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Research Article

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Control of *erwinia* spp. isolated from star-of-bethlehem (*ornithogalum* spp.) using actinomycetes and antiserum prepared from the bacteria using rabbit

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Abstract *Erwinia spp*. causes soft rot in *Ornithogalum spp.*, a flower grown purposely for export in Kenya. As a result the pathogen pauses a great risk to the economy of the country. This study aimed at isolating actinomycetes and *Erwinia spp.* from the soils of Menengai crater and infected *Ornithogalum spp.* respectively. In addition, the study sought to produce antiserum against the pathogen using a rabbit. Isolation of actinomycetes from the soil was carried out using standard methods. *Erwinia spp.* was isolated by first sterilizing the plant tissues using 70% ethanol prior to plating using nutrient agar after cleaning the plant tissues using distilled water. Further sub-culturing on nutrient agar was carried out to obtain pure cultures. The obtained *Erwinia spp.* was used in testing for sensitivity using the actinomycetes isolate and in production of antiserum through injection of the antigen intramuscularly into the rabbit. The antiserum was tested against the pathogen using immunodiffusion technique. Five potent actinomycetes, PAN 12, PAN 30, PAN 35, PAN 50 and PAN 60 were isolated from the soils of menengai crater. The *Erwiniaspp.*obtained had typical cultural and morphological characteristics of *Erwinia spp.* Although there was no significant difference in the zones of inhibition of the *Erwinia spp*. by the actinomycetes isolates, PAN 35 indicated the largest zones of inhibition. The antiserum produced had very high potency of controlling *Erwinia spp.* There is need to control *Erwinia spp.* otherwise horticultural farming in general and growing of the flower in particular will be jeopardized.

Keywords Actinomycetes, antiserum, *Erwinia spp.*, Isolation, *Ornithogalum spp*

Introduction

Ornithogalum spp. is one of the most important ornamental flowers in Kenya and world over [1]. Its commercial value surpasses that of other crops such as wheat, maize and potatoes. With the growing horticultural industry in Kenya, the growing of this flower has witnessed increased cultivation [2].

However, its growth has been curtailed by prevalence of serious pathogens that infect the crop. Of such hindrance to its growth is soft rot disease caused by *Erwiniaspp.* [3]. The pathogen causes the disease before and after harvest of the crop which leads to reduced yield. Its wide host range extending to most members of solanaceae family leads to the pathogens high prevalence in nature [4]. It exists in the soil penetrating the plant body via wounds and natural openings such as lenticels and stomata [5].

The control of *Erwinia spp.* has faced major challenges in the recent past. Normally, the pathogen is controlled using phytosanitary and cultural practices [6]. However, it has developed resistance to commonly used antibiotics [7]. This has probably been due to increased and indiscriminate use of the antibiotics. This has increased the need for development of eco-friendly bio-control methods [8].

Actinomycetes offer a potential solution to this problem [9]. They are a group of bacteria initially thought to be fungi. With in-depth study of the bacteria, it has been established to be a bacteria and not fungi as earlier thought to be [10]. The bacteria are inherently found in the soil and it has been found to be responsible for the earthy smell of rain due to production of a substance referred to as geosmin [11]. Actinomycetes have a high G+C content in their DNA. Most of the known antimicrobials of today have been produced by actinomycetes. Indeed most scholars have found actinomycetes as one of the major solutions to antimicrobial resistance [12].

The discovery of antibody antigen reactions is a great milestone to curbing problems of disease resistance. When a purified antigen is introduced in the body of an animal, the animal automatically produces antibodies against the pathogen [13]. This is the bases of producing antisera that can be used in treating diseases of economic importance today [14]. In testing the effectiveness of an antiserum, ouchterlony double immune-diffusion which is an immunological technique is used [15]. Its main significance lies on the ability to detect, identify and quantify the antibodies and antigens involved in the test [16]. This study was developed to isolate *Erwinia spp.* from the bulb and shoot of *Ornithogalum spp*., carry sensitivity test of the bacteria to actinomycetes and develop antiserum against the pathogen.

Materials and Methods

The study Area

The study was carried out in Ndundori which is located in Nyandarua County in Kenya with an average elevation of 2,354 metres above sea level (Fig. 1). The area is mild densely populated with 328 people per Km^2. The place has a humid climate suitable for growing of horticultural crops. The landscape is mostly covered with mosaic vegetation or croplands. The soils have high lixisols coupled with clay-enriched lower horizon and high saturation of bases. The coordinates of Dondori are 0 °12'S 36° 16' E/ 0.2 °S 36.27 °E. The closest major cities include Nairobi, Eldoret, Kisumu and Arusha [17].

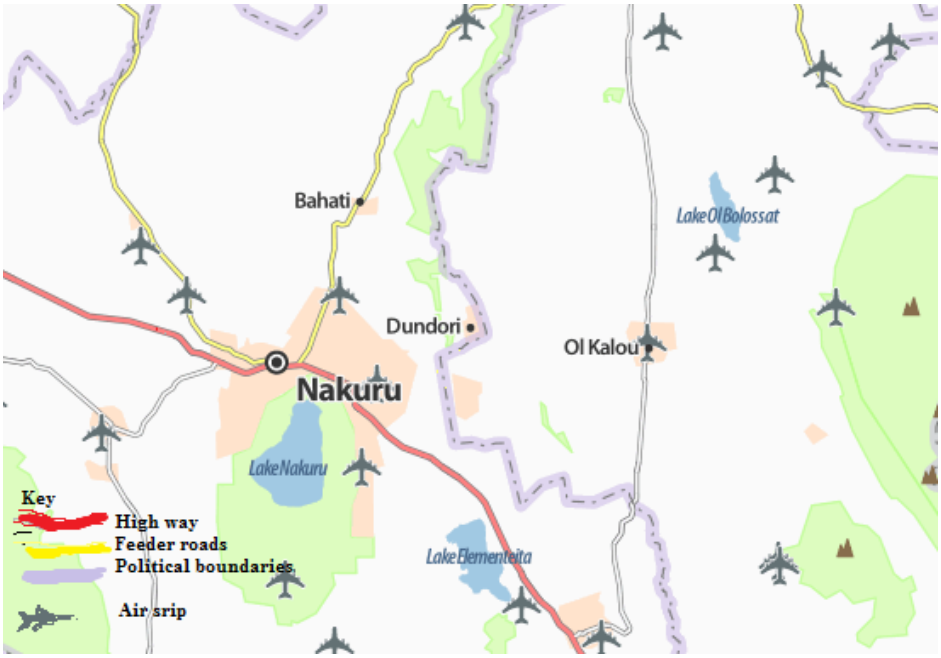

Figure 1: Map of Ndundori, source; Google maps

Collection of soil for isolation of actinomycetes

Soil samples were collected from 8 sampling points in Menengai crater at a depth of up to 5cm from the earth surface. The samples were mixed to form a composite sample before being transported in sterile polythene bags to the Department of Biological Sciences laboratories in Egerton University. In the laboratories, the sample was air

dried on the bench to kill most Gram negative bacteria. In addition the soil was heat treated by placing it in a hot water bath at 50 ^{o}C for 1h.

Isolation of actinomycetes

Actinomycetes were isolated using Starch casein agar (soluble starch: 10 g, K_2HPO_4: 2 g, KNO_3: 2 g, casein: 0.3 g, $MgSO_4.7H_2O$: 0.05 g, $CaCO_3$: 0.02 g, $FeSO_4.7H_2O$: 0.01 g, agar: 15 g, filtered sea water: 1000 ml and pH: 7.0±0.1) supplemented with nalidixic acid and nystatin to kill other bacteria and fungi respectively. I g of soil was placed in a test tube containing 9ml of water to make a stork culture followed by shaking the mixture for 10 min using an orbital shaker. Following this serial dilution was carried out up to 10^{-6}. Spread plate technique was used in plating prior to incubation at 30 ^{o}C for up to 28 days. Colonies showing typical actinomyctes characteristics such as powdery, leathery, partially submerged in culture were sub cultured in yeast extract malt extract agar. Pure colonies were preserved in starch casein slants. Characterization of the actinomycetes was carried out using cultural characteristics, biochemical test and Bergeys manual of systematic bacteriology.

Collection of *Ornithogalum spp.* plant samples

One hundred samples of infected *Ornithogalum spp.* were randomly collected from flower farms in Ndundori area (Fig. 2 and 3). The samples were weighed before being placed in sterile plastic bags followed by transportation to Egerton University, Department of Biological Sciences laboratories. The samples were stored in a deep freezer at 4^{o}C till processing.

Figure 1: Infected bulb

Figure 2: Infected shoot (A) and healthy shoot (B)

Isolation of *Erwinia spp*

A small piece of the infected plant tissues were aseptically removed from the edge of a typical lesion at a boundary with healthy tissues. The tissues were placed in 70 % ethanol for 10 min to surface sterilize them followed by cleaning them using distilled water. The tissues were macerated using a sterilized pestle and a mortar. A small quantity (0.1µl) was placed on previously prepared nutrient agar plates and spread using L shaped glass rod. Incubation was carried out at 37°C for 2-3 days. Sub culturing on nutrient agar was done to obtain pure cultures prior to characterization using biochemical methods.

Sensitivity test for *Erwiniaspp.* to actinomycetes metabolites

The antimicrobial activities of actinomycetes were determined using cross streak method [18]. Mueller-Hinton agar plates were prepared and inoculated with isolated actinomycetes cultures by a single streak and incubated at 30°C for 4 days. The Mueller-Hinton plates will be seeded with *Erwinia spp.* by a single streak at a 90° angle to actinomycete strains. Vancomycine (30µg) was used as a positive control while plain plates were the negative control. Antagonism was measured by determination of the zone of inhibition from the 7th to 13thfollowing incubation of bacteria at 37°C for 24 h.

Production of antisera from a rabbit

The antigen and antigen-adjuvant conjugate were prepare from the isolated *Erwinia spp.* following a protocol described [15]. The antigen-adjuvant conjugate was injected five times into a rabbit at one week interval to initiate an amplified immune response. After five weeks, an area around the rabbit's ear was shaved and a light cut was done using a sterile scalpel. Blood was collected in a clean sterilized test tube .The blood was allowed to stand

under room temperature (28±2 ^{o}C) for 4 h after which the tube was kept overnight without disturbing at 4°C. The clear serum was 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 vials were stored under refrigerated condition.

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. A total of six peripheral wells measuring 3mm in diameter and 4mm apart were made around a center well. Bacterial cells were grown on nutrient agar plate for 4 days and harvested in 0.5 ml of distilled water. The antiserum was serially diluted up to 1:8dilutions. 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 25^{o} C for 2 to 4 days and observed with a stereomicroscope with dark-field illumination for precipiting bands.

Results

Isolation of actinomycetes

Totally, 60 actinomycetes were isolated which were placed in 5 groups based on cultural morphology (Table 1). Among the isolates, 30 isolates were white, orange (15), Grey (10), yellow (3) and blue (2). All the isolates were positive for Gram staining and negative for spore and acid fast staining. All the isolates tolerated 2% NaCl concentration, white, yellow and blue could tolerate 5% NaCl concentration while orange and grey could not. All the isolates could not tolerate 10% NaCl concentration. All the isolates tolerated a pH of 5, white and grey (7) while all the colonies did not tolerate a pH of 9. On temperature tolerance, all the isolates tolerated a temperature of 35 and 55^{o}C while none tolerated a temperature of 5 ^{o}C.

Biochemical characteristics of actinomycetes

All the isolates were positive for catalase, casein hydrolysis, gelatin liquefaction, starch hydrolysis, VogesProskaeur and nitrate reduction tests (Table 2). However, they were negative for oxidase, indole production and H_2S production. The Grey, yellow and blue colonies were positive while the white and orange colonies were negative for methyl red.

Isolation of *Erwinia spp.*

The pure colonies of *Erwinia spp.* had deep cups-like cavities, yellow, circular, convex, smooth and entire margin at initial stages of growth. After 4-5 days, the colonies turned blue and looked like a fried egg. When subjected to biochemical tests, the colonies were Gram negative. In addition, the colonies were negative for starch hydrolysis, nitrate reduction, and Voges Proskaeur tests. The colonies were positive with methyl red reaction, indole formation, when grown under anaerobic conditions. The production of acid from fructose, galactose, and glucose yielded positive results but negative ones for arabinose, lactose, and maltose.

Sensitivity of *Erwinia spp.* to actinomycetes

After incubating the actinomycetes for 7 days, the zones of inhibition varied from 5mm in PAN 35 to 1mm in PAN 60. In addition on day 9 the range of zones of inhibition was 7mm in PAN 35 to 3mm in PAN 60 and PAN 30. On the 11th day the ranges shifted to 8mm in PAN 35 to 3mm in PAN 60. However, on day 13th, the variation in zones of inhibition was 20mm in PAN 35mm to 11mm in PAN 60 while on day 15th the range was 21 in PAN 35 to 10mm in PAN 10.

Table 1: Morphological and physiological characteristics of actinomycetes isolates

Test	**Properties of the isolates**				
Macroscopy and microscopy					
Cultural characteristic	**White colonies**	**Orange colonies**	**Grey colonies**	**Yellow colonies**	**Blue**
Gram staining	+	+	+	+	+
Spore staining	-	-	-	-	-
Acid fast staining	-	-	-	-	-
Physiology					
Nacl(% w/v) tolerance					
2	+	+	+	+	+
5	+	-	-	+	+
10	-	-	-	-	-
pH tolerance					
5	+	+	+	+	+
7	+	-	+	-	-
9	-	-	-	-	-
Temperature tolerance (^{o}C)					
5	-	-	-	-	-
15	-	-	+	+	+
35	+	+	+	+	+
55	+	+	+	+	+

Table 2: Biochemical characteristics of actinomycetes isolates

Biochemical characteristics	**Results**				
	white	**Orange**	**Grey**	**Yellow**	**Blue**
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
Casein hydrolysis	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+
Methyl red	-	-	+	+	+
VogesProskaeur	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Indole production	-	-	-	-	-
H_2S production	-	-	-	-	-

Table 3: Sensitivity test for *Erwiniaspp.* to the leading actinomycetes metabolites producers

Actinomycetes	**Zone of inhibition (mm)**				
	Day 7	**Day 9**	**Day 11**	**Day 13**	**Day 15**
PAN 12	3	4	6	13	13
PAN 30	2	3	4	14	14
PAN 35	5	7	8	20	21
PAN 50	4	6	7	11	11
PAN 60	1	3	3	10	10

Immunodiffusion

The zones of reaction were observed in all the concentrations. The Antigen concentrations were directly proportional to the diameter of the zones.

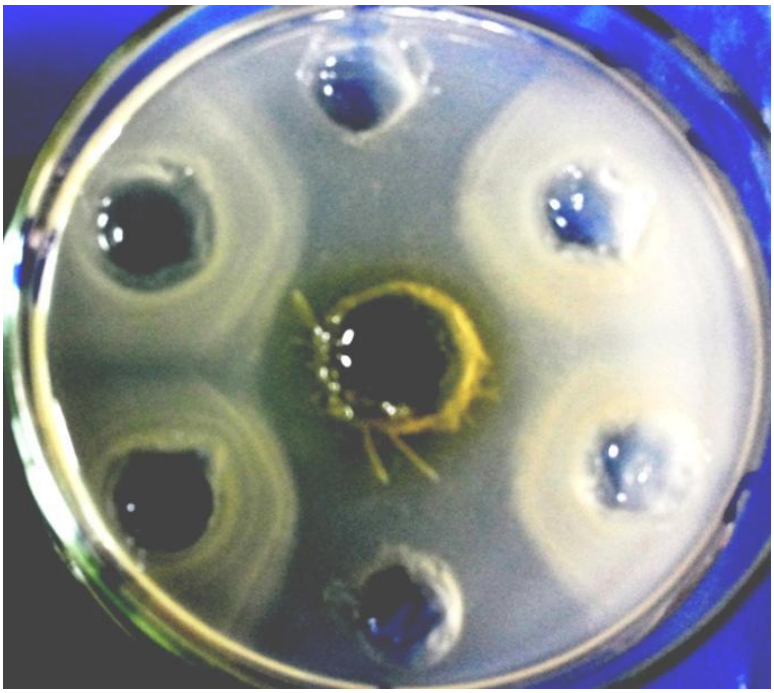

Figure 4: Ouchterlony double-diffusion patterns showing the reactions of antiserum on Erwinia spp.

Discussion

The results of the current study on the isolation of actinomycetes concur with an earlier study by[1] (Table 1). The isolates showed typical characteristics of actinomycetes when subjected to Gram stain. The negative results on spore and acid fast staining agree with a previous study [22]. On tolerance to NaCl the results show that the isolates do well in media with low salinity. This was further confirmed by pH tolerance where all the isolates grew well on media at pH 2. In addition, two isolates grew in media at neutral pH which agrees with a previous study carried out in India [15]. The possible reason for these findings could be physico-chemical characteristics of the volcanic soils from which the actinomycetes were isolated [3].

According to [5] actinomycetes exhibit varied biochemical tests. This was well indicated in the current study (Table 2). The variation in biochemical results may have arisen from the varied metabolic reactions within the many strains of actinomycetes [9].

The results of colonial morphology and biochemical tests of Erwinia spp. obtained in this study are a characteristic of *Erwinia spp.*[8] obtained similar results when carrying out a study on characterization of *Erwinia spp.* The results could have come from the fact that *Erwinia spp.* have fixed morphological and biochemical characteristics in absence of mutation [7].

However, the results on sensitivity of *Erwinia spp.* on actinomycetes observed in this study differed with a previous study [22] (Table 3). Differences in the soil sampling site which determined the strains of actinomycetes could be a contributing factor. The results suggest that PAN 35 is the best stain of actinomycetes due to the high zones of inhibition. Based on the morphological, physiological and biochemical characteristics of the PAN 35, it was similar to a strain isolated by [16]. This could have been contributed by the study areas having the same soil and environmental characteristics [23].

Immunodiffusion test indicated that rabbits have the potential of producing antiserum capable of controlling *Erwinia spp.* (Fig 3). The sensitivity of the antigen to low dilutions of the antiserum attest to this which agreed with an earlier

study carried out in [14]. According to [15], the strain of the antigen from which the antiserum was prepared and its purity during the time of testing determines to a great extent the results of immunodiffusion.

Conclusions

Soft rot of *Ornithogalum spp.*is caused by *Erwinia spp.* The soils of Menengai crater have culturableactinomycetes. *Erwinia spp.*can effectively be controlled by actinomycetesmetabolites and production of antiserum using rabbits.

Recommendations

There is need to control Erwinia spp. that causes soft rot in *Ornithogalum spp.* otherwise horticultural farming in general and growing of the flower will be put to jeopardy. Massive fermentation of actinomycetes metabolites coupled with increased production of antiserum is highly encouraged.

Conflict of interest

The authors declare no conflict of interest.

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