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Original Research Article

Isolation, Control and Development of Antiserum from *Rastonia solanacearum*

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ABSTRACT

Rastonia solanacearum is a major cause of post harvest losses of Irish potatoes in the world today. This study was carried out in order to isolate *Rastonia solanacearum* from infected stems and tubers of Irish potatoes. In addition, the study determined the growth inhibitory activities of essential oils from rosemary, mint, lavender and eucalyptus against the bacterium. Isolation of the pathogen was carried out using serial dilution. Extraction of essential oils was done using distillation method. Sensitivity of the pathogen was carried out using agar well diffusion technique. Antiserum was prepared and tested again the pathogen using immune-diffusion bioassay. The mean number of *Rastonia solanacearum* produced by the infected Irish potato tuber was 1074.000 CFU/g while that of the stem was 328 CFU/g. There was a relationship between heating time and yield of essential oils in rosemary (r=0.99), mint (r=0.99), lavender (r=0.89) and eucalyptus (r=90). Conversely, there was no significant difference in the amount of essential oils produced by rosemary, mint, lavender and eucalyptus (P=0.078). There was a significant difference in the zones of inhibition produced by essential oils from rosemary, lavender, mint and eucalyptus (F=24.97 P=0.0009). Irish potatoes in the study area are highly infected by *Rastonia solanacearum*. Rosemary, mint, lavender and eucalyptus spp. produce essentials oils that are capable of controlling potato wilt bacterial pathogens. The isolated *Rastonia solanacearum* produces antiserum for rapid detection of the pathogen. There is need for mass production of essential oils from rosemary, mint, lavender and eucalyptus for the control of *Rastonia solanacearum*. Large scale production of antiserum for early detection of *Rastonia solanacearum* is highly recommended.

1. INTRODUCTION

Bacteria wilt (Rastonia solanacearum) of potato is generally favored by temperature between 25° and 37°. [10] It does not cause problem in areas where mean soil temperature is below 15°C. [2] Under condition of optimum temperature, infection is favored by wetness of soil. However, once infection has occurred, severity of symptoms is increased by hot and dry conditions which facilitate wilting. [1] Bacteria wilt is a serious problem in many developing countries in the tropical and sub-tropical zones of the world. The disease mainly occurs at latitudes 45°N and 45°S. [3]

Like any other plant, Irish potatoes are infected by different diseases most of which are caused by bacterial, fungal and virus pathogens. [9] Bacteria wilt is responsible for considerable losses in potato industry. In South East Victoria it has caused considerable losses in potatoes growing in the swampy areas. [20]

This disease is symptomized by vane clearing, mosaic, leaf mottling, tip die-back, chlorotic spots and wilting. [15] The disease can cause total loss of crops and prevent land use in potato production for several years. Bacteria wilt is caused by a soil-borne bacterium named *Ralstonia solanacearum*. Based on the type of host plant it attacks it is divided into four biovars. [16]

Bacteria wilt attacks more than 200 species of plants all over the world. [2] These include

economically importance hosts such as tobacco, potato, tomato, eggplant, banana, peanut, beans and pepper. In addition, the bacteria has some weed hosts namely nightshade and thorn apple. [6]

Essential oils (EOs) are extracts of plants prepared by steam distillation. They are generally composed of a combination of substances like terpenes, phenolics, aldehydes, or alcohols. Most of these substances are volatile. [4]

EOs can affect bacterial permeability and survival, either by direct contact or by contact with their vapour. [19] The volatile nature of essential oils could also have a direct application in food preservation and surface disinfection. [5]

The complex composition and different mechanisms of action of EOs may be an advantage over other antimicrobials in preventing development of drug resistance in pathogenic bacteria. [7]

Antibodies in the antiserum bind the infectious agent or antigen. [13] The immune system then recognizes foreign agents bound to antibodies and triggers a more robust immune response. The use of antiserum is particularly effective in recognizing pathogens which are capable of evading the immune system. [4] Stocks of antiserum can be produced from the initial donor or from a donor organism that is inoculated with the pathogen and cured by some stock of preexisting antiserum. [11]

This study was aimed at isolating *Ralstonia solanacearum* from infected potatoes and testing the sensitivity of the bacterium to essential oils obtained from rosemary, mint, lavender and eucalyptus leaves. The study also sought to prepare antiserum for the identification of the bacterium.

2. MATERIALS AND METHODS

2.1. Study area

The research work presentation in this article was carried out in the biological sciences research laboratories in Egerton University Njoro Campus located 30km from Nakuru town. Njoro lies 180km North West of Nairobi town on the Njoro-Mau-Narok road situated on prime Agriculture land at an altitude of 2250m above sea level. [22]

2.2. Collection of infected plant samples

Plant samples from stems and potato tuber (Figure 3.1) with symptoms of potato wilt disease were identified and uprooted from fields A, B, C, D and E. The samples were placed in new sterile polythene bags and transported to Biological Sciences Department, Egerton University laboratories and stored in the refrigerator at -4oC before isolation of *Ralstonia solanacearum*.

2.3. Isolation of *Rastonia solanacearum*

Bacteria wilt of potato was grown on nutrient agar (NA). The medium was dissolved in 250ml distilled water. Autoclaving at 121°c for about 20min was carried out. The medium was allowed to cool to 45oC. The medium was dispensed into sterile Petri dishes. The infected plant samples were cleaned using distilled water. The samples were surface sterilized using 70% ethanol for 3min. The plant samples were cleaned using distilled water to remove the ethanol on the surface. The samples were cut and bacteria picked from the symptomatic regions using a wire loop. Streaking on the medium was carried out. The plates were incubated at 37°C for 24 h. The growing cultures were sub-cultured to obtain pure cultures.

2.4. Characterization of Rastonia solanacearum

The isolate was characterized biochemically using API strips. Using a sterile wire loop, the isolate was inoculated into nutrient agar broth followed by incubation at 28oC for two days. McFarland units were used to standardize the inoculum. The inocula were separately placed in the wells of API strips. The strips were incubated at 37oC for 24h and observed for growth and colour changes of the medium. [2]

2.5. Extraction of essential oils

A sample of 400g of fresh rosemary, mint, lavender and eucalyptus leaves were separately loaded into 2-Litre round bottom flask containing 1.5 litre of water and placed on a heating mantle having power of 450 watt and timed. The samples were boiled with water to release the oil within the leaves. The volatile oils evaporated along with the water into the condenser connected to a flask at 100°C and atmospheric pressure. The condensed steam and oils were collected in a separating funnel after which oil and water were separated. The water was drained off gently and the oils were separately collected in a 10 ml measuring cylinder and measured after every 20 minutes for a period of 3

hours. The traces of water in the essential oils were removed by adding 1 gram of Magnesium Sulphate in the oil as a drying agent after which the yield obtained was calculated. [1]

2.6. Screening for biological control agent of essential oils

Screening for antimicrobial activity was conducted using agar well diffusion method. The bacterial pathogen was inoculated into Mueller Hinton agar using spread plate technique. Agar wells were made using a sterile 8mm cork borer. Each essential oil was separately placed in the wells using a micropipette. [11] The Petri plates were wrapped using parafilm before incubation at 37∘C for 24h. Diameter of zones of inhibition was determined in millimeters.

2.7. Preparation of antiserum using *Ralstonia solanacearum*

The pure culture of *Ralstonia solanacearum* was picked and added to a conical flask containing 0.085% NaCl solution using a wire loop. Sonification for 30mins was carried out to obtain pure a bacterial suspension. The suspension was injected into a chicken in order to produce the antiserum. After five weeks, blood was collected from the chicken in a clean sterilized test tube. The blood was allowed to stand under room temperature (28±2oC) for 4 h after which the tube was kept overnight without disturbance at 4°C. The clear serum was pipetted out and centrifuged at 5000 rpm for 30 minutes at 4°C. The supernatant was pipetted out using a micropipette and dispensed into 1.5 ml eppendorf tubes. A pinch of sodium azide was added to the clarified serum to prevent microbial contamination. The antiserum was stored in vials under refrigerated condition.

2.8. Immunodiffusion assay

The agar plates used for Ouchterlony double-diffusion tests were prepared with 15-ml portions of a preparation containing 1% Difco purified agar, 0.85% NaCl, and 0.02% sodium azide. The medium was dispensed into plastic Petri dishes. Peripheral wells measuring 3 mm in diameter and 4 mm apart were made around a center well. Bacterial cells were grown on a nutrient agar plate for 4 days and harvested in 0.5 ml of distilled water. The antiserum was serially diluted up to 1:8 dilution. A volume of 20µl buffer was placed in each of the vials and mixed thoroughly. The dilution of antiserum in the first vial was 1:1. From this vial, 20µl of 1:1 diluted antiserum was transferred to the second vial to make a dilution of 1:2. This was repeated up to the fourth vial. To the well at the centre, 10µl of the antigen was placed followed by 10µl of the diluted antiserum on the peripheral wells. Two wells acted as control. The plates were incubated at 25o C for 2 to 4 days and observed with a stereomicroscope with dark-field illumination for precipitin bands.

2.9. Data analysis and presentation

Data was analyzed using Microsoft excel spread sheet and statistical package for social sciences (SPSS) Version 17.0 software. The means were compared using ANOVA. The relationship of temperature and time to yield of essential oils was determined using pearsons correlation test. Data was presented using tables and graphs.

3. RESULTS

3.1. Biochemical characteristics of Rastonia solanacearum

The isolate tested positive for catalase, oxidase, maltose, lactose and cellobiose tests (Tables 1). However, the pathogen tested negative for dulcitol, mannitol and sorbitol tests.

432. Number of potato wilt bacteria

The number of Rastonia solanacearum ranged from 950 CFU/g to 1500CFU/g of plant sample in potato tuber (Table 2). In addition, the population of the bacteria varied from 212 CFU/g to 500CFU/g of plant sample. There was a significant difference in the number of Rastonia solanacearum between potato tuber and the stem (Figure 1).

3.3. Extraction of essential oil

The yield of essential oils varied from 0.5% after the samples were heat for 20 minutes to 4.0% after heating for 180 minutes in rosemary (Table 3). On the other hand, the percentage yield in mint ranged from 0.2% after the samples were heat for 20 minutes to 3.3% after heating for 180 minutes. However, the percentage yield varied from 0.7% after heating the samples for 20 minutes to 3.5% after the samples were heat for 180 minutes in lavender. In addition, the percentage yield in eucalyptus ranged from 0.2% after heating the samples for 20 minutes to 3.3% after the samples were heat for 180 minutes. The weights of the plant samples, volume of distilled water and the heating

temperature were maintained constant at 400g, 1.5 L, 100oC respectively. There was a relationship between heating time and yield of essential oils in rosemary (r=0.99), mint (r=0.99), lavender (r=0.89) and eucalyptus (r=90). Conversely, there was no significant difference in the amount of essential oils produced by rosemary, mint, lavender and eucalyptus (P=0.078).

3.4. Sensitivity of the selected pathogens to essential oils from rosemary, mint, lavender and Eucalyptus

The zones of inhibition of the test pathogens by essential oils varied from rosemary (20-26mm), lavender (15-17mm), mint (9-12mm) and eucalyptus (12-18mm) (Table 4). There was a significant difference in the zones of inhibition produced by essential oils from rosemary, lavender, mint and eucalyptus (F=24.97 P=0.0009).

3.4. Immunodiffusion of antiserum

Zones were observed in all the concentrations (Figure 2). The Antigen concentration was directly proportional to the diameter of the zone.

4. DISCUSSION

The isolate tested positive for catalase, oxidase, maltose, lactose and cellobiose tests. However, the pathogen tested negative for dulcitol, mannitol and sorbitol tests.These results are typical of *Ralstonia solanacearum*. The findings agree with those of a previous study by Fefan et al. (2017). The possible reason for the similarity in the findings was isolation of the same stains of *Ralstonia solanacearum*. *Ralstonia solanacearum* is an aerobic nonspore -forming gram-negative pathogenic bacterium. [12] The bacterium is soil-borne and motile. *Ralstonia solanacearum* causes a lot of losses in poorly stored potato leading to huge losses. [17] The isolation of *Ralstonia solanacearum* in this study agrees with findings of Nguyen and Ranamukhaarachchi. [14]

However, the results on extraction of essential oils from rosemary and eucalyptus were higher than in for mint and lavender. This could be attributed to the ecological conditions in which the plants were growing. The results agreed with a previous study. [11] According to Tinatin et al., soil fertility and high rainfall favours the growth of mint, lavender, rosemary and eucalyptus and therefore enhancing production of essential oils. [18]

The inhibitions of the bacterial pathogens by essential oils obtained from rosemary eucalyptus mint and lavender showed that rosemary is effective in inhibiting growth of *Ralstonia solanacearum* than lavender, eucalyptus and mint. These results differed with those of a previous study by Genin and Denny. [8] The composition of the essential oils may have contributed to differences in the results.

Antiserum in the current study was prepared using *Ralstonia solanacearum* isolated from infected potato stem and potato tuber. The antiserum obtained in the current study proven positive in determination of cases of *Ralstonia solanacearum*. This agreed with a previous study by Waithaka et al. [21] The possible reason for the similarity of the results may be similarities in the antiserum and the pathogen isolated. [13]

5. CONCLUSION

Irish potatoes in the study area are highly infected by Rastonia solanacearum. Rosemary, mint, lavender and eucalyptus spp. produce essentials oils that are capable of controlling potato wilt bacterial pathogens. The isolated Rastonia solanacearum produces antiserum for rapid detection of the pathogen.

6. RECOMMENDATIONS

There is need for mass production of essential oils from rosemary, mint, lavender and eucalyptus for the control of Rastonia solanacearum. Large scale production of antiserum for early detection of Rastonia solanacearum is highly recommended.

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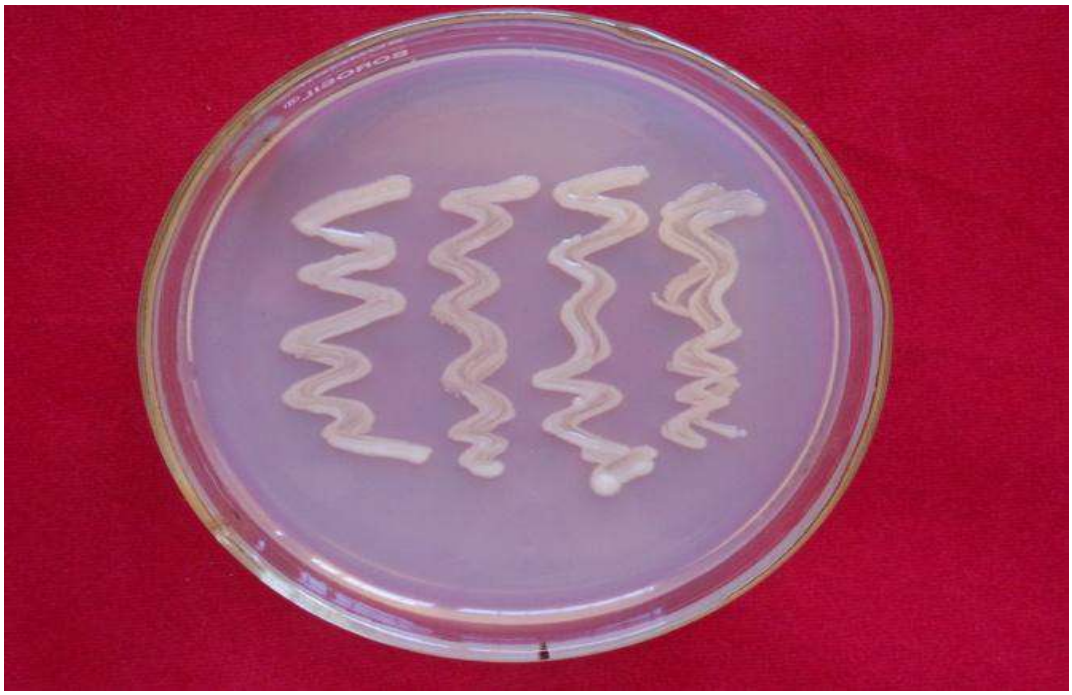

Fig.1. Pure cultures of *Rastonia solanacearum*

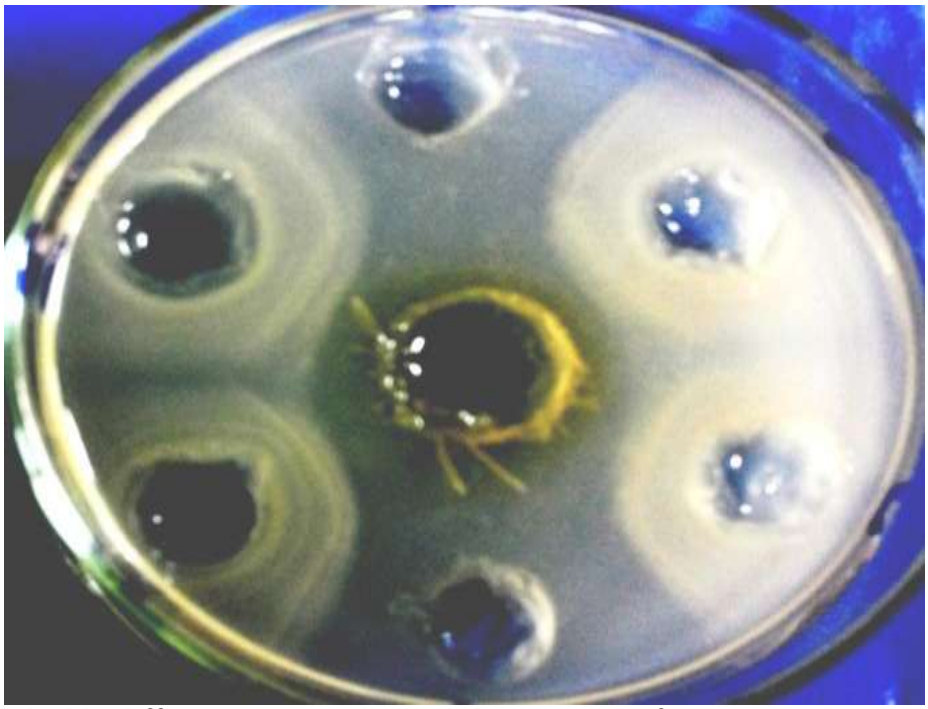

Fig.2.Ouchterlony double-diffusion patterns showing the reactions of antiserum on *Rastonia solanacearum*

Fig.3. Potato plants (a) and potato tuber (b) collected from the field

Table 1: Biochemical characteristics of *Rastonia solanacearum*

Test	Reaction
Catalase	+
Oxidase	+
Maltose	+
Lactose	+
Cellobiose	+
Dulcitol	-
Mannitol	-
Sorbitol	-

+; Positive reaction, -; Negative reaction

Table 2: Number of *Rastonia solanacearum* in CFU/g of sample

Serial Number	Number of potato wilt bacteria	
	Potato tuber (10^{-4})	Stem (10^{-4})
1	1000	500
2	1500	350
3	900	347
4	950	233
5	1020	212
Mean	1074.00	328.40

Table 3: Yield of essential oils from rosemary, mint, lavender and eucalyptus

Plant	Weight (g)	Distilled H_2O (L)	Heating time (Min)	Temperature (^oC)	Yield (%)
Rosemary	400	1.5	20	100	0.5
	400	1.5	40	100	0.8
	400	1.5	60	100	1.4
	400	1.5	80	100	1.6
	400	1.5	100	100	2.4
	400	1.5	120	100	2.8
	400	1.5	140	100	3.0
	400	1.5	160	100	4.0
	400	1.5	180	100	4.3
Mint	400	1.5	20	100	0.2
	400	1.5	40	100	0.3
	400	1.5	60	100	0.7
	400	1.5	80	100	1.0
	400	1.5	100	100	1.6
	400	1.5	120	100	1.8
	400	1.5	140	100	2.6
	400	1.5	160	100	2.8
	400	1.5	180	100	3.3
Lavender	400	1.5	20	100	0.7
	400	1.5	40	100	0.9
	400	1.5	60	100	1.5
	400	1.5	80	100	1.0
	400	1.5	100	100	1.8
	400	1.5	120	100	2.0

	400	1.5	140	100	2.2
	400	1.5	160	100	3.1
	400	1.5	180	100	3.5
Eucalyptus	400	1.5	20	100	0.2
	400	1.5	40	100	0.3
	400	1.5	60	100	0.4
	400	1.5	80	100	1.1
	400	1.5	100	100	1.5
	400	1.5	120	100	1.8
	400	1.5	140	100	2.6
	400	1.5	160	100	2.8
	400	1.5	180	100	3.3

Table 4: Zones of inhibition of the selected pathogens by essential oils from rosemary, mint, lavender and eucalyptus

Pathogen	**Zone of inhibition (mm)**			
	Rosemary	**Mint**	**Lavender**	**Eucalyptus**
Staphylococcus aureus	26	12	17	18
Bacillus subtilis	24	11	16	16
Escherichia coli	20	8	17	13
Klebsiella pneumoniae	22	9	15	12

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