# Potential application of actinomycetes as natural fungicide

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# Potential application of actinomycetes as natural fungicide

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**Abstract**. Fungal diseases in green leafy vegetables are commonly under-estimated. However, the application of agrochemical fungicide has raised multiple drawbacks such as the higher amount of chemical residues and intensified microbial community stress. There is an urgent need to establish a bio-control antifungal agent with minimal toxicity threat, which sourced from natural environment. This study aimed to isolate soil-borne fungi from soil underneath infected green leafy vegetables and identify the antifungal activities of actinomycete strains on the isolated fungi strain. Fungi were isolated from 3 soils collected underneath the infected green leafy vegetables. Actinomycete strains were screened for antifungal potential against selected fungi strains using agar plug assay. Forty-one fungal strains were isolated from the soil samples. Actinomycetes strain C.D6.5 exhibited the largest inhibition zone on five selected fungal strains, whereas actinomycetes strain C.KSJ 13.3 produced the broadest spectrum of antifungal activities. It can be concluded that actinomycetes strain have significant antagonistic activity against fungi and pure antifungal component can be extracted as an effective and environmental friendly bio-control agent on plant fungi.

## 1. Introduction

Fungi have been identified as the major culprit for more than 70% of the plant diseases [1]. This diverse group of plant pathogens managed to survive in quiescent state on both living and dead plant tissues and proliferated under favourable conditions [2]. These soil-dwelling phytopathogens interfere with plant immune response to trigger infection which may result in crop failure and detrimentally affect the population whom highly dependent on a sole crop as major food source. The emerging condition of dramatic climate change also furthered imposed elevated stress on plant communities. Plant pathogens' ability to cause disease increased rapidly when the host plants are under stress, which eventually makes them a significant threat [3]. Chemical fungicide utilization has been advocated back in Green Evolution era attributed to its fast working mechanism and work effectively with minimal concentration [4]. Nevertheless, it is still undisputable that long-term fungicide application in agriculture sector does bring drawbacks such as ecological contamination and potential human health risk through occupational exposure. Plant pathogens' biocontrol was initiated as an environmental friendly substitution for agrochemicals and recent study has been emphasized on biological control technique on plant protection [5]. Biocontrol antifungal agent, which employed exudates of beneficial living microorganisms, was proposed to repress pathogenic fungi' activity on plant. Actinomycetes are one of the most abundant nature microorganisms and easily accessible as it can be commonly obtained from the soil from different sources. This high guanine (G)-cytosine (C) base characterized bacteria constitute a significant group among the soil microbial community attributed to its soil fertility

enhancement through nitrogen fixation and dead organic matter recycle characteristics [6]. Actinomycetes improved the organic matter cycle in agricultural soil by generated degradative enzymes such as protease, cellulases, chitanases and ureases for nutrient bioavailability [7, 8]. The bioremediation of pollutants in contaminated soils resulted from the collaboration of actinomycetes and other microorganisms that helped maintain the soil biological buffering capacity [6]. More than 60% of the isolated actinomycetes strains from Riyadh soil were effective in growth inhibition of *Aspergillus alternata* in tomoto blight disease [9]. Antifungal studies conducted by Shah *et al*., 2017 [10] proposed that the secondary metabolites produced by actinomycete strains induced it to perform antifungal activity that inhibits hyphal growth of the pathogenic fungi. Therefore, this study was carried out to investigate the fungi communities in infected soil and antifungal activities of actinomycete strains on infected green leafy vegetables contributing to the development of natural fungicide.

## 2. Material and Methodology

### *2.1. Soil Sample*

The sampling was carried out at the Agropark Universiti Malaysia Kelantan, Jeli campus (GPS coordinate 5°44'46.4" N 101°52'03.5" E). The soil samples were collected (5-10 mg) at the plantation site of infected green leafy vegetables were collected at a depth of 2 to 3 cm and kept in a labelled zipped-lock bag. The soil samples were subjected to slow air-drying for 7 to 10 days at warm temperature between 28 °C to 32 °C to mimic conditions that will occur during natural drying and kept at room temperature of 25 °C for future use.

### *2.2. Soil-borne Fungal Isolation*

Fungi communities in the infected soil were isolated using ten-fold dilution series method. The soil sample was diluted at 1:100 by placing 1 g of the soil sample into an Erlenmeyer flask containing 100 mL sterile distilled water. The Erlenmeyer flask was sealed before vigorously shaken at 120 rpm for 30 minutes. The resultant supernatant was serially diluted up to $10^{-3}$ with sterile distilled water. 200 $\mu$L of the diluted suspension were surface-plated onto HiMedia potato dextrose agar (PDA) (39 g/L PDA powder) supplemented with Streptomycin (50 µg/ mL). The plates were incubated at 28 °C for 5 days.

### *2.3. Actinomycetes Culture*

Thirteen actinomycetes strains which were previously isolated from flooded soil near Dabong, Jeli Kelantan were selected randomly and pieces of strain were transferred to Humic Acid-Vitamin Agar (HV Agar) (1 g/L humic acid, 1.7 g/L potassium chloride, 0.5 g/L magnesium sulphate, 0.02 g/L calcium carbonate, 0.01 g/L ferrous sulphate, 0.5 g/L disodium phosphate and 10 g/L agar) before incubation for 3 weeks at 30 °C.

### *2.4. Fungal Morphological Identification*

The fungi were picked from the isolation plate and individually transferred onto Oxoid Sabouraud Dextrose Agar (SDA) (65 g/L SDA powder) as a lawn. The plates were incubated at 28 °C for another 5 days. All fungi growth was observed and comparison was made according to their morphological characteristics such as margin and colour of aerial and substrate mycelium.

### *2.5. Screening of Actinomycetes Strain for Antagonistic Activity against Isolated Fungi*

The antifungal activities of selected actinomycetes strain against the isolated fungi were tested using agar plug method. The selected actinomycetes strain was streaked on NA agar and the plates were incubated at 30 °C until full mature lawn produced. A sterile 10 mm cork borer was used to prepare actinomycetes agar plugs from the mature actinomycete lawn. Sterile distilled water was used to scrape off the spores off the fungi and the spore suspensions were transferred and spread onto SDA plates. Actinomycetes plug were then placed on top of the fungi plate. 1 mg/mL cycloheximide and

sterile distilled water were used as positive and negative controls, respectively. The plates were incubated at 30 °C for 2 days. The antifungal assay of selected actinomycetes was conducted in triplicate to obtain a mean reading. The formation of inhibition zones were observed daily and measurement was recorded in the form of mean ± standard deviation (SD).

## 3. Results and Discussion

### *3.1. Samples Collection*

The soil samples were originated from green leafy vegetables cultivation of Green Pak Choy (Soil 1), Curly Wrap Wong King Pak Choy (Soil 2) and Chinese Kale (Soil 3). These green leafy vegetables were observed to have fungal disease as in Figure 1. The soil samples were freshly collected after morning watering took place and left for slow air dry under warm temperature for 14 days to imitate natural physiological drying process and reduced excess moisture. Desiccation diminished bacterial growth in soil samples but fungal growth remained constant [11, 12]. The drought resistance of fungal was mainly attributed to the presence of thick cell wall [13]. The strength and integrity of this chitin composed structure can be enhanced through the incorporation of β 1, 3-glucan carbohydrate which cross linked with chitin [14].

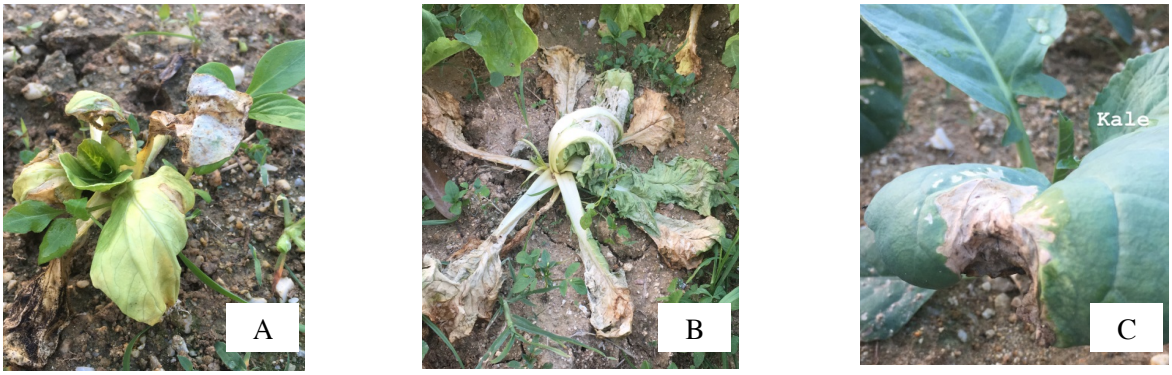


**Figure 1.** Infected leafy vegetables. A) Green Pak Choy that suffered from stunted growth and downy, B) Collapsed Curly Wrap Wong King with wilted and papery leaves and C) Brown leaf spot present in the edge of Chinese kale leaves.

### *3.2. Isolation of Fungi*

A total of 41 fungal strains were isolated from the three air-dried soil samples by serial dilution up to $10^{-3}$ and inoculated on PDA supplemented with streptomycin plates. Serial dilution was conducted in order to prevent overpopulation of fungal colonies on PDA culture media [15]. The macerated potatoes derived nutrient media with rich carbon: nutrient ratio promotes good sporulation and pigmentation for an extensive fungal range [16, 15]. Fourteen strains were isolated from both Soils 1 and 2 respectively, whereas 13 fungi strains were recovered from Soil 3. Figure 2 and 3 illustrated the countable fungal community on PDA isolation plates of $10^{-1}$ and $10^{-2}$ serially diluted soil suspension. No fungal growth was detected on the $10^{-3}$ serially diluted soil suspension isolation plate, as the soil liquid suspension can be considered too diluted that did not contain any of the fungal strain.


**Figure 2**. Fungi on $10^{-1}$ serially diluted soil suspensions isolation plates.


**Figure 3**. Fungi on $10^{-2}$ serially diluted soil suspensions isolation.

*3.3. Fungi Morphological Characteristics*

All isolated fungal strains were sub-cultured onto SDA agar because diminished pigmentation can be observed on PDA cultured fungi attributed to its low copper level as laccase utilized this cofactor in pigment formation of fungi conidia [16]. General identification of fungal could be interrupted as colony morphology features were prioritized in species recognition despite microscopic examinations [17]. The main remarkable characteristics used in this morphological selection through were macroscopic observations for conidia and reverse colours, texture, topography and soluble pigments (Table 1).

**Table 1**. Morphological Characteristics of Isolated Soil Fungi Based on Macroscopic Observation. ND = Not detected. Identical text colour highlight indicates fungi morphologies similarity.

| Fungal Isolate | Macroscopic Observation (SDA plate) | | | | |
|---|---|---|---|---|---|
| | Colour of aerial mycelia | Reverse Colour | Texture | Topography | Soluble Pigment |
| S1C1 | Orange | Orange-black | Velvety | Filamentous, crateriform, filiform | ND |
| S1C2, S2C11 | Black | Yellow | Cottony | Rhizoid, flat, filiform | ND |
| S1C3 | Brownish-grey | Black | Velvety | Rhizoid, raised, filiform | Black |
| S1C4, | Light yellow | Bright yellow | Velvety | Filamentous, raised, filiform | Light yellow |
| S1C10 | Light yellow | Bright yellow | Powdery | Filamentous, raised, filiform | Light yellow |
| S1C9 | Brownish-white | Yellow | Powdery | Filamentous, raised, filiform | ND |
| S1C5 | Brownish-white | Yellow | Cottony | Filamentous, raised, filiform | ND |
| S2C3, S2C5, S1C6 | White | Yellow | Powdery | Filamentous, flat, filiform | ND |

| | | | | | |
|---|---|---|---|---|---|
| S1C11, S1C14 | White | Yellow | Powdery | Filamentous, flat, entire | ND |
| S3C4 | White | Yellow | Powdery | Irregular, raised, filiform | ND |
| S1C7 | Yellowish-brown | Bright yellow | Powdery | Rhizoid, flat, filiform | Light yellow |
| S2C13 | Greyish-brown | Dark yellow | Powdery | Filamentous, flat, undulate | ND |
| S2C14 | Greyish-brown | Dark brown | Powdery | Filamentous, flat, filiorm | ND |
| S2C6 | Greyish-brown | Dark yellow | Powdery | Rhizoid, raised, filiform | Light brown |
| S2C7 | Greyish-white | Dark yellow | Powdery | Irregular, flat, filiform | ND |
| S3C11 | Greyish-white | Yellow | Powdery | Rhizoid, raised, filiform | ND |
| S1C8 | Greyish-green | Light brown | Velvety | Rhizoid, flat, filiform | Light brown |
| S1C12, S3C7, S3C8 | Greyish-white | Maroon-yellow | Velvety | Filamentous, crateriform, filiform | Maroon |
| S1C13 | Yellowish-white | Bright yellow | Powdery | Filamentous, flat, filiform | Light yellow |
| S2C1 | Black | Yellow | Cottony | Filamentous, flat, filiform | ND |
| S2C2 | Greyish-white | Black | Powdery | Rhizoid, raised, filiform | ND |
| S2C4 | Brown-white | Blackish-brown | Powdery | Filamentous, flat, filiform | Brown |
| S2C8 | Greyish-white | Black | Powdery | Rhizoid, flat, undulate | ND |
| S2C9, S2C10 | Black | Yellow | Powdery | Filamentous, flat, filiform | ND |
| S2C12 | Blackish-white | Yellow | Powdery | Filamentous, flat, filiform | ND |
| S3C1, S3C5 | White | Yellow | Velvety | Filamentous, flat, filiform | ND |
| S3C2,S3C3, S3C13 | White | Yellow | Powdery | Filamentous, raised, filiform | ND |
| S3C6 | Light brown | Bright yellow | Velvety | Circular, flat, filiform | Bright yellow |
| S3C9 | Light brown | Yellow | Powdery | Filamentous, raised, filiform | ND |
| S3C10 | Grey with white beads | Black-orange | Velvety | Filamentous, flat, filiform | ND |
| S3C12 | Brownish-white | Yellow | Velvety | Rhizoid, raised, filiform | ND |

It was observed that the conidia colour of isolated fungi occupied a wide range of colours from orange, black, brown to the most frequent white whereas the reverse colours only available in orange, black, maroon and yellow. Most of the isolated fungi exhibited powdery and velvety texture but a few owned cottony textures due to dense amount of spores and aerial hyphae formed. In terms of topography, majority of the isolated fungi strains have filamentous or rhizoid form, either flat or raised elevation and entire or filiform margin. The soluble pigment fungi strain produced which eventually altered the medium colour varied according to the isolates and ranged from black, brown, maroon to the most frequent yellow. The media composition significantly affects production of fungal pigment [18].

*3.4. Antifungal Assay*

All 11 revived actinomycetes strains were used as test organisms against the selected fungi strains for antifungal assay. The fungi strains were selected based on the difference in the morphological characteristics. Only 5 actinomycete strains; C.D 6.5, C.D 13.4, CFHV D8.12, C.KSJ 7.1, C.KSJ 13.3 and H.D 288 showed antagonistic activity against at least one of the selected fungal strains. Actinomycete strain C.D 6.5 exhibits the most significant antifungal effect against the isolated soil fungal strain. This actinomycetes strain, which previously identified as Streptomyces sp. formed largest inhibition zone of 10.67 ± 1.15 mm on tested fungi strain S3C9. The potential of Streptomyces sp. as bio-control antifungal agent was supported by few similar studies on plant fungal pathogens included *Alternaria solani* and *Verticillium dahliae* [19], *Fusarium* spp. [20] and *Aspergillus flavus* [21]. Actinomycetes strain formed inhibition zones ranged from 3.67 mm to 10.67 mm (Table 2) indicated that the actinomycetes strains have moderate inhibiting responses on tested fungi strains. Actinomycetes strain C.KSJ 13.3 was also worth focused in this study despite strain C.D 6.5 as it displayed the broadest spectrum of antifungal activities. Inhibition zones were formed on 3 fungi strains out of 5 strains tested. The average inhibition zones established were smaller compared to those of C.D 6.5 as a trade-off of cumulative inhibition capacity and growth efficiency [22].

**Table 2**. Diameter of inhibition zone formed on fungi strains by actinomycetes strains. '-' indicates no inhibition zones formed. SDW = sterile distilled water.

| Actinomycetes strains | Average Inhibition Zone (mean ± SD mm) | | | | |
|---|---|---|---|---|---|
| | Fungi strains | | | | |
| | S1C2 | S1C8 | S2C13 | S3C9 | S3C12 |
| C.D 6.5 | - | - | - | 10.67 ± 1.15 | - |
| C.D 8.3 | - | - | - | - | - |
| C.D 13.4 | 9.67 ± 0.29 | - | - | 3.67 ± 6.35 | - |
| CFHV D8.1.2 | - | 8.33 ± 1.53 | - | - | - |
| C.KSJ 2.3 | - | - | - | - | - |
| C.KSJ 7.1 | - | - | - | 10.00 ± 0.00 | - |
| C.KSJ 12.3 | - | - | - | - | - |
| C.KSJ 13.3 | - | - | 7.33 ± 6.43 | 9.67 ± 1.53 | 6.33 ± 5.51 |
| H.D 288 | 7.67 ± 6.66 | - | - | 9.67 ± 0.58 | - |
| H.D 628 | - | - | - | - | - |
| H.D 642 | - | - | - | - | - |
| Cycloheximide (+ve control) | 10.91 ± 0.18 | 9.91 ± 0.07 | 8.64 ± 0.16 | 7.09 ± 0.24 | 3.18 ± 0.29 |
| SDW (-ve control) | - | - | - | - | - |

## 4. Conclusion

In conclusion, 41 fungal strains were successfully isolated from the infected soil samples. Two actinomycete strains; C.D 6.5 and C.KSJ 13.3 showed antagonistic activities against 5 isolated fungi strain. Further research utilizing these actinomycete strains is needed to uncover the potential for developing natural fungicide.

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