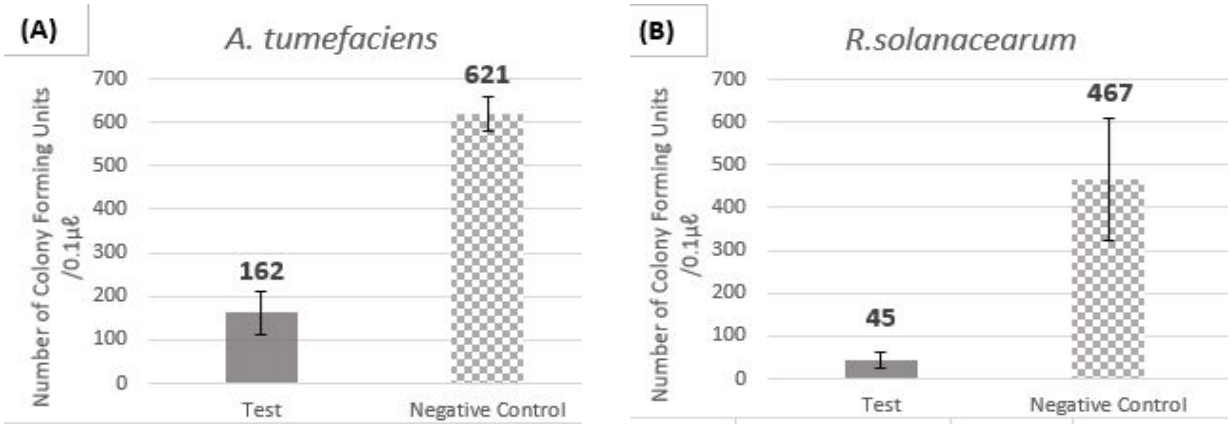

Fig. 3.2: Number of colony forming units recorded from Colony Count Test for *A.tumefaciens* **(A)**, P=0.002, and *R.solanacearum* **(B),** P=0.03. Setups comprised of 9mℓ broth media, 0.5mℓ bacterial culture and 0.5mℓ extract, concentration of test extract for colony count test was 0.1 g/mℓ. A total of 15 replicates were conducted.

As seen in Fig. 3.2, the number of colony forming units recorded for the test setup ( ) was much lower than the negative control setup ( ). The clear reduction in the number of bacterial colonies in the test setup compared to negative control setup indicated that the Spent Coffee Ground Extract had killed many of the bacterial colonies, displaying bactericidal properties. The results were significant as the p-values were below 0.05. It was concluded that Spent Coffee Ground Extract has bactericidal properties against *A. tumefaciens* and *R. solanacearum*.

The inhibition and reduction of number of colonies for both bacteria was likely due to the presence of nitrogenous compounds like chlorogenic acids, which are abundant in spent coffee grounds (Ballesteros *et. al*, 2014) and exhibit strong bacteriostatic and bactericidal behaviour (Sousa *et al.*, 2015). Chemical analysis of the Spent Coffee Ground Extract can be conducted to

determine if the Spent Coffee Ground extract contains sufficient concentrations of those nitrogenous compounds to inhibit the growth of bacteria.

3.3 Minimum Bactericidal Concentration Test

Bacteria	Minimum Bactericidal Concentration
A. tumefaciens	>0.5 g/mℓ
R. solanacearum	0.25g/mℓ

Fig. 3.3:Minimum Bactericidal Concentration (MBC) for *A. tumefaciens* and *R. solanacearum* obtained using the broth microdilution method with concentrations from 0.5g/mℓ to 0.015625 g/mℓ.

As shown in Fig 3.3, *R.solanacearum* has a low minimum bactericidal concentration of 0.25g/mℓ, showing the potential of using the aqueous extract of spent coffee grounds as an antibacterial agent.

A. tumefaciens had a higher minimum bactericidal concentration than *R. solanacearum.* This might be due to the *A. tumefaciens* having a high minimum bactericidal concentration for many antibacterial compounds (Sobota, 1976). Research from Kalogeraki, Jun, Eberhard, Madsen, and Winans (2002) showed that the VirH2 protein, encoded by the bicistronic *virH* operon in the Ti-plasmid of *A.tumefaciens*, enabled *A.tumefaciens* to catalyse the O-demethylation of ferulic acid, an antibacterial compound found in spent coffee grounds, into caffeic acid, which was less toxic than ferulic acid. This allowed *A.tumefaciens* to better survive in the presence of the extract.

3.4 Fungi Growth Test

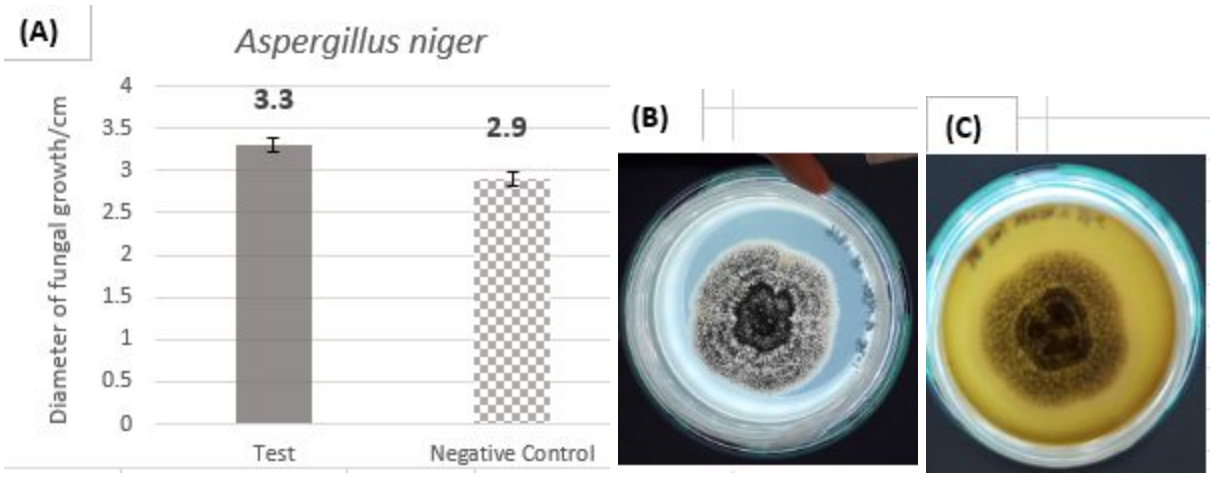

Fig.3.4: Diameter of fungal growth recorded from Fungi Test for *Aspergillus niger* **(A)**, P=0.051. Diameter of fungal growth for *Aspergillus niger* negative control setup **(B)**, and test setup **(C)**. 50µℓ of fungi culture was pipetted in the center of the agar plate. A total of 15 replicates were conducted.

The results in figure 3.4 seem to suggest that test setup ( ) had a larger diameter of growth than the negative control setup ( ). The results were not significant as the p-value was greater than 0.05. Thus, it was concluded that Spent Coffee Ground Extract did not have fungistatic properties against *Aspergillus niger*. This finding contradicted the findings by Ballesteros *et. al* (2014), which showed that the aqueous extract of spent coffee grounds did have antifungal properties against wood-decaying fungi. The difference in results were likely due to preparation techniques of the spent coffee grounds. The spent coffee grounds used in Ballesteros *et. al* (2014) were pretreated to remove a large proportion of low molecular weight products like sugars, unlike this project which directly sourced spent coffee grounds from Starbucks without treatment. The presence of carbohydrates, like sugars might have also promoted the growth of *A.niger*, and hindered the antifungal activity of other chemicals in the extract (Ballesteros *et. al*,

2014). The lack of fungicidal properties may be attributed to a low caffeine concentration. According to research conducted by Arora and Ohlan (1997), a caffeine concentration of 0.5% was required to fully inhibit fungal growth. However, the concentration of caffeine in the extract was less than 0.1%, which was significantly lower than 0.5%. This demonstrates that the aqueous extract of spent coffee grounds would not be viable as a fungicide.

3.5 Chemical Analysis

The concentration of caffeine in the extract was determined to be 20 parts per million.

4. Conclusion and Future Work

Conclusion and Future Work

Aqueous extract of spent coffee grounds (*Coffea arabica*) exhibited *in vitro* protection against *A.* tumefaciens and *R. solanacearum* but did not exhibit *in vitro* protection against *Aspergillus niger*. This project had demonstrated the potential of using spent coffee grounds, an abundant waste material, as a bactericide instead of conventional agrochemicals, benefiting the environment by preventing loss in biodiversity and reducing wastage. Doing so also has economic benefits as producers are able to market their produce to consumers as organic or eco friendly. Further work could include testing if spent coffee grounds offer *in vivo* protection against agriculturally harmful bacteria and fungi, testing the antibacterial and antifungal properties against different types of agriculturally harmful bacteria, such as gram-positive bacteria, and testing the antibacterial and antifungal properties of the aqueous extract of spent coffee grounds (*Coffea canephora*).

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